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Towards a mechanistic understanding

of telomere loop structures

in *S. cerevisiae*

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Summary

Only with the evolution from circular to linear genomes, allowing easy exchange of genetic information by sexual reproduction, complex organisms with large genomes could evolve. But the linearity of a chromosome inherits a major problem, namely two chromosome ends that have to be protected. Telomeres at the very end of the chromosomes ensure cell survival. These non-protein coding DNA repeats are essential features of chromosomes, as their loss leads to irreversible cellular senescence and chromosome loss. Paradoxically, telomeres resemble DNA double-strand breaks (DSBs), however, unlike DSBs, they are refractory to repair events. This so-called “end protection” function carried out by telomeres ensures that chromosomes do not fuse together in an end-to-end manner and avoids the DNA damage response machinery from being activated, leading to cell cycle arrest. Although end protection has largely been attributed to the major telomere binding complexes such as shelterin in mammals and the CST complex in yeast, it has also been proposed that a three-dimensional structure at the telomere may contribute to safeguarding telomeres. These so called telomere loops (t-loops) have been demonstrated via electron- and super-resolution-microscopy in mammalian cells, however, the short length and base composition of yeast telomeres prevent such approaches. By using a combination of Chromatin Immunoprecipitation (ChIP) and transcriptional readouts it has been demonstrated that yeast telomeres loop back onto their respective subtelomeres; however, both methods are indirect and unsatisfactory in terms of analyzing the dynamic regulation of loop structures.

In this study we have established a new assay based on Chromosome Conformation Capturing (3C) to directly detect and quantify interactions between a telomere and its subtelomeric region in *S. cerevisiae*, as a measure of telomere looping. In this manner we could exploit the genetic advantages of the yeast system to understand the mechanistic details of telomere loop formation and maintenance.

Since telomere shortening leads to an unprotected telomere, we wondered whether telomere length may have an impact on looping. We were able to show a significant looping defect in cells that lack telomerase as well as in other mutants that harbor short telomeres. On the contrary, elongated telomeres were able to maintain the looped structure. This suggests that a critical telomere length is essential to maintain the telomere loop and that telomeres in senescent cells

are likely in an open conformation, rendering them susceptible to nucleolytic end resection and unscheduled DNA repair events.

Gene looping is another kind of looped chromatin that brings promoter and terminator together. Gene loops depend on transcription, a functional transcription initiation complex and several components of the mRNA processing machinery. Indeed, we could detect a telomere looping defect upon loss of RNA polymerase II and in a mutant of the transcription preinitiation complex (*sua7-1*). It has been shown that certain chromatin loops that bring promoter and enhancer regions in close proximity depend on a non-coding RNA species that interacts with components of the promoter associated mediator complex. Depletion of total RNA levels or mutation of mediator resulted in a telomere looping defect, indicating a similar regulation at the telomere.

Zusammenfassung

Erst mit der Evolution von zirkulären zu linearen Genomen, die einen einfachen Austausch von genetischen Informationen durch sexuelle Reproduktion erlaubten, konnten sich komplexe Organismen mit großen Genomen entwickeln. Die Linearität eines Chromosoms verursacht allerdings ein großes Problem, nämlich zwei Chromosomenenden, die geschützt werden müssen. Telomere am äußeren Ende der Chromosomen gewährleisten das Überleben der Zellen. Diese nicht für Proteine kodierenden, sich wiederholenden DNA-Sequenzen sind essenzielle Merkmale von Chromosomen, da ihr Wegfall zu zellulärer Seneszenz und Chromosomenverlust führt. Paradoxerweise ähneln Telomere DNA-Doppelstrangbrüchen, allerdings sind sie, im Gegensatz zu Doppelstrangbrüchen, renitent gegenüber Reperaturvorgängen. Diese Schutzfunktion von Telomeren verhindert die Fusion von Chromosomen und die Aktivierung der DNA Schadensreaktion, die zu einer Zellzyklus-Arretierung führen würde. Obwohl diese Schutzfunktion vor allem den wichtigsten Telomer-Bindekomplexen wie *shelterin* in Säugetieren und dem CST Komplex in Hefe zugeschrieben wird, wurde ebenfalls eine dreidimensionale Struktur am Ende der Telomere vorgeschlagen, die die Telomere schützt. Diese sogenannten Telomerschleifen (telomere loops oder t-loops) wurden mittels Elektronen- und hochauflösender Mikroskopie in Säugerzellen gezeigt, die kurze Länge und Basenkomposition der Telomere in Hefe erlauben diesen Ansatz allerdings nicht. Mittels einer Kombination von Ko-Immunpräzipitation und eines transkriptionellen Auslesesystems wurde gezeigt, dass auch Telomere in Hefe auf ihre jeweiligen Subtelomere zurückfalten. Nichtsdestotrotz sind beide Methoden indirekt und nicht zufriedenstellend bezüglich der Analyse der dynamischen Regulation von Schleifenstrukturen.

In dieser Studie haben wir ein neues Testverfahren basierend auf Chromosomen-Konformations-Erfassung (chromosome conformation capturing; 3C) etabliert, um mit seiner Hilfe Interaktionen zwischen einem Telomer und seiner subtelomeren Region, als Maß für Telomer-Rückfaltung in Hefe, direkt zu detektieren und zu quantifizieren. Auf diese Art und Weise konnten wir die genetischen Vorteile des Hefe-Systems nutzen, um die mechanistischen Details der Telomerschleifenbildung und –erhaltung zu verstehen.

Da eine Telomerverkürzung ein ungeschütztes Telomer zur Folge hat, stellten wir die Frage, ob die Telomerlänge Einfluss auf die Telomer-Rückfaltung haben könnte. Wir konnten einen erheblichen Rückfaltungsdefekt in Zellen mit fehlender Telomerase und in weiteren Mutanten

mit kurzen Telomeren feststellen. Im Gegensatz dazu waren verlängerte Telomere weiterhin in der Lage Schleifen zu bilden. Dies suggeriert, dass eine kritische Telomerlänge essentiell für die Erhaltung der Telomerschleife ist und das Telomere in seneszenten Zellen wahrscheinlich in einer geöffneten Konformation vorliegen, was sie zugänglich für nukleolytischen Verdau und irreguläre DNA Reparaturvorgänge macht.

Genschleifen (*gene loops*) sind eine weitere Art von rückgefaltetem Chromatin, die Promotoren und Terminatoren zusammen bringen. Genschleifen hängen sowohl von Transkription und einem funktionellen Transkriptions-Initiations-Komplex als auch von einigen Komponenten der mRNA Prozessierungsmaschine ab. Tatsächlich konnten wir eine defekte Bildung von Telomerschleifen nach Wegfall der RNA Polymerase II und in einem Mutanten des Transkriptions-Initiations-Komplex feststellen. Es konnte gezeigt werden, das bestimmte Chromatinschleifen, die Promotoren und Transkriptionsverstärker in nahe räumliche Nähe bringen, von einer nicht-kodierenden RNA Art abhängen, die mit Komponenten des Promotor-assoziierten Mediator Komplex interagieren. Der nukleolytische Abbau von RNA oder eine Mutation des Mediator Komplexes führte zu reduzierter Telomerschleifenbildung, was eine ähnliche Regulation an Telomeren andeutet.

List of Publications

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Table of Contents

1 Introduction 17
1.1 Telomeres 17
1.1.1 An introduction to telomeres – starting with the end 17
1.1.2 The end protection problem and telomere looping 18
1.1.3 The end replication problem and telomere maintenance 23
1.1.4 TPE and TPE-OLD 26
1.1.5 Transcription at the telomere - TERRA 28
1.2 RNA Polymerase II transcription in yeast 30
1.2.1 A short overview – from the beginning to the end 30
1.2.2 Getting to know - The Mediator complex 32
1.3 A looped genome 34
1.3.1 Gene loops 35
1.3.2 Chromatin loops 38
1.4 Looking at loops – a comparison 40
1.4.1 Microscopy 40
1.4.2 A genetic readout – “Construct2” 42
1.4.3 Chromatin immunoprecipitation (ChIP) 43
1.4.4 Chromosome Conformation Capturing (3C) 44
1.5 Aims of this study 45
2 Materials and Methods 48
2.1 Materials 48
2.1.1 Yeast strains 48
2.1.2 Plasmids 48
2.1.3 Oligonucleotides 49
2.1.4 Yeast media and plates 49
2.1.5 Buffers and stock solutions 51
2.1.6 Other materials 54
2.2 Methods 58
2.2.1 Yeast growth 58
2.2.2 Arrest of yeast cells in different cell cycle phases 58
2.2.3 Transformation of yeast 58

2.2.4 Spotting experiments 59
2.2.5 Genomic DNA extraction 59
2.2.6 Telomere length measurement by Telomere PCR 59
2.2.7 RNA extraction 59
2.2.8 TERRA purification 60
2.2.9 Denaturing Agarose gel for RNA 60
2.2.10 Reverse Transcription of RNA/TERRA 61
2.2.11 Quantitative RT-PCR for TERRA levels 61
2.2.12 DNA staining and flow cytometry 61
2.2.13 Protein extraction 62
2.2.14 SDS-PAGE 62
2.2.15 Western Blotting 63
2.2.16 Chromosome conformation capturing (3C) 63
2.2.17 Quantitative PCR on 3C samples 64
2.2.18 Statistical analysis 64
3 Results 65
3.1 Detecting telomere loops with 3C in yeast 65
3.1.1 Telomeric 3C using DpnII 66
3.1.2 Telomeric 3C using NcoI 67
3.1.3 Telomeric 3C can confirm published looping defects 68
3.2 A relationship between telomere length regulation and telomere looping in *S. cerevisiae* 70
3.2.1 Telomere looping is not required for bulk telomere length regulation 71
3.2.2 Shortened telomeres are looping defective 72
3.3 Regulation and Maintenance of telomere looping in *S. cerevisiae* 76
3.3.1 Telomere loop formation is independent of homologous recombination 76
3.3.2 Telomere loop formation requires the transcription machinery 77
3.3.3 Cell cycle regulation of telomere looping 81
3.3.4 Telomere looping is independent of G4-Quadruplexes 83
3.4 On the functions of telomere looping in *S. cerevisiae* 84
3.4.1 TERRA levels are not regulated by telomere looping 84
4 Discussion 86
4.1 What has been known before this study 86
4.2 3C – a new tool to understand telomere looping 86
4.3 Short telomeres adopt an open conformation 89

4.4 Starting to understand the mechanisms 93
4.4.1 Homologous recombination is not involved in yeast telomere looping 93
4.4.2 Only a transcribed telomere is forming a fold-back 95
4.4.3 Temporal telomere loop regulation 101
4.4.4 (Temporo-) Spatial telomere loop regulation 103
4.4.5 More on telomere loop regulation 105
4.5 Why do telomeres loop? 106
4.5.1 Telomere protection 106
4.5.2 Transcription regulation and telomere position effect 107
4.6 Concluding remarks and future perspectives 111
5 References 113
6 Table of Figures 130
7 Abbreviations 131
8 Appendix 134
9 Acknowledgements 141

1 Introduction

1.1 Telomeres

1.1.1 An introduction to telomeres – starting with the end

With the evolution of eukaryotic cells the genome organization changed dramatically from a single circular DNA molecule to a set of linear chromosomes, allowing easy exchange of genetic information by sexual reproduction. This design paved the way for the development of higher eukaryotes, capable of adapting to ever-changing environments.

Eukaryotic linear chromosomes, each comprising two physical ends, terminate in nucleoprotein-structures called telomeres, which consist of non-protein coding DNA repeats and a set of dedicated telomere binding proteins. Telomeres are essential features of chromosomes as their loss leads to either a permanent cell cycle arrest, called cellular senescence, or apoptosis. This is not surprising, given their conservation across species. Sequences may vary in a species-specific manner, but the overall structure and composition of telomeres are conserved from yeast to human.

Telomeres in *S. cerevisiae* consist of heterogeneous, double-stranded, non-nucleosomal TG_{1-3} repeats (Shampay et al. 1984; Wang and Zakian 1990; Wright et al. 1992), while human telomeres are made up of TTAGGG repeats and considered to be heterochromatic. Yeast telomeres are made up of 250 - 350 base pairs (bp) whereas their human counterparts can reach a length of up to several thousand bp. Yeast telomeres end in a single-stranded (ss) 3’ overhang of 10 - 15 bp formed on the G-rich lagging strand template (Wellinger et al. 1993; Wellinger et al. 1993), which is elongated to 50 - 100 bp in late S phase (Larrivee et al. 2004). Overhangs in human cells range from 50 - 200 bp (McElligott and Wellinger 1997). With their G-rich sequence telomeres have been shown to form so-called G-quadruplexes (Tang et al. 2008). These structures consist of planar G-squares, containing four guanine bases each that stack on top of each other (also see Figure 28).

Yeast telomeres are categorized into two subtypes, X-only and Y` telomeres (Figure 1). The two classes are based on the composition of the telomere associated sequences (TAS) in the subtelomeric region (Chan and Tye 1983). While containing a low gene density, all subtelomeres harbor a TAS called X element whereas only about 50 % of telomeres have an additional Y´

element (Figure 1). The X element contains a core X element and additional subtelomeric repeated elements (STR). The Y' elements vary in copy number (up to 4 copies) and appear in two sizes of 5.2 kb or 6.7 kb. Y' elements are located between X elements and the telomere (Figure 1) (Wellinger and Zakian 2012).

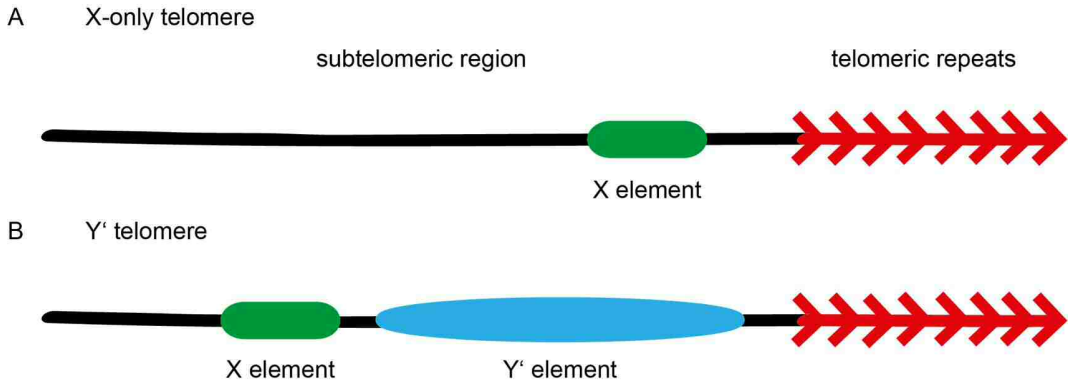

Figure 1: Natural yeast telomeres and their associated repetitive sequences
A) X-only telomere with the G-rich telomeric repeats (red) and the X element (green). Both are found at all telomeres. B) Y' telomere with G-rich telomeric repeats (red), the X element (green) and the large Y' element (blue). Y' elements can be found at approximately 50 % of the telomeres. (adapted from (Lydall 2003))

1.1.2 The end protection problem and telomere looping

1.1.2.1 Telomere binding proteins

DNA double-strand breaks (DSBs) in the genome lead to the activation of a DNA damage response (DDR). In mammalian cells, checkpoint activation is mediated by ATM and ATR (Yang et al. 2004). In yeast, the MRX (Mre11, Rad50 and Xrs2) and Ku (Ku70 and Ku80) complexes are recruited to the site of damage, which leads to the activation of Tel1 by MRX. Tel1 and Mec1, the ATM/ATR homologs in yeast (Craven et al. 2002), mediate a cell cycle arrest that usually persists until recovery, when the damage is repaired either by a fusion event via non-homologous end joining (NHEJ) or by homology directed repair. NHEJ predominantly occurs during G1 phase, whereas during S/G2 phases, when a homologous chromosome is available, the HR dependent repair pathway is favored (Teixeira 2013). As part of the HR pathway the 5' ends get extensively resected by MRX as well as other nucleases including: Sgs1, Dna2 and Exo1. The remaining long 3' ssDNA overhang can then be bound by RPA, recruiting the HR proteins Rad51 and Rad52 and promoting the checkpoint activation (Teixeira 2013) followed by further repair events i.e. strand invasion.
Since chromosome ends structurally resemble one half of a DSB, they have to be protected from such end processing and from DNA repair that would lead to fusion events with other free DNA

ends. Chromosome end fusions would have deleterious effects for the cell in upcoming mitosis since dicentric chromosomes would arise, leading to genome instability (de Lange 2009). Telomeres are the cellular measure to prevent such events.

Specific telomere binding protein complexes have been shown to be essential to overcome the "end protection problem" at linear chromosome ends (see Figure 2). These complexes include the human "shelterin" complex (Palm and de Lange 2008) and the conserved CST trimer (Grandin et al. 2001). Indeed, theses complexes have been demonstrated to 1. minimize nuclease access to the telomeres, 2. directly inhibit NHEJ events and 3. prevent checkpoint activation and hence senescence (Bonetti et al. 2010; Anbalagan et al. 2011). The shelterin complex found in vertebrates consists of six subunits, TRF1, TRF2, TIN2, RAP1, TPP1 and POT1. TRF1 and TRF2 directly bind ds telomeric sequences (see Figure 2). Both recruit TIN2, which itself binds to the heterodimer of TPP1 and ss telomere binding protein POT1. RAP1 is exclusively recruited by TRF2 and has no DNA binding activity (Palm and de Lange 2008). Shelterin also regulates telomerase and deletion of TRF1 results in telomere elongation while TPP1-POT1 seem to stimulate telomerase (Zaug et al. 2010). The shelterin complex is furthermore essential to prevent a DNA damage response at mammalian telomeres. TRF2 represses ATM-dependent signaling and classical NHEJ, which would lead to telomere end-to-end fusions (Capper et al. 2007). TRF1 prevents replication fork stalling in telomeric DNA and represses the accompanying telomere fragility (Sfeir et al. 2009). POT1 prevents ATR kinase signaling at telomeres by preventing the binding of RPA to the ss telomeric repeats, especially in G2, when ss telomeric DNA levels are increased (Hockemeyer et al. 2006). POT1 is furthermore required for the correct formation of the 3' telomeric overhang after replication (Churikov et al. 2006). In addition, telomere binding proteins prevent homologous recombination at the telomeres (Wu et al. 2006). Consistent with its essential functions at the telomere, the disruption of shelterin leads to p53-dependent senescence.

In analogy to the shelterin complex the double-stranded region of yeast telomeres is bound by Rap1 (see Figure 2) (Conrad et al. 1990) and associated proteins. The single-stranded overhang region is bound by the CST complex, similar to its homolog in human cells in a replication protein A–like (RPA) manner (see Figure 2) (Gao et al. 2007). Yeast CST consists of the three essential proteins Cdc13, Stn1 and Ten1 (Grandin et al. 2001). In human cells, Cdc13 is replaced by CTC1

(see Figure 2) and functions of the complex seem to differ. Cdc13 coordinates the C-strand fill in after replication (Qi and Zakian 2000). While Cdc13 interacts with Est1 to recruit telomerase in yeast (Nugent et al. 1996), the CST complex has also been shown to negatively regulate telomere elongation (Hang et al. 2011). CST furthermore protects the telomere from degradation by exonucleases like Exo1 (Nugent et al. 1996) that would otherwise lead to RPA bound ssDNA, a DNA damage response and Mec1-dependent checkpoint activation (Maringele and Lydall 2002; Vodenicharov and Wellinger 2006). In addition, CST might directly compete with RPA binding (Wellinger and Zakian 2012).

Rap1 can be bound by Rif1 and Rif2 (Wotton and Shore 1997), which are involved in telomere length regulation (see Figure 2) (Marcand et al. 1997) and form a shelterin-like complex. Together with Rif2, Rap1 protects the telomere by inhibiting the recruitment of the MRX nuclease complex and RPA, and thereby preventing Mec1-dependent checkpoint activation and end-to-end fusions (Pardo and Marcand 2005; Bonetti et al. 2010). Alternatively, Rap1 can be bound by the Sir2/3/4 complex (Cockell et al. 1995), which has a histone deacetylase activity to promote the heterochromatic nature of the telomeres. A further complex that binds and protects telomeres is the Ku complex, consisting of Ku70 and Ku80 (Feldmann and Winnacker 1993; Polotnianka et al. 1998). This complex also localizes to DNA ends at DSBs to promote repair by NHEJ. At telomeres, however, it has different functions. Here it affects telomerase and telomere maintenance as well as replication (Bertuch and Lundblad 2003; Fisher et al. 2004). Interestingly, it has been shown that the CST complex is essential during S phase when telomeric overhangs are long and exonucleases are predominantly active. During the remaining cell cycle, Rap1 with Rif1 and Rif2 as well as the Ku complex are necessary for telomere protection (Vodenicharov et al. 2010).

In human cells critically short and dysfunctional telomeres cannot maintain their protective function, leading to ATM- and ATR-dependent checkpoint responses and the formation of telomere-induced foci (TIF). After activation of CHK1 and CHK2 kinases, p53 is phosphorylated and p21 kinase gets expressed, leading to a cell cycle arrest in G1 (Teixeira 2013). Interestingly, such telomeres experience a 53BP1 mediated increase in mobility that promotes NHEJ (Dimitrova et al. 2008).

In yeast, a similar DDR is activated by critically short telomeres. During replication, MRX and Tel1 associate with short telomeres, leading to resection of the 5’ end by the endonuclease Sae2, the

helicase Sgs1, the helicase/nuclease Dna2 and exonuclease Exo1. But Cdc13, a telomere specific protein that binds the 3' overhang, blocks recruitment of RPA and further activation of Mec1. Instead of repair by HR, telomerase mediated telomere elongation is promoted. In a telomerase negative background, yeast cells with critically short telomeres accumulate in G2/M. The checkpoint is similar to a DSB as the same protein factors are necessary. Uncapped telomeres also elicit a checkpoint arrest in G2/M phases, however the response is different to eroded telomeres.

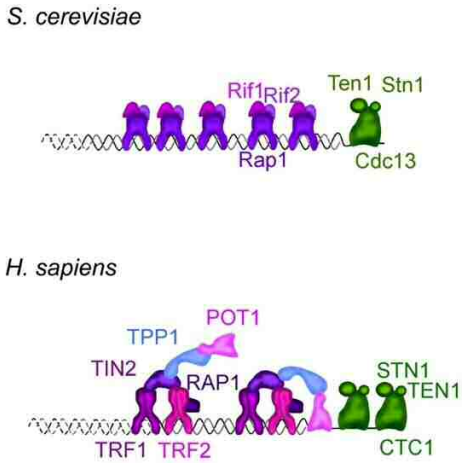

Figure 2: Telomere binding proteins
(Adapted from (Teixeira 2013)) Telomere binding proteins in *S. cerevisiae* and *H. sapiens.* See text for details.

1.1.2.2 Telomere looping

Apart from telomere binding proteins, another (less characterized) factor that may contribute to end protection is a lariat-like telomere loop structure that has first been demonstrated by electron microscopy in mammalian cells (Figure 3A) (Griffith et al. 1999; Doksani et al. 2013). It is formed at the very end of the chromosome and might be important to hide the free DNA end from being recognized by the DNA damage response machinery. So far it has been shown in a variety of species such as mammals, *Trypanosoma brucei*, garden pea or *Caenorhabditis elegans* (Griffith et al. 1999; Munoz-Jordan et al. 2001; Cesare et al. 2003; Raices et al. 2008). The loop presumably participates in telomere maintenance, since its loss appears to be correlated with rapid senescence and telomere dysfunction (Poschke et al. 2012). However, failure to resolve t-loops can also lead to t-loop sized telomere deletions (Wang et al. 2004; Sarek et al. 2015).

In higher eukaryotes the so called t-loop is formed exclusively by telomeric repeats and TRF2 was sufficient to induce *in vitro* loop formation of an artificial telomeric sequence (Griffith et al. 1999). At the junction of the t-loop the 3' telomeric overhang seems to invade the double-stranded

telomeric repeats to form a so-called displacement loop (D-loop; see Figure 3B) that leads to the formation of ssDNA at the junction of the loop. This is supported by *in vitro* studies that could detect binding of p53, which has a preference for Holliday junctions and ssDNA (Stansel et al. 2002), as well as the *Escherichia coli* (*E.coli*) single-stranded DNA binding protein (SSB) to the junction of the loop (Griffith et al. 1999). Consistently, the single-stranded overhang was required for *in vitro* t-loop formation (Griffith et al. 1999). TRF2 was shown to assist the strand invasion and TRF1 might additionally promote the fold-back by bending the DNA (Bianchi et al. 1997). In the fission yeast *Schizosaccharomyces pombe* the telomeric protein Taz1 (the TRF2 orthologue) has also been demonstrated to remodel telomeric model DNA of 518 bp into loops (Tomaska et al. 2004). Importantly, loop formation was dependent on a telomeric overhang, suggesting that a homology directed strand invasion was required and promoted by Taz1.

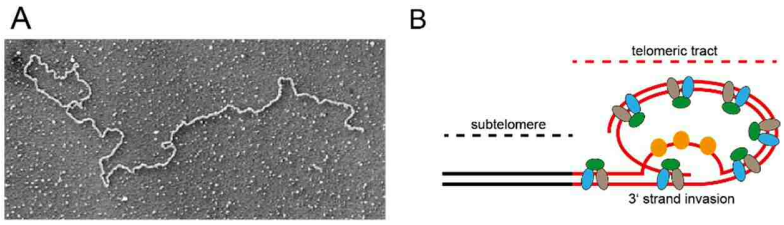

Figure 3: Telomere looping
A) Electron microscopy of t-loops from HeLa cells (adapted from (Griffith et al. 1999)). B) Scheme of a human telomere forming a t-loop where telomeric proteins (blue, grey and green) are required to form a fold-back structure within the telomeric tract (red). The 3' overhang invades the double-stranded telomeric repeats forming a displacement loop that is bound by ssDNA (orange) binding proteins.

In budding yeast telomere looping has been shown via a chromatin immunoprecipitation (ChIP) -based approach and a genetic construct (also see section 1.4) since the prerequisites for microscopic detection are not met (Strahl-Bolsinger et al. 1997; de Bruin et al. 2000). On the one hand the sequence of yeast telomeres does not contain the TA-step that is necessary for psoralen-mediated DNA crosslinking to stabilize the loop. On the other hand yeast telomeres are difficult to isolate and image due to their short length. The heterogeneity of telomeric repeats in budding yeast (Wang and Zakian 1990) indicates that homology directed displacement loops within the telomeric tract might be difficult to form. Consistently, experimental evidence suggests that yeast telomeres loop back to a subtelomeric portion of the chromosome since telomere binding proteins could coimmunoprecipitate subtelomeric DNA (Poschke et al. 2012), rendering yeast telomere loops different to a classical t-loop. Furthermore, a TRF-like protein that seems to be

required for t-loop formation has not been identified in yeast so far. Interestingly, the optimal length for protein-free DNA-loops appears to be 400 bp or higher (Shimada and Yamakawa 1984; Amouyal 2014), which is in support of a fold-back structure into the subtelomere, taking into account a yeast telomere length of 250-350 bp. However, DNA binding proteins are able to increase DNA bendability (Perez et al. 2014).

Pryde and Louis (1999) speculate that the subtelomeric X element might be involved in loop formation (Pryde and Louis 1999). Interestingly, both TAS contain mammalian telomeric or telomere-like repeats that are potential binding sites for the transcription factor Tbf1, whose sequence is related to that of the human telomeric proteins TRF1 and TRF2 (Koering et al. 2000). As of yet, mechanisms for the maintenance of the loop structure in yeast are unknown. They may be maintained by protein-protein interactions (de Bruin et al. 2000) or strand invasion, as shown for human cells. A genetic screen using the viable yeast deletion collection (containing 4921 strains) to find mutants that were defective in forming a fold-back structure at their telomeres, performed in our lab, identified 112 non-essential genes that were required, to various extents, for telomere fold-back formation/maintenance (Poschke et al. 2012). Amongst the most highly represented protein families histone deacetylases appeared. A ChIP based assay could confirm the telomere looping defect in mutants of both the Sir2 and Rpd3 histone deacetylase complexes (Poschke et al. 2012), providing a direct link between histone modifications or heterochromatin and telomere looping. Also in *Drosophila* a link between telomere protection and histone deacetylases (HDAC) could be demonstrated, since loss of the Rpd3 complex was accompanied by increased telomere fusions (Burgio et al. 2011).

Interestingly, the telomeric G4-quadruplexes (Paeschke et al. 2011; Bochman et al. 2012) have been speculated to additionally protect telomeres.

1.1.3 The end replication problem and telomere maintenance

Replication of yeast telomeres has been shown to occur late in S phase (Raghuraman et al. 2001), which seems to be dependent on telomere binding proteins (Stevenson and Gottschling 1999). Additionally, the repetitive sequence as well as secondary structures at the telomere (also see 1.1.2) represent obstacles for the replication machinery. When it reaches the end of the C-rich leading strand, a blunt ended telomere is generated by polymerase ε. Tel1-dependent 5’ to 3’ end resection of the leading strand follows to generate the 3’ overhang. A C-strand fill-in reaction

ensures proper length of the overhang, resulting in a telomere shortening of 5-10 nucleotides with every round of DNA replication (Lingner et al. 1995; Soudet et al. 2014). Resection is mediated by redundant pathways involving Sae2, the MRX complex and Sgs1 (Wellinger and Zakian 2012). Replication of the lagging strand is discontinuous, with the last RNA primer binding to the very end of the lagging strand to allow replication start. Upon removal of the approximately 10 nt primer (by an unknown mechanism), the 3' overhang is reestablished without sequence loss (Soudet et al. 2014). Telomere shortening during replication is hence a leading strand problem and called the "end replication problem" of the chromosome.

To prevent loss of coding regions, telomeres represent a buffer that allows complete replication of the coding sequences until a critical telomere length has been reached. Critically short telomeres lose their protective function and become dysfunctional (see 1.1.2), triggering a DNA damage response accompanied by genomic instability (Hackett et al. 2001) as well as a permanent cell cycle arrest called senescence (Lundblad and Szostak 1989). Hence, in the absence of a telomere maintenance mechanism, telomere length can contribute to determining the lifetime of a cell, also called the Hayflick-limit (Hayflick 1965), which represents a potent tumor suppressor mechanism.

Nevertheless, cells have evolved strategies to overcome this limitation. Cells of renewal tissue and the majority of cancer cells (Shay and Bacchetti 1997), and importantly also yeast, solve this "end replication problem" by expressing the enzyme telomerase. Telomerase is a reverse transcriptase that uses its internal RNA component as a template to add terminal repeats to the 3' end of a telomere, thereby compensating for sequence loss and maintaining a constant telomere length (Greider and Blackburn 1985; Lingner et al. 1995; C.I.Nugent and V.Lundblad 1998; Teixeira et al. 2004; Hug and Lingner 2006). The ribonucleoprotein telomerase in yeast consists of a catalytic core containing the reverse transcriptase Est2 (TERT in humans) (Lingner et al. 1997) and the RNA template Tlc1 (TERC in humans) (Singer and Gottschling 1994). Furthermore, accessory factors Est1 and Est3 are involved in the formation of the active telomerase holoenzyme (Lendvay et al. 1996). Human telomerase additionally contains dyskerin (Cohen et al. 2007). To elongate telomeres, telomerase has to be recruited by telomere binding proteins. Human TPP1 has been shown to recruit telomerase via its OB-fold domain (Zhong et al. 2012), which is crucial for telomere length maintenance. In contrast, in yeast Cdc13 is responsible

for telomerase recruitment. Cdc13 interacts with the telomerase subunit Est1 to recruit telomerase to its site of action specifically in late S phase (Tseng et al. 2006). Consistently, telomerase activity could be shown to be restricted to late S phase (Gallardo et al. 2011). Interestingly, Cdc13 in association with its binding partners in the CST complex is also able to inhibit telomerase (Hang et al. 2011).

Since only short telomeres represent a threat to cell survival, there is no need to elongate all telomeres at once in each cell cycle. In fact, it has been demonstrated that telomerase preferentially elongates the shortest telomeres (Teixeira et al. 2004), probably due to a protein counting mechanism in which the decrease of binding sites at short telomeres causes reduced levels of telomere binding proteins. Consistently, reduced levels of Rap1 at short telomeres allow telomerase activity (Marcand et al. 1997), and the deletion of Rap1 binding proteins Rif1 and Rif2 also causes telomere elongation, resembling the short telomere phenotype (Wotton and Shore 1997). In the absence of telomerase, for example after deletion of the gene encoding for the catalytic subunit of telomerase (*EST2*), yeast cells undergo a constant, telomere length-independent but replication-dependent, telomere shortening (Marcand et al. 1999; Marcand et al. 2000). This shortening finally leads to a permanent cell cycle arrest and cellular senescence when telomeres are critically short; however, not all yeast cells remain arrested or senescent. In rare cases, so called survivors arise from telomerase negative senescing cells. Their formation is dependent on homologous recombination (HR) that mediates elongation preferentially of short telomeres (Fu et al. 2014) and leads to heterogeneous telomere length (Lundblad and Blackburn 1993).

Yeast survivors can occur in two types that are characterized by the proteins involved in survivor formation and the telomeric sequences that are amplified (Lundblad and Blackburn 1993; Teng and Zakian 1999). Type I survivors substantially amplify subtelomeric Y' repeats, whereas the TG repeats remain relatively short (Lundblad and Blackburn 1993). Type II survivors arise by amplification of the terminal TG repeats, resulting in exceptionally long telomeres. Survivor formation in both cases depends on the HR protein Rad52. However, the pathways to type I and type II are distinct. Type I survivors additionally depend on Rad51, Rad54, Rad57 and probably Rad55 (Le et al. 1999; Chen et al. 2001), whereas type II depend on the MRX complex, the helicase Sgs1, as well as Rad50 and Rad59 (Le et al. 1999; Teng and Zakian 1999; Chen et al. 2001; Fu et al.

2014). Pol32, a subunit of polymerase δ, is also necessary for survivor formation (Lydeard et al. 2007), suggesting that replication is involved in recombination-mediated telomere elongation. Cells do not form exclusively one type of survivor. Type I occurs more frequently, but it is not stable. In fact, type I can convert to type II survivors, which grow faster and may eventually take over liquid cultures (Teng and Zakian 1999). Interestingly, survivors are so frequent that spontaneous mutations cannot explain their occurrence.

A similar telomerase-independent process of telomere maintenance was also described in 10 - 15 % of human tumour cells and termed alternative lengthening of telomeres, ALT (Bryan et al. 1995; Bryan et al. 1997). ALT cells are also dependent on HR and share the heterogeneous telomere length of type II survivors. Interestingly, HR elsewhere in the genome is not elevated in ALT cells compared to telomerase positive cells, indicating specificity for telomeres (Bechter et al. 2003). The ALT phenotype does not seem to be a mutation, since back-crossing with telomerase positive cells resulted in loss of the ALT phenotype (Perrem et al. 1999). It possibly represents a general mechanism of telomere maintenance, since ALT has also been demonstrated in normal mammalian somatic cells (Neumann et al. 2013).

1.1.4 TPE and TPE-OLD

The telomere and its adjacent subtelomeric part is heterochromatic and represses the transcription of genes in its vicinity (called telomere position effect). Whereas the telomeric sequences in yeast are free of nucleosomes, the telomere structures can be influenced via post-translational modifications of histones located in the subtelomere. HDACs impede transcription by increasing the affinity between histones and the DNA and by facilitating methylation of deacetylated lysines, leading to formation of heterochromatin.

Telomeres have been shown to exert a repression on genes placed in the subtelomere adjacent to the telomeric repeats (Gottschling et al. 1990). This so-called telomere position effect (TPE) silences their expression and has been extensively studied in yeast. A variety of proteins are involved, including the SIR HDAC protein family, Rap1 and histones H3 and H4 directly associated with the telomeric regions (Aparicio et al. 1991; Liu et al. 1994; Cockell et al. 1995; Strahl-Bolsinger et al. 1997). Sir4 binds to telomeric Rap1 and recruits the HDAC Sir2 to initiate heterochromatin assembly by deacetylating histones 3 and 4 (Luo et al. 2002). This stimulates the spread of further Rap1-Sir complexes. Sas2, a histone acetylase (HAT), counteracts Sir2 to generate an acetylation

gradient that represents the boundary between active and inactive telomeric chromatin (Suka et al. 2002).

Initially, it had been proposed that SIR proteins spread into the subtelomere for 2 - 4 kb, and so would the heterochromatin. In this scenario silencing would decrease with increasing distance from the telomere (Gottschling et al. 1990). However, the corresponding studies used truncation mutants that lacked parts of the natural subtelomeric sequences. Later it could be shown that silencing at native telomeric ends is discontinuous (Pryde and Louis 1999), reaching a maximum in the subtelomeric core X element (Figure 1). Y' elements, on the other hand, turned out to be resistant to silencing, carrying more euchromatic marks then X elements (Zhu and Gustafsson 2009). The repressive nature is hence reestablished at the centromere proximal X element. Since Y' and other silencing affecting elements are only found in a subset of subtelomeres, the telomere position effect is largely heterogeneous. Importantly, induced transcription of the telomeric tract reduced silencing (Sandell et al. 1994) and increased histone acetylation (de Bruin et al. 2000). Since shortening of the transcribed telomere correlated with induced transcription, loss of silencing could be an effect of either the induced transcription or the shortening of the telomere. It has also been suggested that TPE depends on telomere looping. Upon induced transcription, accompanied by loss of silencing, telomere binding protein Rap1 was lost in the subtelomeric region. This was interpreted as a loss of telomere looping, a structure that was therefore speculated to initiate TPE (de Bruin et al. 2000).

Another phenomenon could be observed by Robin et al. (2014) in human myoblasts. They showed that telomeres could silence genes up to 10 Mb away from the telomeric tract by forming a chromatin loop to locate the telomere close to the regulated genes. They termed their finding telomere position effect over long distances (TPE-OLD). Furthermore, they could show that TPE-OLD is telomere length dependent, where short telomeres long before reaching critically short length already show reduced interaction with distant genes (Robin et al. 2014). Consistently, it was demonstrated that human cancer cell lines show altered gene expression profiles in response to telomere length changes (Hirashima et al. 2013). In contrast to previously published data, the reported genes were silenced upon telomere elongation. Ultimately, it has also been shown that the mediator complex (see section 1.2.2) is necessary for telomere silencing (Zhu et al. 2011).

1.1.5 Transcription at the telomere - TERRA

As described before, telomeres are heterochromatic and genes in their vicinity are silenced (also see section 1.1.4); nevertheless telomeres are transcribed into the telomeric repeat-containing RNA (TERRA) that belongs to the class of long non-coding RNAs (lncRNAs) (Azzalin et al. 2007; Luke et al. 2008). TERRA is strand-specific, G-rich, comprises of subtelomeric and telomeric sequences and is transcribed by RNA polymerase II towards the telomeric end (Azzalin et al. 2007; Luke et al. 2008; Schoeftner and Blasco 2008). Its length is very heterogeneous and reaches from 100 to >9000 bp in mammals (Azzalin et al. 2007) and an average length of 380 bp in *S. cerevisiae* (Luke et al. 2008). The majority of TERRA in yeast (Luke et al. 2008) but only 7 % in human cells is polyadenylated (Azzalin and Lingner 2008). Polyadenylation increases TERRAs half-life and in yeast is performed by the canonical polyadenylation polymerase Pap1 (Luke et al. 2008). Human TERRA contains a canonical 7-methylguanosine cap (Porro et al. 2010). TERRA can be found exclusively in the nucleus, where it exists as a free and a telomere associated fraction (Azzalin et al. 2007; Porro et al. 2010), the latter most likely via the formation of RNA:DNA-hybrids (Luke et al. 2008). In human cells it is the non-polyadenylated fraction that is associated with the chromatin (Porro et al. 2010). Transcriptional start sites (TSS) have been identified in the subtelomere of human and yeast cells (Nergadze et al. 2009; Pfeiffer and Lingner 2012). In yeast the TSS of TERRA has been mapped to the core X element in the 1L-subtelomere (Pfeiffer and Lingner 2012). Transcription of TERRA is cell cycle dependent with the lowest levels present in S phase (Porro et al. 2010), and can be influenced by the chromatin state of the subtelomere (Arnoult et al. 2012).

Different and sometimes contradictory roles have been addressed to TERRA. Levels differ in cell types and even depend on the differentiation status (Azzalin and Lingner 2008), indicating that TERRA has important functions in a cell and during cell development. As an ncRNA it might be involved in chromatin regulation at the telomere in analogy to Xist at the inactivated X-chromosome and other lncRNAs (Brockdorff and Duthie 1998; Deng et al. 2009). Furthermore, TERRA might directly inhibit telomerase. Endogenous TERRA is bound to telomerase in mammalian cell extracts (Redon et al. 2010), and in *in vitro* assays TERRA-mimicking oligonucleotides were shown to bind to telomerase RNA and inhibit telomerase activity (Schoeftner and Blasco 2008; Redon et al. 2010). In a *rat1-1* mutant, with reduced exonucleolytic

activity of the Rat1 exonuclease, TERRA has been shown to be upregulated and telomeres at the same time shorten in a telomerase dependent manner, suggesting a telomerase inhibiting function of TERRA *in vivo* (Luke et al. 2008). Interestingly, induction of TERRA transcription in yeast induced telomere shortening of the transcribed telomere in an Exo1- and replication-dependent manner (Sandell et al. 1994; Maicher et al. 2012; Pfeiffer and Lingner 2012), however, the shortening was independent of telomerase. Therefore *in vivo* TERRA likely does not regulate telomere length via telomerase inhibition. In agreement, an induced TERRA transcribing telomere could be efficiently elongated by telomerase (Farnung et al. 2012). The variety of TERRA binding proteins might alleviate TERRAs effect on telomerase, for example by sequestration of TERRA by hnRNPA1 (Lopez de Silanes et al. 2010; Redon et al. 2013). In contrast to shortening effects of TERRA, Cusanelli et al. (2013) demonstrated that yeast express high levels of TERRA at short telomeres, accumulating in foci that may recruit telomerase RNA in early S phase to form so called T-Recs. These TERRA-telomerase clusters are then recruited to the short telomere where TERRA was transcribed, probably to ensure the elongation of specifically short telomeres (Cusanelli et al. 2013). Additionally, the natural fluctuation in TERRA levels could be responsible for orchestrating the change in telomere binding proteins by providing an alternative binding partner. For example the switch between ss binding proteins POT1, RPA and hnRNPA1 during and after replication has been proposed to be TERRA regulated (Flynn et al. 2011).

It has been speculated that TERRA at telomeres can form RNA:DNA hybrids (Luke et al. 2008; Balk et al. 2013; Arora et al. 2014) that eventually form R-Loops, higher order chromatin structures consisting of the hybrid and the displaced DNA strand (Aguilera and Garcia-Muse 2012). The purpose of TERRA might be the formation of R-loops or hybrids that can influence different mechanisms at the telomere and have been shown to affect telomere length by telomerase inhibition (Luke et al. 2008) and also in an Exo1-dependent manner (Pfeiffer et al. 2013). RNase H depletion, which results in an upregulation of hybrid occurrence in yeast, led to a higher frequency of recombination at the telomere, leading to telomere elongation (Balk et al. 2013). Yeast type II survivor formation in a telomerase negative background has also been demonstrated to be connected to hybrids (Yu et al. 2014) as well as the recombinogenic nature of telomeres in ALT cancer cells (Arora et al. 2014). TERRA therefore seems to be involved in telomere

maintenance in the absence of telomerase probably by hybrid formation. Consistently, ALT cells show high levels of TERRA expression (Episkopou et al. 2014).

The conserved nature of TERRA implies that it has an important role to fulfill, however, it is also evident that upregulation of TERRA or defects in its displacement can have deleterious effects on chromatin integrity. Mutants of the non-sense mediated RNA decay machinery with upregulated TERRA foci at the telomere experience a rapid loss of entire telomeric tracts (Azzalin et al. 2007). Also the accumulation of hybrids at the telomere leads to fast telomere loss in the absence of telomerase or HR (Balk et al. 2013).

1.2 RNA Polymerase II transcription in yeast

1.2.1 A short overview – from the beginning to the end

Transcription by yeast RNA polymerase II (Pol II) is a topic that has been addressed in many reviews and publications and only a short overview including some relevant aspects can be given here. The first step of RNA Pol II transcription is the assembly of the preinitiation complex (PIC) at the promoter consisting of the polymerase itself, a set of general transcription factors (GTF; TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH) that load the polymerase onto the DNA, and probably the mediator complex (see section 1.2.2) (Sikorski and Buratowski 2009). The TATA-box binding protein (TBP), together with TBP-associated factors, make up the transcription factor II D (TFIID) that binds the DNA and interacts with TFIIB to position the transcription initiation complex. TFIIB acts as a bridge and physically interacts with Pol II (Kostrewa et al. 2009). Next, the transcription bubble forms and transcription gets initiated (Cheung et al. 2011). After initiation, a scaffold consisting of the mediator complex and a subset of the TFs remains at the promoter to facilitate the following transcription rounds. Yeast TFIIB could not be found in this remaining scaffold (Yudkovsky et al. 2000). The elongation step or the progression of the polymerase shows a characteristic phosphorylation profile of the C-terminal domain (CTD) of the Pol II subunit Rpb1. This profile has recently been studied in detail by different groups, and even though their interpretations differ slightly, the main concepts are similar (Kim et al. 2010; Mayer et al. 2010; Tietjen et al. 2010; Bataille et al. 2012). Via a variety of modifications, mainly phosphorylation, the CTD plays a pivotal role in the regulation of transcription initiation, elongation, mRNA processing and termination (Hsin and Manley 2012). Initially non-phosphorylated, serine 5 (Ser5) and probably also Ser7 of the CTD are phosphorylated by Kin28 in promoter regions to allow promoter escape and

transcription initiation (Komarnitsky 2000; Akhtar et al. 2009). Ser2 is phosphorylated mainly by Ctk1 but also Bur1, promoting transcription elongation (Cho et al. 2001). At the end of the transcription cycle, Ser5 and Ser7 are dephosphorylated by the phosphatase Ssu72 (Krishnamurthy et al. 2004; Bataille et al. 2012) and Ser2 by Fcp1 (Cho et al. 2001), preparing the polymerase for another round of transcription (Bataille et al. 2012). Ssu72, as a component of the cleavage and polyadenylation factor (CPF), interacts with and is stimulated by Pta1 (Dichtl et al. 2002; Ghazy et al. 2009). After dephosphorylation the polymerase either dis- and reassociates or it might directly be recycled to the promoter via gene looping (see section 1.3.1). But not only the polymerase is subjected to cotranscriptional modifications, also the transcript gets processed prior to termination. As the first step, mRNA undergoes 5' capping as soon as the 5' end is available. The involved capping enzymes are recruited by the phosphorylated CTD of Pol II (Suh et al. 2010); in fission yeast, this is the only essential purpose of Ser5 phosphorylation (Schwer and Shuman 2011). Upon detection of a cleavage and polyadenylation signal on the pre-mRNA transcript, the CPF and cleavage factor I (CFI) are responsible for endonucleolytic cleavage and polyadenylation of the nascent mRNA (Kim et al. 2004). Again, the responsible factors are recruited in a phosphorylation dependent manner by the CTD. It has been shown that the CTD with its phosphorylated Ser2 is bound by Pcf11, a subunit of the CPF (Meinhart and Cramer 2004). In many experiments it has been demonstrated that 3' end processing is also required for proper termination of transcription. Pcf11 has been shown to dismantle the Pol II/DNA/RNA complex *in vitro* (Zhang et al. 2005) and *pcf11* mutants caused termination defects (Sadowski et al. 2003). Interestingly, the same study showed an uncoupling of termination and cleavage, since a cleavage defective *pcf11* mutant was still able to properly terminate mRNA transcription (Sadowski et al. 2003). The fact that reverse characteristics have been published for snoRNAs, underlines the difficulties in deciphering the requirements for termination (Kim et al. 2006). However, several other 3' cleavage factors have been shown to be required for proper mRNA termination such as Rna14, Rna15 and Pcf11 (Birse et al. 1998), or Hrp1 (Greger et al. 2000). Polyadenylation factors are not involved, but a proper polyadenylation signal and other cis elements on the transcript are necessary for termination (Whitelaw and Proudfoot 1986; Logan et al. 1987; Russo and Sherman 1989). For the termination of at least specific mRNA transcripts, the Ser2 and Ser7 phosphatase Ssu72 is required (Ganem et al. 2003), and in some instances also Ref2 and Pta1 (Nedea et al.

2003). The CTD of Pol II is additionally required, probably as the organizing unit that for example directly interacts with Pcf11 (McCracken et al. 1997). Also required for termination is the 5' to 3' ssRNA exonuclease Rat1 in yeast (Kim et al. 2004). The so called torpedo model proposes that upon cleavage of the RNA, Rat1 degrades the downstream transcript and somehow signals the polymerase to terminate (Luo et al. 2006). The fact that a cleavage proficient *pcf11* mutant is termination defective might be explained by the interaction of Pcf11 and Rat1 that is necessary to recruit Rat1 to active genes.

1.2.2 Getting to know - The Mediator complex

The mediator complex is a conserved multiprotein complex consisting of up to 25 identified proteins in *S. cerevisiae* with a weight of over 1 MDa and a complex and dynamic architecture (Robinson et al. 2015). The mediator complex is subdivided into 4 modules, the head, middle, tail, and kinase or Cdk8 module Figure 4) (Dotson et al. 2000; Kang et al. 2001). The head module, and probably also the middle module, interact with Pol II, the tail module with transcription factors. The kinase module contains the components with enzymatic activity (Wang et al. 2014).

Mediator is a transcriptional coactivator and key regulator of RNA Pol II-dependent genes, and as such affects and coordinates many steps of transcription at many different genomic loci (Zhu et al. 2006). Mediator not only regulates mRNA-coding genes but also some Pol II transcribed ncRNAs (Thorsen et al. 2012), and more and more appears to be the central coordinator of transcription. It is implicated in the initiation of activator-dependent transcription and activator–independent basal transcription (Lacombe et al. 2013). Mediator promotes the establishment of the PIC (Baek et al. 2002; Esnault et al. 2008) and its interaction with Pol II (Imasaki et al. 2011). Another function of mediator is the actual transition to transcriptional elongation, which might be facilitated by dissociation of mediator and Pol II upon CTD phosphorylation (Donner et al. 2010). Mediator could also be demonstrated along the gene body and is involved in elongation (Zhu et al. 2006) as well as in termination (Mukundan and Ansari 2011) and reinitiation (Yudkovsky et al. 2000). It can also link transcription and DNA repair (Eyboulet et al. 2013) and functions have also been shown in development and cell cycle regulation (Szilagyi and Gustafsson 2013).

Initially, the mediator complex was identified as a mediator of transcription enhancement by activator proteins bound to distant enhancer elements (Kelleher et al. 1990; Flanagan et al. 1991).

Mediator bridges between activators at regulatory DNA elements and the transcription machinery at the promoter, integrating regulatory signals to either enhance or repress transcription. To perform its task, several mediator subunits are able to physically interact with the CTD of RNA Pol II (Nonet and Young 1989; Thompson et al. 1993), forming the Pol II holo-enzyme and recruiting the polymerase to the promoter. To communicate an enhancing effect of distant activators to the polymerase, long-range chromatin interactions, or chromatin loops have been identified that are promoted by mediator (Saramaki et al. 2009) (see section 1.3).

Even though mediator exerts its main activities at the promoter, it could also be detected at the terminator region of certain genes (Zhu et al. 2006), mediated by promoter terminator contacts, so-called gene loops (Mukundan and Ansari 2013). It could be demonstrated that mediator helps to recruit the CPF and CF1, explaining the termination defect in a *med18* mutant (Mukundan and Ansari 2011). The same mutant was shown to have a gene looping defect, indicating that mediator also affects short-range DNA conformation (see section 1.3). As noted before, mediator interacts with activators to enhance target gene transcription. In some cases an ncRNA species can represent the actual activator and interaction between ncRNA and mediator brings distant loci, where the RNA is transcribed, into proximity of the mediator bound promoter, to enhance or repress transcription (Lai et al. 2013).

Mediator not only binds to gene coding and enhancer regions, but has also been shown at centromeres and telomeres (Zhu et al. 2011; Peng and Zhou 2012; Thorsen et al. 2012). As identified in genome-wide screens, mediator mutations affect telomere length in *S. cerevisiae* (Suzuki and Nishizawa 1994; Askree et al. 2004), and also the heterochromatic nature of telomeres is under the control of mediator. Mediator mutations have been shown to lead to increased histone acetylation and derepression of subtelomeric genes (Suzuki and Nishizawa 1994; Zhu et al. 2011). In this context, mediator has an impact on the boundary between active and inactive chromatin by balancing the HDAC Sir2 and the HAT Sas2. Loss of mediator leads to increased Sas2 levels, which finally causes the increased acetylation (Zhu et al. 2011).

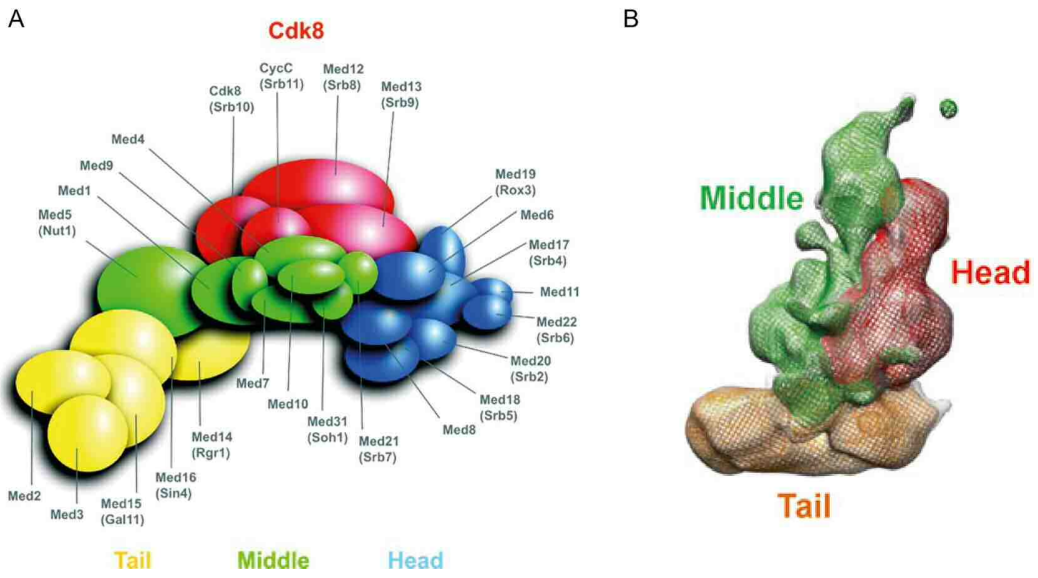

Figure 4: The mediator complex
A) Previously defined architecture of *S. cerevisiae* mediator with head (blue), middle (green), tail (yellow) and kinase (Cdk8, red) modules based on interaction studies (adapted from (Guglielmi et al. 2004). B) Redefined modular organization of the core yeast mediator complex consisting of middle, head and tail modules (adapted from (Wang et al. 2014))

1.3 A looped genome

Three-dimensional loop- or fold-back structures are not restricted to telomeres but can be found throughout the genome to bring distant regions on the linear genome into close physical proximity. They can occur via long-range inter- or intrachromosomal contacts, as in chromatin loops (e.g. enhancers and promoters; see section 1.3.2) (Marsman and Horsfield 2012), or short-range contacts, as in the case of gene loops (see section 1.3.1). They can regulate transcription of single genes, they can bring together or form clusters of similarly regulated genes (multigene loops or topologically associated domains - TADs) and they can influence chromatin conformation. Looped conformations have already been described in a variety of organisms, ranging from viruses (Perkins et al. 2008) and yeast cells (Ansari and Hampsey 2005; Duan et al. 2010), to fly (Cleard et al. 2006; Ahanger et al. 2013), mouse (Nemeth et al. 2008) or human cells (Osborne et al. 2004; O'Reilly and Greaves 2007). Looping structures have been demonstrated at many genes already, but are absent at others. Taken together, the results obtained so far indicate that gene- and chromatin loops have different regulatory roles in a variety of processes. Whether telomere looping shares aspects of these structures remains to be determined.

1.3.1 Gene loops

Gene loops juxtapose intragenic promoters and terminators (O'Sullivan et al. 2004; Tan-Wong et al. 2008), and have been associated with a variety of functions. They have been shown to confer transcriptional memory to some genes, where recent transcription is memorized by looping (Laine et al. 2009). Within a short-term repression period, the loop remains intact and retains the transcription machinery at the promoter region, allowing an efficient restart of gene expression (also called memory gene loops; MGL). Interestingly, these MGLs associate with the nuclear pore complex (NPC) via the nuclear envelope protein Mlp1 and even after repression they stay there for a period of time. In an *mlp1* strain, NPC-association and transcriptional memory are lost, gene loops disassemble after gene repression. Mlp1 just seems to stabilize the loops since a looping defective *sua7-1* mutant also loses memory even in presence of Mlp1 and NPC association (Tan-Wong et al. 2009). Gene looping has furthermore been associated with transcriptional bursts, where looped and non-looped states are part of a 2-state model in which one of them, likely the looped state, is the “on” state in which transcription occurs (Hebenstreit 2013). Instead of constant transcript levels, these bursts lead to a transcriptional noise that has been shown for a subset of genes in different species (Newman et al. 2006; Raj et al. 2006; Zenklusen et al. 2008; Suter et al. 2011). Leading to phenotypic variability in a cell population, transcriptional bursts likely confer an advantage in an ever-changing environment.

In addition, the recruitment of the chromatin remodeling enzyme Isw2 to non-canonical target sites was demonstrated to depend on gene looping (Yadon et al. 2013). The transcription factor (TF) Ume6 binds to its target site and recruits Isw2. By Ume6 dependent DNA looping, Isw2 comes also in proximity with the terminator region as well as other genomic loci, where it can exert its activity. In other words, gene and also chromatin loops indirectly affect the chromatin state of regions close by, mainly repressing transcription. Interestingly, formation of these loops is, like other gene loops, Sua7 dependent (Yadon et al. 2013).

Tan-Wong et al. (2012) further identified an enhancement of transcriptional directionality at bidirectional promoters via looping structures, thereby reducing ncRNA transcription and increasing mRNA level, probably by positioning and recycling RNA Pol II (Tan-Wong et al. 2012). The same group showed a repressing activity of a gene loop at the human and mouse BRCA2 gene (Tan-Wong et al. 2008).

The formation of gene loops in yeast requires at least one initial round of transcription, as well as an intact transcription and RNA processing machinery (Ansari and Hampsey 2005). Looping thus requires the phosphatase activity of Ssu72 (Pappas and Hampsey 2000; Ansari and Hampsey 2005) and Pta1 (Ghazy et al. 2009). Both are interaction partners (He et al. 2003) and components of the CPF (also see 1.2.1). Ssu72 dephosphorylates the hyperphosphorylated elongation competent form of RNA Pol II at Ser5 and participates in the formation of the hypophosphorylated and initiation competent form. Ssu72 can exhibit its CTD phosphatase activity only in association with Pta1 (Krishnamurthy et al. 2004), explaining the looping defect of a *pta1* mutant (Ansari and Hampsey 2005). Moreover, a transcription dependency of gene looping was demonstrated, since a temperature sensitive mutant of an RNA Pol II subunit, *rpb1-1* (Nonet et al. 1987), loses the fold-back conformation after 1 hr at non-permissive temperature (Ansari and Hampsey 2005). It has also been demonstrated that the mutant allele *sua7-1*, which encodes for the transcription factor IIB (TFIIB), is defective in gene looping (Singh and Hampsey 2007). *sua7-1* contains a glutamic acid to lysine replacement at position 62 (E62K) that does not affect transcript levels (Pinto et al. 1992) or PIC assembly (Cho and Buratowski 1999). A *sua7-1* mutant therefore affects gene looping independent of its role in transcription initiation. One explanation could be that TFIIB, even though part of the initiation complex, normally also interacts with RNA Pol II at the terminator. The affinity of this complex for the promoter bound scaffold could close the loop and bring the polymerase back to the promoter for another round of transcription. The mutated B finger of *sua7-1*, which was shown to interact with RNA Pol II (Bushnell et al. 2004), would explain the declined interaction at the terminator only, since *sua7-1* only led to diminished TFIIB-terminator crosslinking and not promoter crosslinking (Singh and Hampsey 2007). In this context, Ssu72 might be necessary to convert the polymerase to the hypophosphorylated initiation-competent form (Singh and Hampsey 2007). Also the inactivation of yeast kinase Kin28, involved in transcription initiation by phosphorylating Ser5 of the RNA Pol II CTD, also caused loss of gene looping (O'Sullivan et al. 2004). Interestingly, Kin28 as well as Sua7 have been shown to physically interact with Ssu72 (Wu et al. 1999; Ganem et al. 2003). In addition, transcriptional activators can affect looped conformations of yeast genes. The absence of the activators Met28, Ino2 or Gal4 led to loss of looping at their respective target genes. Even though they could not be shown to associate with the terminator region, and hence did not directly partake in the loop,

looping correlated with activation of the genes. In one of the studies it was furthermore shown that gene looping was also lost in an *rna15-2* mutant. Rna15, another component of the CFI, also physically interacts with TFIIB and at the non-permissive temperature *rna15-2* shows reduced transcript levels at the tested genes (El Kaderi et al. 2009). Rna15 was furthermore shown to interact with Sub1, a transcriptional coactivator involved in transcription initiation and termination (Calvo and Manley 2001) that also interacts with Pta1 (He et al. 2003). Later it was shown that yeast TFIIB also interacts with other subunits of CFI (Rna14, Rna15, Clp1, Hrp1 and Pcf11) and the poly (A) polymerase (Pap1), forming a holo-TFIIB complex that is devoid of other TFs or the RNA Pol II. In the gene looping defective *sua7-1* mutant, this complex cannot form and activated transcription exhibits a kinetic lag. Mutation of a subset of these interaction partners of TFIIB, namely in *rna14-1*, *pcf11-1* and *pap1-1*, also exhibited a gene looping defect (Medler et al. 2011). Interestingly, in mammalian cells interaction of TFIIB with terminator bound complexes with roles in cleavage and polyadenylation could also be shown (Wang et al. 2010). Perkins et al. (2008) found gene looping in the HIV-1 provirus in human cells. Interestingly, its formation could be shown to be dependent on the polyadenylation signal of the gene (Perkins et al. 2008).

It is not surprising that the mediator complex is also involved in gene looping, as it affects all main steps of transcription (see 1.2.2). Termination of transcription at a subset of genes is dependent on Med18. Consistently, *med18* mutants show defects in termination and interestingly also in gene looping at these genes (Mukundan and Ansari 2013).

A different aspect of gene looping in yeast was revealed by Posas and colleagues. Upon osmostress, the stress-activated protein kinase Hog1 activates several genes and also the transcription of lncRNAs (Proft et al. 2006; Nadal-Ribelles et al. 2014). Hog1 was found at the 3' region downstream of CDC28 and not at the 5' region. However, to exert its activating activity Hog1 needs to be close to the promoter region to recruit the RSC chromatin remodeler that finally leads to an increase in transcription (Mas et al. 2009). A gene loop was shown to provide this proximity. Interestingly, the loop, and hence Cdc28 induction, was dependent on an antisense transcript of *CDC28* as well as on Ssu72 and Sua7 (Nadal-Ribelles et al. 2014).

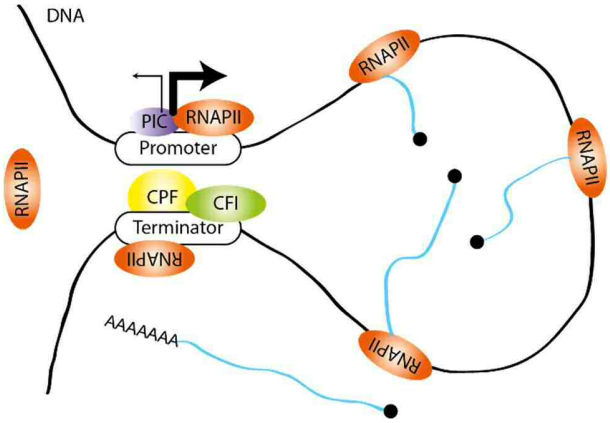

Figure 5: Gene looping
Scheme of a looping gene. Formation of such a gene loop depends on RNA polymerase II (RNAPII) transcription, a functional preinitiation complex (PIC), factors of the cleavage and polyadenylation factor (CPF) and cleavage factor I (CFI) and on a polyadenylation signal. It allows fast RNA polymerase II recycling from terminator to promoter and confers transcriptional directionality.

1.3.2 Chromatin loops

Chromatin loops describe the juxtaposition of two or several chromatin loci that do not belong to one gene. Screens using methods based on chromosome conformation capturing identified a plethora of different chromatin contacts (Sanyal et al. 2012; Jin et al. 2013; Rao et al. 2014) that can be mediated by a variety of protein factors. The contact map is dynamic, can be cell- (Phillips-Cremins et al. 2013) and context-specific as, for instance, it can change in response to certain signals like hormones (Hsu et al. 2010). One common type of chromatin loop brings distant enhancer/silencer regions close to promoters of positively or negatively regulated genes (Bulger and Groudine 2010; Krivega and Dean 2012). Transcription factors at enhancer regions associate with coactivators that bind and recruit the transcription machinery to the promoter region, closing a DNA loop structure (Vakoc et al. 2005). The yeast upstream activating sequences, functional equivalents to enhancers, seem to function in a similar manner by loop formation (Petrascheck et al. 2005). Chromatin loops can also influence a whole set of genes to ensure their coregulated transcription. By bringing a set of distant genes into close proximity, forming a multigene complex or topologically associated domain (TAD), a transcription factor can exert its effects on all genes at once. These hot-spots allow efficient and coordinated transcriptional control (Schoenfelder et al. 2010).

Chromatin looping has also been discussed as a mean to allow fast movement of chromatin binding proteins along the DNA, also termed intersegmental hopping (Pollak et al. 2014). Further, the formation of heterochromatic clusters also depends on looped chromatin (Miele et al. 2009). One of the coactivators identified in chromatin looping is the multi-subunit mediator complex, which is involved in the establishment as well as the maintenance of chromatin looping. Knockdowns of several components have been shown to exert a looping defect. *Med1* mutants have been shown to lose enhancer-promoter interactions at the mouse CREBP1 (Park et al. 2005) and also at the human p21 gene (Saramaki et al. 2009). In mouse embryonic stem (ES) cells, mediator has been demonstrated to establish chromatin looping with the help of cohesin, with which it was shown to physically interact. Consistently, a looping defect was shown in *med12* as well as in *smc1a* mutants, a cohesin component (Kagey et al. 2010). These interactions occurred over distances >100 kb, but also smaller chromatin loops have been identified (600-1000 bp) that are promoted by mediator and are independent of cohesin (Phillips-Cremins et al. 2013).

Another factor that is able to affect the chromatin structure is the vertebrate CCCTC-binding factor (CTCF) (Wallace and Felsenfeld 2007). CTCF is a protein responsible for transcriptional insulation, where it protects promoters from the effects of enhancers or silencers (Bell et al. 1999). CTCF furthermore functions at boundary elements that flank regions of different chromatin architecture. Rao et al. (2014) found CTCF and cohesin at 86 % of chromatin loop anchors of all loops they detected in the human genome (Rao et al. 2014). Many other proteins are involved in CTCF mediated looping, like for instance endonucleases of the nucleotide excision repair pathway (Le May et al. 2012). By applying ChIA-PET, a combination of ChIP and chromosome conformation capturing (3C), in mouse embryonic stem cells Handoko et al. (2011) analyzed the whole CTCF interactome and identified 5 different functional chromatin domains arranged in a looped conformation (Handoko et al. 2011). 12 % of the loops contained active genes whereas 11 % contained inactive genes and repressive chromatin marks. 19 % of the loops blocked spreading of Histone methylation marks and 31 % blocked activated from inactivated genes outside the loop. In 27 % of the loops no pattern could be identified. These studies emphasize the multitude of functions looped chromatin structures can exert.

Whereas loop formation in most cases depends on protein-protein interactions or in the case of mammalian t-loops on DNA-DNA contacts, another type of interaction has been revealed by Lai

et al. (2013). Before, a class of activating non-coding RNAs, termed ncRNA-a or enhancer RNAs (eRNA), transcribed at a distal locus, has been shown to activate neighboring protein coding genes like enhancers (De Santa et al. 2010; Kim et al. 2010; Orom et al. 2010; Yang et al. 2016), probably by chromatin loop formation (Pnueli et al. 2015). Whereas the exact mechanism was unclear before, Lai et al. detected an interaction of an ncRNA-a with mediator to recruit it to the target sites in an RNA-dependent manner increasing target gene transcription. A chromatin loop was demonstrated between the ncRNA-a locus and the target gene, which was dependent on both the RNA and the mediator complex. Consistently, depleting Med12 of mediator or expression of siRNAs against the ncRNA-a, led to diminished chromatin looping and decreased activation of ncRNA-a regulated genes (Lai et al. 2013). The ncRNA-a was furthermore shown to activate the histone H3 kinase activity of mediator (Lai et al. 2013). ncRNA-a species or eRNAs involved in chromatin architecture might be a general means to regulate gene expression and chromatin conformation as could be shown in a mouse model (Pefanis et al. 2015).

Importantly, telomeres can also be involved in chromatin looping. Using 3D-FISH (three-dimensional fluorescence in situ hybridization), it could be shown that human myoblast telomeres associate with chromosomal loci to regulate expression of genes up to several megabase pairs (Mb) away from the respective telomere (Robin et al. 2014). Interestingly, this phenomenon, termed TPE-OLD (also see section 1.1.4), was telomere length dependent.

1.4 Looking at loops – a comparison

To study a phenomenon like telomere looping in *S. cerevisiae* one needs assays that allow to look at or measure directly or indirectly the occurrence of the structure. The methods described below have been applied to study telomere loop or gene loop structures in different model organisms.

1.4.1 Microscopy

Griffith et al. (1999) were the first to use electron microscopy to look at looped telomeres in human and mouse cells (Griffith et al. 1999) (also see Figure 6A+B). Prior to imaging, isolated nuclei were psoralen and UV treated to introduce inter-strand crosslinks to avoid resolution of the telomeric loop structures. After endonuclease treatment of the chromatin, the large telomeric fragments can be separated from the smaller chromatin fragments by size exclusion, followed by electron microscopy. While being a direct way to look at mammalian t-loops, it is

highly laborious to look at a population of telomeres. Unfortunately, it is not feasible in *S. cerevisiae* since the telomeric sequence is missing the TA step, which is the substrate for psoralen crosslinking. Furthermore, yeast telomere restriction fragments are of similar size compared to other chromatin fragments and therefore difficult to isolate (de Lange 2004). Apart from showing t-loops in human and mouse cells, electron microscopy was also applied to detect telomere loops in *Trypanosoma brucei* (Munoz-Jordan et al. 2001), in *Pisum sativum* (Cesare et al. 2003) or in *Kluyveromyces lactis* with overelongated telomeres (Cesare et al. 2008). In *Oxytricha fallax*, t-loops could be shown via electron microscopy even without prior crosslinking (Murti and Prescott 1999).

Doksani et al. (2013) made use of another microscopic approach based on super-resolution fluorescence imaging, called STORM for stochastic optical reconstruction microscopy (Doksani et al. 2013; Benarroch-Popivker et al. 2016) (also see Figure 6C). By using a FISH probe that binds to the telomere, the STORM technique enabled them to visualize t–loops in mouse splenocytes and mouse embryonic fibroblasts (MEFs). For higher stability of the structure they also used psoralen and UV crosslinking.

Another microscopic approach to assess higher order telomere structures has been performed by Robin et al. (2014) in which 3D-FISH was used to show colocalization of a telomeric and a subtelomeric probe as far as 10 Mb away from the telomere (Robin et al. 2014) (also see Figure 6D). When the probes where detected in close proximity, it was proposed that this represents a long range telomere loop. These interactions over long distances correlated with a telomere position effect, prompting them to call their observation TPE-OLD (see section 1.1.4). Whereas this fold-back is not representing a t-loop, it remains to be determined if it is homologous to the telomere looping observed in yeast. This approach has been employed in many cases already to verify data obtained in screens using the 3C technique.

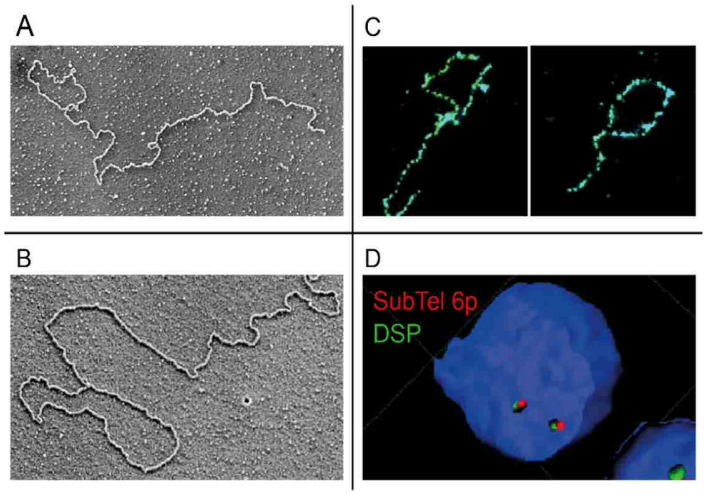

Figure 6: Different methods to visualize telomeric fold-back structures.
A+B) Electron microscopy of t-loops from HeLa cells (A) and mouse liver cells (B). (adapted from (Griffith et al. 1999)) C) STORM images of t-loops from MEF cells after chromatin spreading (adapted from (Doksani et al. 2013)) D) Confocal images of 3D-FISH showing colocalization of the fluorescence probe targeting the gene DSP in 7.5 Mb distance of its telomere 6p and the probe targeting the 6p subtelomere in 200 kb distance of the telomere 6p. (adapted from (Robin et al. 2014))

1.4.2 A genetic readout – "Construct2"

One way to study telomere looping in yeast is based on a transcriptional readout system (de Bruin et al. 2001). In this so-called construct2, a Gal4 activated upstream activating sequence (UAS) (Guarente et al. 1982) is positioned downstream of a *URA3* reporter gene (Figure 7). Whereas Gal4 fails to activate the *URA3* promoter when construct2 is positioned everywhere else in the yeast genome (Guarente and Hoar 1984), it is capable of activating *URA3* transcription when the whole construct is located at the telomere (de Bruin et al. 2001). The explanation for this phenomenon is telomere looping, where the UAS is looped back towards the *URA3* promoter to allow activation of the reporter in the presence of Gal4. Cells expressing *URA3* die on 5-FOA containing plates (Boeke et al. 1984), since it is converted to toxic metabolites, allowing a fast and easy readout for looped telomeres.

While offering a channel for large scale screening of yeast mutants that have a looping defect (Poschke et al. 2012), a genetic readout inherits some major drawbacks. As an indirect method, growth on 5-FOA containing medium could be due to other reasons, for example mutations affecting the 5-FOA metabolism pathways would lead to the detection of false positive looping mutants. More specifically, the phenomenon of transcriptional silencing at the telomere (TPE) or desilencing in specific mutants would provoke false positive or false negative hits, respectively. A

genetic readout might be a first approach to identify candidates, but verification should be performed in a more direct way (Poschke et al. 2012).

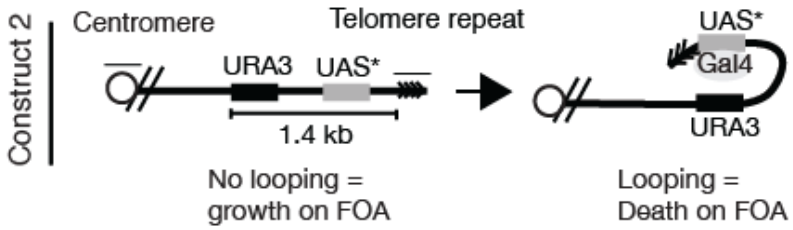

Figure 7: Scheme of construct2.
Only in the presence of Gal4 and in a looped conformation, expression of the reporter gene URA3 can occur. This expression allows a readout on 5-FOA containing plates, a drug that gets converted into a toxic reagent by URA3. (from (Poschke et al. 2012))

1.4.3 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) has been used to study the association of a variety of proteins like Sir-proteins or Rap1 with the telomere (Hecht et al. 1996; Strahl-Bolsinger et al. 1997). Rap1 DNA binding had been extensively studied, and was supposed to bind sequence-specific to telomeric repeats, not to the subtelomere (Shore and Nasmyth 1987; Shore et al. 1987; Buchman et al. 1988; Buchman et al. 1988). Hence, it was expected that only telomeric DNA would be precipitated after crosslinked chromatin was sheared to sizes of 0.5-1.0 kb (Conrad et al. 1990; Wright et al. 1992). Surprisingly, Rap1 also precipitated DNA of the subtelomere, and thus a fold-back structure bringing Rap1 in proximity to subtelomeric regions was assumed to be the cause (Strahl-Bolsinger et al. 1997; de Bruin et al. 2000). Later, this observation was used to control for telomere looping in yeast, where the loss of a Rap1 signal in the subtelomere indicated a looping defect (Poschke et al. 2012). The same ChIP setup was applied to a TAP-tagged Cdc13 protein, which binds to the 3' single-stranded overhang of telomeres. Cdc13-TAP was able to precipitate DNA 1 kb upstream of the telomeric tract, despite fragment sizes of about 300 bp after shearing, indicating telomere looping into the subtelomere. Of note, however, the major drawback of ChIP to look at telomere looping structures is its indirect nature. There is the potential for false positive results due to the relocalization of proteins used in the immunoprecipitation in different mutant backgrounds, something that is expected in most cases where ChIP is used. Rap1 signals in the subtelomere could be due to Rap1-Sir3 binding, and changes in Sir3 acetylation levels might lead to Rap1 dissociation, being interpreted as a looping defect. Cdc13 in the subtelomere might bind

to ssDNA that occurs where RNA:DNA hybrids are formed. Therefore, methods directly showing the interaction of two genomic loci, in other words telomere and subtelomere, should be preferred.

1.4.4 Chromosome Conformation Capturing (3C)

Chromosome conformation capturing (3C) is a method that was published in 2002 (Dekker et al. 2002). It allows the analysis of interaction frequencies between two distant genomic loci. Similar to microscopy, it is a direct method to measure chromatin looping. In contrast to the analysis of a single cell or a single DNA molecule, 3C however, allows analysis of a whole cell population. 3C is based on proximity ligation of DNA fragments. Proteins and DNA of a cell population are crosslinked by formaldehyde addition, resulting in a stabilized chromatin conformation and a snapshot of current interactions. In this way even distant DNA loci on the linear genome can be in close proximity of each other due to DNA folding. The fixed chromatin then gets digested by a restriction enzyme and afterwards the sticky ends get ligated in a highly diluted solution. The dilution ensures that only genomic fragments that are crosslinked can be ligated, in other words DNA fragments interconnected by proteins. The ligated products then represent the three-dimensional structure of the genome. PCR amplification over the ligated site can only happen upon ligation, and signal strength indicates the interaction frequency of the two loci tested. Therefore primer pairs have to be designed, near and towards the ends of the restriction sites to look at. The PCR readout can occur semi-quantitative by quantifying ethidium bromide incorporation into the DNA in an agarose gel (Dekker et al. 2002) or by quantitative PCR methods (Splinter et al. 2006). Since its development, 3C has been applied to the study of a plethora of chromatin interactions, be it gene loops or chromatin loops. However, 3C has not yet been used to analyze the telomere fold-back structure.

Up until now many variants of 3C have been developed that go beyond the analysis of two known loci. In the variants, including 4C, 5C, HiC or ChIA-PET, all cellular chromatin contacts or all contacts of one locus can be determined. Many screens have been published using these methods to allow a broad overview of genome contact maps in different species. For an overview of 3C and 3C derived variants see Figure 8 (also see (de Wit and de Laat 2012)).

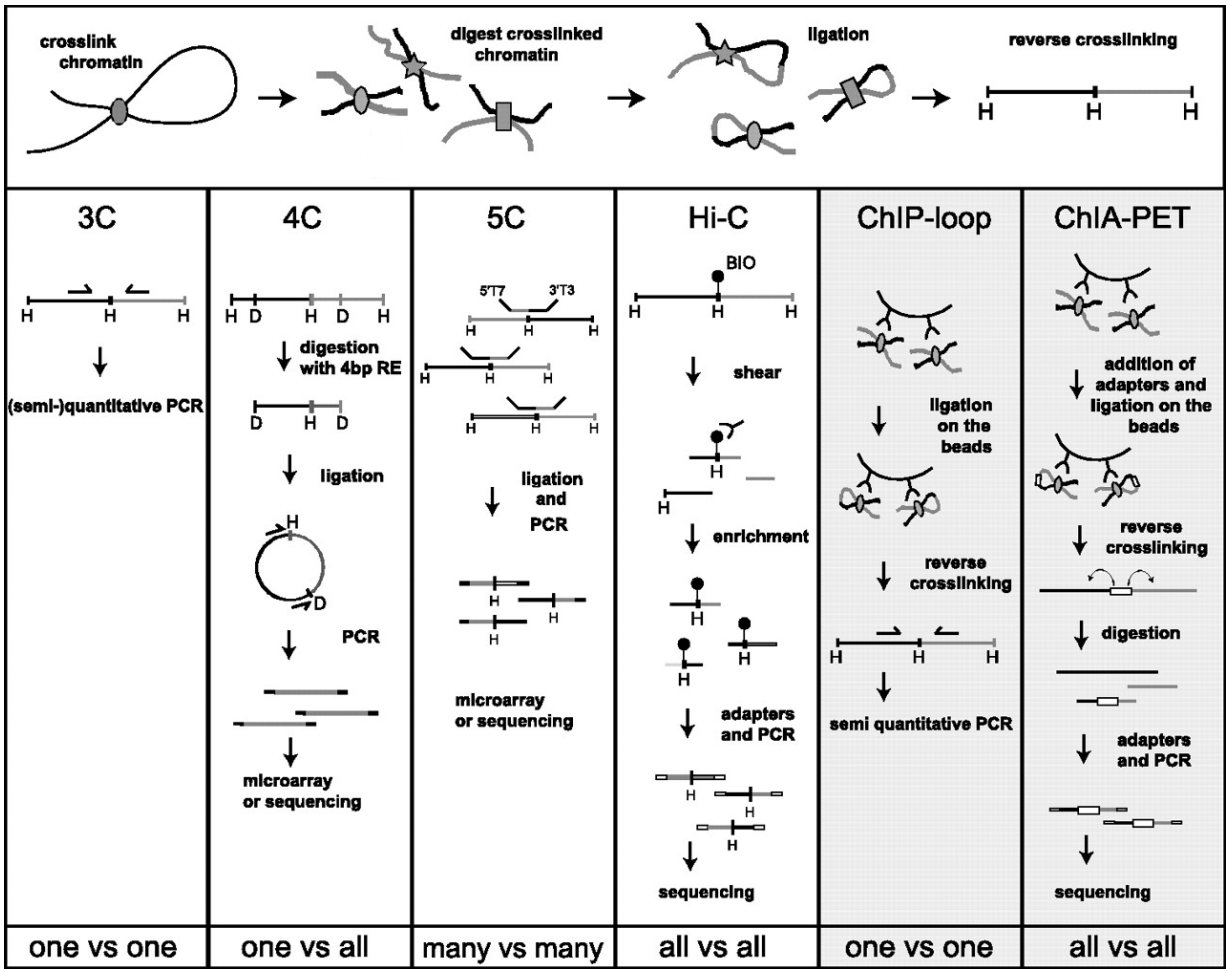

Figure 8: Chromosome conformation capturing (3C) and its variants
The upper panel describes the common steps to all 3C variants. Details to every single procedure is shown in the respective column. The lower panel names the characteristic of the respective variant, if a single locus or all possible genomic loci are measured against one or all possible loci. (adapted from (de Wit and de Laat 2012))

1.5 Aims of this study

Even though looping at mammalian telomeres has been identified via electron microscopy almost 15 years ago (Griffith et al. 1999) only little is known about these three-dimensional structures. In the following years, they were identified in several organisms, but owing to a lack of appropriate assays characterization of the loops progressed only slowly. Nevertheless, telomere loops seemed to be an inherent feature of telomeres and the goal of this study was to understand more about mechanistic and structural features of this telomere fold-back in the model organism budding yeast, *S. cerevisiae*.

We wanted to investigate the relationships between telomere looping and telomere length as well as the requirements and regulation of telomeric loops with a focus on the transcription

machinery and RNA species. Our lab identified 112 looping defective yeast mutants (Poschke et al. 2012) and taking their respective telomere lengths into account should give us an idea of a potential influence of the telomere length on telomere looping. By employing yeast mutants with either elongated or shortened telomeres, in the absence or presence of a catalytically inactive telomerase, we wanted to further elucidate the interdependence between length and looping and also assess the influence of telomerase.

Gene loops have been shown to be dependent on transcription and different components of complexes sitting at promoter and terminator regions (Singh and Hampsey 2007). A genetic screen of the viable yeast deletion collection performed in our lab (Poschke et al. 2012) already identified mutants of several components of the transcription and RNA processing machinery as looping defective (*set2*, *rpb9*, *ctk1*). By employing mutants of the transcription and RNA processing machinery we wanted to analyze the transcription dependency of telomere looping. Certain chromatin loops have been shown to depend on ncRNA and the mediator complex (Lai et al. 2013), and the genetic screen also identified components of the mediator complex (*nut1*, *med1*). So far no connection between telomere looping structures and transcription, mediator or RNA has been drawn. We would therefore like to understand if telomere loops have similar requirements as existing three-dimensional chromatin structures.

As a tool we wanted to establish a new assay based on the chromosome conformation capturing (3C) technique that we adapted for telomeric structures to measure the interaction frequency between the chromosome end and the respective subtelomere.

We also wanted to understand how telomeric loops in S. cerevisiae were maintained. *In vitro* studies suggest that in human cells the so called t-loops are dependent on homologous recombination (Verdun and Karlseder 2006) and form via DNA-DNA base pairing. By using mutants of the HR machinery, *rad51* and *rad52* strains, we wanted to assess if this holds true for *S. cerevisiae* as well.

Another interesting aspect was a more detailed view on the lncRNA TERRA with respect to telomere looping. Gene looping had been shown to reduce aberrant ncRNA species (Tan-Wong et al. 2012) and some telomere looping defective mutants had been shown to contain increased TERRA levels (de Bruin et al. 2001; Iglesias et al. 2011). This might suggest a possible negative effect of telomere looping on TERRA expression. On the other hand, as mentioned before, some

chromatin loops are dependent on certain ncRNA species that interact with the mediator complex (Lai et al. 2013). By affecting RNA/TERRA levels or by assessing TERRA level in looping defective mutants, we want to elucidate the effect of a loop structure on TERRA and vice versa to understand a potential relationship.

2 Materials and Methods

2.1 Materials

2.1.1 Yeast strains

(For the 112 looping defective strains see Supplemental table 1 8.Appendix)

Code	Name	Genotype	Source
yBL7	wild type	*MAT*a *his3 leu2 met15 ura3*	Euroscarf
yMD195	*sin3*	*MAT*a *his3 leu2 met15 ura3 sin3::KAN*	
yBL530	*sir2*	*MAT*a *his3 leu2 met15 ura3 sir2::KAN*	
Derived from yMD1243	*est2*	*his3 leu2 ura3 est2::NAT*	
yBL301	*rad51*	*MAT*a *his3 leu2 met15 ura3 rad51::KAN*	
yBL276	*rad52*	*MAT*a *his3 leu2 met15 ura3 rad52::KAN*	
yRS185	*sua7-1*	*MAT*α *cycl-5000 cyc7-67 ura3-52 leu2-3,112 cyh2* sua7-1	Gift from Eulalia de Nadal
yRS186	wild type of sua7-1	*MAT*α *cycl-5000 cyc7-67 ura3-52 leu2-3,112 cyh2*	Gift from Eulalia de Nadal
yBL37	*upf1*	*MAT*α *his3 leu2 ura3 upf1::KAN*	
yBL307	*ku70*	*MAT*a *his3 leu2 lys2 ura3 ku70::KAN*	
yAM408	*sum1*	*MAT*a *his3 leu2 met15 ura3 sum1::KAN*	
yRS218	*hrs1*	*MAT*α *his3 leu2 met15 ura3 hrs1::KAN*	
yBL312	*rad50*	*MAT*a *his3 leu2 lys2 ura3 rad50::KAN*	
yKB244	AFB2	*MAT*a *his3 leu2 lys2 ura3* AFB2	
yRS229	AFB2 *rpb1-AID*	*MAT*a *his3 leu2 lys2 ura3* AFB2 *rpb1*::*rpb1*-AID-FLAG(Hyg)	
yAM208	*bar1*	*MAT*a *his3 leu2 met15 ura3 bar1::KAN*	
yRS219	*tel1est2*	*his3 leu2 ura3 est2::NAT tel1::KAN*	

2.1.2 Plasmids

Code	Name	Plasmid	Source
pBL97	Empty vector	pRS316, *URA3*	Gift from Matthias Peter
pBL191	Cdc13-Est2-fusion	pRS415, *URA3*	Gift from Victoria Lundblad
pBL390	*EST2*	pSE358-*EST2*, *TRP1*	Gift from Mike Chang
pBL391	*est2*D670A	pSE358-est2D670A, *TRP1*	Gift from Mike Chang
pBL354	*EST2*	pRS416-*EST2*, *URA3*	Gift from Robert Knieß
pBL215	Empty vector	pVS45, *LEU2*	Gift from Mike Chang
pBL214	pGal-Pif1	pVS45-pGal-*PIF1*, *LEU2*	Gift from Mike Chang

2.1.3 Oligonucleotides

Code	Name/Description	Sequence	Experiment
oBL207	TERRA RT	CACCACACCCACACACCACACCCACA	Rev. transcription
oBL293	Actin RT	TTTGTTGGAAGGTAGTCAAAGAAGCC	Rev. transcription
oBL295	1L	CGGTGGGTGAGTGGTAGTAAGTAGA	qRT-PCR
oBL296	1L	ACCCTGTCCCATTCAACCATAC	qRT-PCR
oBL258	6R	GTGTGTAGTGATCCGAACTCA	qRT-PCR
oBL259	6R	GCATATTGATATGGCGTACGCACACGT	qRT-PCR
oLK57	15L	GGGTAACGAGTGGGGAGGTAA	qRT-PCR
oLK58	15L	CAACACTACCCTAATCTAACCCTGT	qRT-PCR
oLK49	6Y'	GGCTTGGAGGAGACGTACATG	qRT-PCR
oLK50	6Y'	CTCGCTGTCACTCCTTACCCG	qRT-PCR
oBL292	Actin	CCCAGGTATTGCCGAAAGAATGC	qRT-PCR
oBL293	Actin	TTTGTTGGAAGGTAGTCAAAGAAGCC	qRT-PCR
oBL359	polyG primer	CGGGATCCGGGGGGGGGGGGGGGGG	Telomere PCR
oBL358	Telomere 1L	GCGGTACCAGGGTTAGATTAGGGCTG	Telomere PCR
oAM26	Telomere 7L	CGGATCCCAGAGTAGAGGTAG	Telomere PCR
oBL361	Telomere 6Y'	TTAGGGCTATGTAGAAGTGCTG	Telomere PCR
oRS39	Loading control fwd	GGCTGTCAGAATATGGGGCCGTAGTA	3C qPCR
oRS40	Loading control rev	CACCCCGAAGCTGCTTTCACAATAC	3C qPCR
oRS64	P5	CCACCACCACATGCCATACT	3C qPCR
oRS66	P6	CCCGTTCGTAAAATTGGCGT	3C qPCR
oRS68	P7	AGGTACCCTGTTTGAACCAACT	3C qPCR
oRS79	P8	TATCGCTTCCGCCAAGATCC	3C qPCR
oRS80	P9	TCACATTGCGTCCAACTCCA	3C qPCR
oRS48	P1	TACTACCACTCACCCACCGT	3C
oRS46	P2	ACTCATGCGGGTGCTATGAT	3C
oRS44	P3	AGAACACTAACCCCTCAGCTT	3C
oRS42	P4	TTGCGAAGCCGCACATTTC	3C
oRS23	Gene loop *BLM10* fwd	TGCTATGTCCGAGTTCAATAG	Gene looping
oRS24	Gene loop *BLM10* rev	TGAAGCACAGCTACTGTTGG	Gene looping

2.1.4 Yeast media and plates

Agar-Plates (1L)	YPD	YPD + KAN	YPD + NAT	YPD + HYG
YPD Agar	65 g	65 g	65 g	65 g
dd H_2O	1 L	1 L	1 L	1 L
Autoclave	20 min 121°C	20 min 121°C	20 min 121°C	20 min 121°C
KAN (G418) 50 mg/ml		5 ml		
NAT 100 mg/ml			1 ml	
HYG 100 mg/ml				3 ml

Plates (1 L)	SD complete	SD –AA
Yeast Synthetic Dropout Medium Supplement without amino acids	1,92 g	1,92 g
Yeast Nitrogen base without amino acids	6,7 g	6,7 g
Agar	24 g	24 g
dd H_2O	950 ml	960 ml
100x Amino acids	10 ml	
Autoclave	20 min 121°C	20 min 121°C
50 % Glucose	40 ml	40 ml

Liquid medium (1 L)	SD complete	SD –AA
Yeast Synthetic Dropout Medium Supplement without amino acids	1,92 g	1,92 g
Yeast Nitrogen base without amino acids	6,7 g	6,7 g
dd H_2O	950 ml	960 ml
100x Amino acids	10 ml	
Autoclave	20 min 121°C	20 min 121°C
50 % Glucose	40 ml	40 ml

Plates (1 L)	Pre-Sporulation Plates
Standard nutrient broth	30 g
Yeast extract	10 g
Agar	20 g
dd H_2O	360 ml
Autoclave	20 min 121°C
50 % Glucose	100 ml

Liquid medium (100 ml)	Sporulation medium
Potassium acetate	1 g
Zinc acetate 5 mg/ml	1 ml
dd H_2O	100 ml
Autoclave	20 min 121°C

Liquid medium (1 L)	LB	LB carbenicillin
Yeast extract	5 g	5 g
Tryptone	10 g	10 g
NaCl	10 g	10 g
dd H_2O	1 L	1 L
Autoclave	20 min 121°C	20 min 121°C

Liquid medium (1 L)	LB	LB carbenicillin
Carbenicillin (100 ng/ml)		2 ml

Plates (1 L)	LB	LB ampicillin
LB agar	40 g	40 g
dd H_2O	1 L	1 L
Autoclave	20 min 121°C	20 min 121°C
Ampicillin		100 µg/ml

2.1.5 Buffers and stock solutions

10 x PBS (1 L)	
NaCl	80 g
KCl	2 g
Na_2HPO_4 – 7 x H_2O	26.8 g
KH_2PO_4	2.4 g
Adjust to pH 7.4	HCl
dd H_2O	1 L
Autoclave	20 min 121°C

10 x TBE (1 L)	
Tris base	108 g
EDTA (0.5 M), set to pH 8 with NaOH, autoclaved	20 ml
Boric acid	55 g
dd H_2O	1 L
Autoclave	20 min 121°C

TE-buffer (1 L)	
Tris base 1 M pH 8	100 ml
EDTA (0.5 M), set to pH 8 with NaOH, autoclaved	20 ml
dd H_2O	880 ml
Autoclave	20 min 121°C

AE-buffer (1 L)	
Sodium acetate anhydrous	4.1 g
EDTA	2.92 g
dd H_2O	1 L
Adjust to pH 5.3	NaOH
Autoclave	20 min 121°C

SDS	10 %	20 %
SDS	20 g	40 g
dd H_2O (sterile)	200 ml	200 ml
Sterilization	Millipore filter 0.22 µm	Millipore filter 0.22 µm

LiAc-Mix (100 ml)	
1 M LiAc (sterile)	10 ml
10 x TE (sterile)	10 ml
dd H_2O (sterile)	80 ml

PEG-Mix	
PEG 4000	40 g
LiAc-Mix (sterile)	100 ml
Autoclave	20 min 121°C

Solution 1 (20 ml)	
10 M NaOH	3.7 ml
β-Mercaptoethanol	1.52 ml
dd H_2O	14.78 ml

Solution 2 (20 ml)	
TCA (100 %)	10 ml
dd H_2O	10 ml

Solution 3 (20 ml)	
Acetone	20 ml

Urea loading buffer	
1 M Tris-HCl pH 6.8	1.2 ml (120 mM final)
70 % Glycerol	7.14 µl (5 % final)
Urea	4.8 g (8 M final)
β-Mercaptoethanol	100 µl (143 mM final)
20 % SDS	4 ml (8 % final)
dd H_2O	4 ml
Add Bromophenol blue	

10 x Telomere-PCR buffer (10 ml)	
Tris-HCl pH 8.8	810 mg (670 mM final)

10 x Telomere-PCR buffer (10 ml)	
$(NH_4)_2SO_4$	211 mg (160 mM final)
70 % Glycerol	7.1 ml (50 % final)
Tween 20	10 µl (0.1 % final)
dd H_2O	2.9 ml
Adjust to pH 8.8 with 37 % HCl	
Store 1 ml aliquots at -20°C	

10 x FA gel buffer (1 L)	
MOPS free acid	41.85 g (200 mM final)
NaAc	4.1 g (50 mM final)
EDTA	2.92 g (10 mM final)
Adjust to pH 7.0	NaOH

FA running buffer (1 L)	
10 x FA gel buffer	100 ml
37 % Formaldehyde	20 ml
dd H_2O	880 ml

5 x RNA loading buffer (10 ml)	
Saturated aqueous bromophenol blue solution	16 µl
EDTA (0.5 M), set to pH 8 with NaOH, autoclaved	80 µl (40 mM final)
37 % Formaldehyde	720 µl (2.664 % final)
100 % Glycerol	2 ml (20 % final)
Formamide	3.08 ml (30 % final)
10 x FA gel buffer	4 ml
RNase free dd H_2O	Add to 10 ml

FA lysis buffer (1 L)	-SOD	+SOD
HEPES-KOH pH 7.5	50 mM	50 mM
NaCl	140 mM	140 mM
EDTA (0.5 M), set to pH 8 with NaOH, autoclaved	1 mM	1 mM
Triton X-100	1 %	1 %
Sodium deoxycholate		0.1 %

Blocking solution (100 ml)	
1 x PBS	100 ml
Tween-20	100 µl (0.1 % final)
Skim milk powder	5 g (5 % final)

Washing solution (100 ml)	
1 x PBS	100 ml
Tween-20	100 µl (0.1 % final)

2.1.6 Other materials

Enzymes	Source
RNase A solution	AppliChem
Proteinase K	Qiagen
2x Q5 Mastermix	New England Biolabs
Q5 DNA Polymerase	New England Biolabs
Terminal Transferase	New England Biolabs
Zymolyase yeast lytic enzyme	New England Biolabs/ Zymo Research
2x Phusion HF Mastermix HF buffer	New England Biolabs
2x Phusion HF Mastermix GC buffer	New England Biolabs
Phusion HF DNA Polymerase	New England Biolabs
Calf Intestinal Alkaline Phosphatase (CIP)	New England Biolabs
DNaseI (RNase free)	New England Biolabs
SuperScript III Reverse Transcriptase	Invitrogen
T4 DNA Ligase	New England Biolabs
Restriction enzymes (DpnII, NcoI-HF)	New England Biolabs
LightCycler 480 SYBR Green I Master	Roche

Ladders	
1 kb DNA ladder	New England Biolabs
100 bp DNA ladder	New England Biolabs
ssRNA ladder	New England Biolabs
Prestained Protein Marker, Broad Range (7-175 kDa)	New England Biolabs

Kits	
Puregene Yeast/Bact. Kit B	Qiagen
QIAquick PCR purification Kit	Qiagen
RNeasy MinElute Cleanup Kit	Qiagen
Rnase free DNase Kit	Qiagen
Zero Blunt TOPO PCR Cloning Kit	Invitrogen
QIAquick Gel Extraction Kit	Qiagen
DyNAmo Flash SYBR Green qPCR Kit	Thermo Scientific
QIAprep Spin MiniPrep Kit	Qiagen
In-Fusion HD cloning Kit	Clontech

Primary antibodies	Source	Host	Concentration
α-Pgk1	Invitrogen (459250)	mouse (monoclonal)	1:20000
α-FLAG M2	Sigma Aldrich (F3165)	mouse	1:1000
α-Sic1	Gift from E. Schiebel	rabbit	1:2000

Secondary antibodies	Source	Host	Concentration
α-Mouse IRDye 800CW	LI-COR	Goat (polyclonal)	1:10000
α-rabbit	Bio-Rad	Goat (polyclonal)	1:3000

Additional materials	
0.1 M DTT	Invitrogen
5-FOA	Zymo Research
6x Loading buffer	New England Biolabs
Agarose	AppliChem
Bradford Solution	AppliChem
Bromophenol blue indicator	Sigma-Aldrich
CPD-Star	Roche
DMSO	Sigma-Aldrich
dNTPs	New England Biolabs
Ethanol	Sigma-Aldrich
Formaldehyde 37 %	AppliChem
Formamide	AppliChem
G418	Sigma-Aldrich
Glycine	Sigma-Aldrich
Glycogen	Roche
Hydroxyurea	Sigma-Aldrich
Light Cycler 480 Sealing Foil	Roche
Lysing matrix C tubes	MP Biomedicals
Mini Protean TGX Precast gels 4-15 %	Bio-Rad
Molecular grade water	Sigma-Aldrich
NEB buffer 2.1	New England Biolabs
NEB buffer 4	New England Biolabs
NEB cutsmart buffer	New England Biolabs
Nocodazole	AppliChem
Nourseothricin	WERNER BioAgents
Phase Lock Gel tubes	5 PRIME
Phenol (liquid)	Sigma-Aldrich
Phenol (solid)	Fluka
Phenol:Chloroform:Isoamyl alcohol	AppliChem
Ponceau S solution	Sigma-Aldrich
Protease Inhibitor Mix complete Mini tablet EDTA-free	Roche

Additional materials	
qPCR microwell plates 384 clear	Roche
qPCR microwell plates 384 white	Bio-Rad
Qubit HS reagent	Life Technologies
RedSafe™ Nucleic Acid Staining Solution	iNtRON-Biotechnology
RNaseOUT™	Invitrogen
SDS	Fluka
Sodium deoxycholate	Sigma-Aldrich
Super Signal West Pico Chemiluminescent Substrate	Thermo Scientific
SYTOX® Green	Life Technologies
TCA	Sigma-Aldrich
Trans-Blot nitrocellulose membrane	Bio-Rad
Triton X-100	Sigma-Aldrich
Tween-20	Sigma-Aldrich
Yeastmaker™ carrier DNA	Clontech
α-Factor	Zymo Research
β-Mercaptoethanol	Fluka

Electronic devices	
BD FACS Canto II	Becton Dickinson
BD FACS Verse	Becton Dickinson
Bioruptor Pico	Diagenode
Bioruptor Twin XD10	Diagenode
C1000 Touch ThermalCycler	Bio-Rad
Centrifuge 5417R	Eppendorf
Centrifuge 5810R	Eppendorf
Centrifuge Pico 17	Thermo Scientific
CFX384 Real-Time System	Bio-Rad
ChemiDoc Touch Imaging System	Bio-Rad
Cooling centrifuge 5430R	Eppendorf
dd H_2O machine Astacus	Membra pure
Dissection Microscope MSM 400	Singer Instruments
Dissection Microscope MSM manual	Singer Instruments
Excella E24 Incubator Shaker	New Brunswick Scientific
FastPrep24	MP Biomedicals
Hybridization oven MS incubator	UniEquip
Ice machine MF41	Scotsman
Ice machine Micro Cubes 120L	Wessamat
Incubator Heratherm IMC18	Thermo Scientific
Incubator Heratherm IMH60	Thermo Scientific
Incubator Shaker Infors HT Multitron	Infors
Incubators MIR154	Sanyo

Electronic devices	
LAS4000	Fujifilm
Leica DM1000 LED	Leica
LightCycler 480	Roche
Microfuge MCF-2360	LMS
Microscope	Optech Technology
Nanodrop 2000	Thermo Scientific
Nanodrop 2000C	Thermo Scientific
Odyssey	LI-COR Biosciences
Odyssey Clx 9140	LI-COR Biosciences
pH meter PB-11	Sartorius
Photometer Ultrospec 2100pro	Amersham Biosciences
Photometer Ultrospec 3100pro	Amersham Biosciences
PowerPac Basic	Bio-Rad
Qubit	Invitrogen by Life Technologies
Reinstwassersystem (dd H_2O) GenPure (TKA)	Thermo Fisher Scientific
Scale ED822-OCE	Sartorius
Termoblocks 2-2404	Neolab
Test Tube Rotator	Labinco
Thermocycler C1000	Bio-Rad
ThermoMixer f1.5	Eppendorf
Trans-Blot Turbo	Bio-Rad
Western Blot running chambers	Bio-Rad

Software	
Adobe Illustrator	Adobe
Adobe Photoshop	Adobe
End Note X5/X6	Thomson Reuters
FACS Diva™	Becton Dickinson
FileMaker Pro 10	FileMaker Inc.
FlowJo	Miltenyi Biotec
Gimp	The Gimp Team
Image Lab	BioRad
Image Studio Ver3.1	LI-COR Biosciences
Microsoft Office 2013/ for Mac (Word, Excel)	Microsoft
Multi-Gauge 3.2	Fujifilm
Prism 6	GraphPad
Serial Cloner	Serial basics

2.2 Methods

2.2.1 Yeast growth

Yeast cultures were grown in/on YPD at 30°C unless stated otherwise. Standard liquid cultures were incubated at 230 rpm using flasks without baffles.

2.2.2 Arrest of yeast cells in different cell cycle phases

An overnight culture of *bar1* cells was diluted to OD_{600} 0.1 (OD_{600} = optical density at 600 nm), grown to OD_{600} 0.5 and treated to arrest them at different cell cycle phases.

2.2.2.1 Arrest with α-Factor

To arrest the culture in G1, α-factor was added to a final concentration of 0.2 µg/ml. Cells were grown for 150 min and synchrony was monitored microscopically. After measuring the OD_{600} samples were taken for further experiments.

2.2.2.2 Arrest with nocodazole

To arrest the culture in G2, nocodazole was added to a final concentration of 15 µg/ml. Cells were grown for 150 min and synchrony was monitored microscopically. After measuring the OD_{600} samples were taken for further experiments.

2.2.2.3 Arrest with hydroxyurea

To arrest the culture in S phase, hydroxyurea was added to a final concentration of 0.2 M. Cells were grown for 150 min and synchrony was monitored microscopically. After measuring the OD_{600} samples were taken for further experiments.

2.2.3 Transformation of yeast

25 ml of an exponentially growing yeast culture (OD_{600} = 0.4 – 0.8) were pelleted at 3000 rpm at room temperature (RT) and resuspended in 5 ml LiAc-Mix. After pelleting again, the cells were resuspended in 250 µl LiAc-Mix and 100 µl of the cell suspension was used for transformation. 100 µg yeastmaker carrier DNA was added as well as 0.2 – 0.5 µg plasmid DNA, or 1-2 µg of a DNA fragment (either cut out of a plasmid by restriction digest or PCR amplified). After further addition of 700 µl PEG-Mix the cells were incubated at RT for 30 min on a rotating wheel. Cells were heat shocked at 42°C for 15 min. After pelleting the cells for 1 min at 3000 rpm at RT they were resuspended in 300 µl YPD medium. Cells were incubated at 30°C for 30 min in the case of plasmid

DNA and 120 min for integration and spread on appropriate selective medium using glass beads. Plates were incubated for approximately 2 days at 30°C.

2.2.4 Spotting experiments

An overnight culture of yeast cells was diluted to an OD_{600} of 0.5 in 100 µl H_2O. 10-fold serial dilutions in H_2O were prepared, and 5 µl of each was spotted onto appropriate agar plates and incubated at the indicated temperatures for up to three days.

2.2.5 Genomic DNA extraction

Genomic DNA was extracted following the protocol of the Puregene Yeast/Bact. B Kit (Qiagen).

2.2.6 Telomere length measurement by Telomere PCR

Employing terminal transferase to add a C-tail to the 3' overhang of the telomere allows us to use a G-rich primer together with a subtelomere-specific primer to amplify the telomeric repeats. On an agarose gel we are then able to compare telomere length of the mutants and the wild type. 150 ng genomic DNA in 9 µl 1 x NEB buffer 4 were denatured at 96°C for 10 min and rapidly cooled down to 4°C in a thermocycler. For the C-tailing reaction, 1 µl solution containing 0.4 Units terminal transferase in 1x NEB buffer 4 and 1 mM dCTPs was added and incubation for 30 min at 37°C, 10 min at 65°C and 5 min at 96°C followed. After cooling down to 65°C, 30 µl of preheated PCR-Mix (1 mM oligo-dG Primer, 1 mM telomere-specific forward primer, 0.267 µM dNTPs and 0.083 U/ml Phusion Q5 polymerase in PCR buffer containing 89.11 mM Tris-HCl at pH 8.8, 21.28 mM $(NH_4)_2SO_4$, 6.65 % glycerol, 0.0133 % Tween-20) were added. Amplification occurred with the following profile: 3 min 95°C; 45 cycles: 30 sec 95°C, 15 sec 63°C, 20 sec 72°C; 5 min 72°C; hold at 4°C. The samples were separated on a 1-1.5 % agarose gel prepared with TBE-buffer and containing 0.005 % RedSafe at 100 V for 30 min. Pictures were taken with either a LAS4000 or a ChemiDoc Touch Imaging System. Telomere length was analyzed with the help of MultiGauge or ImageLab and visualized in Prism. For the length of the 112 looping defective mutants compared to wild type, 8 independent wild type samples were measured and the sample representing the mean was run next to the mutant samples.

2.2.7 RNA extraction

10 - 15 ml of an exponentially growing yeast culture (OD_{600} = 0.4 – 0.8) was pelleted for 5 min at 3000 rpm and at 4°C and resuspended in 400 µl AE-buffer. 40 µl of 10 % SDS were added and the

tube was vortexed. 500 µl pre-equilibrated phenol was added and incubation for 5 min at 65°C followed. To equilibrate phenol, it was mixed with an equal volume of AE-buffer followed by inverting several times. The top aqueous phase was removed and the process was repeated another two times. The top phase was removed again and the pH checked (should be around 5.3). After incubation the samples were cooled for 5 min on ice and centrifuged for 5 min at 13000 rpm and at 4°C. The aqueous phase was transferred to a new reaction tube and 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) was added. After mixing and incubation for 5 min at RT the sample was centrifuged again for 5 min at 13000 rpm at RT. The aqueous phase was transferred to a new reaction tube and mixed with 40 µl 3 M Sodium acetate (pH 5.3). 1 ml 100 % ethanol (-20°C) was added and the sample was inverted and incubated for 20 min on ice. After pelleting the nucleic acids for 5 min at 13000 rpm at 4°C, they were washed with 1 ml 80 % ethanol (RT). The pellet was air dried for 10 min and resuspended in 87 µl RNase free water. DNA was digested by addition of 3 µl DNase and 10 µl RDD buffer of the RNase-free DNase Kit for 30 min at 37°C. 1 µl was measured at the Nanodrop. RNA samples were stored at -80°C.

2.2.8 TERRA purification

To purify TERRA RNA molecules the samples obtained with protocol 2.2.7 were further processed. 50 µg, assessed via Nanodrop, were purified with the RNeasy MinElute Cleanup Kit (Qiagen) according to the protocol but eluted with 38 µl RNase free dd H_2O.The samples were again DNase treated by adding 50 µl RNase free dd H_2O, 10 µl buffer RDD, 3 µl DNase and 3 µl RNaseOUT. After incubation for 30 min at 37°C another RNeasy Cleanup with elution in 38 µl followed. DNase treatment and Cleanup were repeated a third time and elution was done in 30 µl RNase free dd H_2O. Samples were stored at -80°C until gel analysis or quantitative RT-PCR.

2.2.9 Denaturing Agarose gel for RNA

RNA after RNase A treatment of spheroplasts was analyzed on either a non-denaturing 1.5 % agarose gel (also see 2.2.6) or on a denaturing agarose gel that helps to separate RNA species by size without the influence of RNA conformation. Therefore RNA samples in RNA loading buffer were denatured at 65°C for 5 min and cooled on ice before they were loaded on a 1.2 % agarose gel containing 0.0067 % formaldehyde and 0.005 % RedSafe™. The gel was equilibrated in FA

running buffer and RNA was separated for 90 min at 65 V. Pictures were taken with a ChemiDoc Touch Imaging System.

2.2.10 Reverse Transcription of RNA/TERRA

For reverse transcription 6 µl oligo-Mix (0.4 µl 25 mM dNTPs, 1.0 µl 10 µM oBL207, 0.4 ml 10 µM oBL293, 4.2 µl H_2O) was added to 7 µl purified RNA diluted to a total amount of 3 µg. The RNA was denatured for 1 min at 90°C and then quickly cooled down to 55°C (0.8°C/sec). 7 µl RT-Mix were added (1 µl 0.1 M DTT, 1 µl SuperSkript III Reverse Transcriptase, 1 µl RNaseOUT, 4 µl 5x first strand buffer) to reach a final volume of 20 µl. As a negative control the Reverse Transcriptase was replaced with water. Reverse transcription was performed for 60 min at 55°C and 15 min at 70°C. Samples were cooled to 4°C and 30 µl dd H_2O was added. cDNA was stored at -20°C.

2.2.11 Quantitative RT-PCR for TERRA levels

qRT-PCR was performed with the DyNAmo Flash SYBR Green qPCR Kit on a C1000 Touch ThermalCycler with a CFX384 Real-Time System. One reaction consisted of 2 µl dd H_2O, 1 µl of each primer (see Table 1), 1 µl of cDNA and 5 µl SYBR Green Mix. The DNA was denatured for 10 min at 95°C followed by quantification in 40 cycles of 15 sec 95°C, 1 min 60°C. ΔCt values were calculated as Ct_{TERRA}-Ct_{actin}. ΔΔCt = ΔCt_{mutant}-$\Delta Ct_{wild\ type}$. Fold enrichment compared to wild type was calculated as follows: $2^{-\Delta\Delta Ct}$.

Table 1 Primer sets used in quantitative RT-PCR

Target	Primer set	End concentration
TERRA Telomere 1L	oBL295 + oBL296	0.5 µM
TERRA Telomere 6R	oBL258 + oBL259	0.5 µM
TERRA Telomere 15L	oLK57 + oLK 58	0.1 µM
TERRA Telomeres 6Y'	oLK49 + oLK50	0.3 µM
Actin mRNA	oBL292 + oBL293	0.5 µM

2.2.12 DNA staining and flow cytometry

1 ml of an exponentially growing yeast culture (OD_{600} = 0.4 – 0.8) was pelleted for 5 min at 3000 rpm at RT and fixed in 1 ml cold 70 % ethanol. Cells were stored at 4°C or further processed by centrifugation for 5 min at 3000 rpm at RT. The pellet was resuspended in 1 ml H_2O, pelleted again and resuspended in 0.5 ml 50 mM Tris-HCl (pH 8.0) containing 0.2 mg/ml RNase A followed

by incubation for 3 hrs at 37°C. Cells were centrifuged for 5 min at 3000 rpm at RT and resuspended in 0.5 ml 50 mM Tris-HCl (pH 7.5) containing 1 mg/ml Proteinase K followed by incubation for 45 min at 50°C. Cells were again pelleted 5 min at 3000 rpm at RT and resuspended in 0.5 ml 50 mM Tris-HCl (pH 7.5). Either cells were stored at 4°C or 100 µl of the suspension was transferred to a new reaction tube and sonicated (20 sec at low intensity with Bioruptor Twin XD10 or 2x 10 sec and 30 sec in between with Bioruptor Pico). 50 µl of the cells were transferred into a FACS tube and DNA was stained by addition of 1 ml 50 mM Tris-HCl (pH 7.5) containing 1 µM SYTOX Green. Cells were kept in the dark and were immediately analyzed on a flow cytometer (either a BD FACS Canto II or a BD FACS Verse). FSC and SSC were detected with a 488 nm laser with detector setting of 370.9 V and 362.8 V, respectively. Slight adjustments were performed if necessary. SYTOX Green signal was detected with a 502 nm longpass filter and a 530/30 nm bandpass filter at 466 V when the FACS Canto II was used. In case of the BD FACS Verse, a 527/32 nm bandpass filter was used with a 507 nm longpass filter at 388 V. 20000 events were analyzed in each run. For analysis BD FACSDiva™ and FlowJo were used.

2.2.13 Protein extraction

For protein extraction 2 OD_{600} units of an exponentially growing yeast culture (OD_{600} = 0.4 – 0.8) were collected by centrifugation for 2 min at 13000 rpm at RT. The cell pellet was resuspended in 150 µl of solution 1 and incubated on ice for 10 min. 150 µl solution 2 was added and incubation for further 10 min on ice followed. Cells were centrifuged for 2 min at 13000 rpm at 4°C and the pellet resuspended in 1 ml solution 3. The samples were centrifuged again for 2 min at 13000 rpm at 4°C and the pellet resuspended in 100 µl urea buffer. After incubation for 5 min at 75°C the samples were stored at -20°C or loaded onto a Mini Protean TGX Precast Gel.

2.2.14 SDS-PAGE

For protein separation of samples obtained after protein extraction (2.2.13), samples were incubated for 5 min at 75°C and centrifuged for 10 min at 13000 rpm. 5-15 µl were loaded onto a Mini Protean TGX Precast Gel (4-15 % gradient). Separation occurred for approximately 2 hrs at 100 V at RT.

2.2.15 Western Blotting

Gels were blotted onto a nitrocellulose membrane using the high molecular weight program (HMW) of the Trans-Blot-Turbo system. Transfer and separation of proteins was visualized using Ponceau S solution and imaged at either a LAS4000 or a ChemiDoc Touch Imaging System. The membrane was blocked for 1 hr at RT in blocking solution. Incubation with the primary antibody diluted in blocking solution followed over night at 4°C. The membrane was washed four times for 15 min in washing solution and then incubated with the secondary antibody diluted in blocking solution at RT. Incubation occurred for 1 hr with enzyme coupled secondary antibodies and for 2 hrs in the dark with LI-COR secondary antibodies. The membrane was then washed three times for 15 min in washing solution and once in 1x PBS. If LI-COR antibodies were used, the membrane was kept in the dark during all steps. Horseradish peroxidase (HRP)-coupled antibodies were detected by incubating the membrane with the Super Signal West Pico Chemiluminescent Substrate (mixed 1:1) and imaged at a LAS4000. LI-COR fluorescent antibodies were detected at the Odyssey Clx 9140 system.

2.2.16 Chromosome conformation capturing (3C)

25 ml of an exponentially growing culture, adjusted to OD_{600} 0.4, was crosslinked for 20 min with 1.2 % formaldehyde (0.83 ml of a 37 % stock solution) on a rotating wheel at RT. The reaction was quenched for 5 min rotating at RT by the addition of 360 mM glycine. Cells were cooled on ice and washed twice with 20 ml 1x PBS. The cells were pelleted for 3 min at 3900 rpm at 4°C and resuspended in 200 µl FA-lysis buffer -SOD containing one tablet Protease Inhibitor Cocktail (Roche cOmplete™ Mini) per 10 ml buffer. Cell lysis was performed in Lysing matrix C tubes (MP Biomedicals) with a FastPrep®-24 instrument at 6.5 m/s for 2x 30 sec and 1 min on ice between runs. The cell extract was recovered with 800 µl FA-lysis buffer +SOD containing one tablet Protease Inhibitor Cocktail (Roche cOmplete™ Mini) per 10 ml buffer and pelleted for 15 min at 4°C at 13000 rpm. The pellet was washed with 500 µl FA-lysis buffer -SOD and centrifuged again for 5 min at 4°C. The extract was resuspended in 200 µl 20 mM Tris-HCl pH 7.5 and 20 µl were used for digestion with 100 units of restriction enzyme in a final volume of 100 µl. The digestion was performed over night at 37°C, shaking at 900 rpm. Digestion was stopped with 5 µl of 20 % SDS and incubation at 65°C for 20 min. 75 µl Triton X-100 was added, followed by addition of 570 µl 20 mM Tris-HCl pH 7.5. The chromatin was pelleted for 15 min at 13000 rpm at 4°C and

resuspended in 675 µl 20 mM Tris-HCl pH 7.5. For ligation, 75 µl T4 ligase buffer and 3000 units of T4 ligase were added and incubation for 4 - 5 hrs on a rotating wheel at RT followed. After treatment with 20 µg of DNase-free RNase for 20 min at 37°C, the chromatin was decrosslinked over night at 65°C in the presence of 150 µg Proteinase K and 0.1 % SDS. DNA was extracted using an equal volume of phenol:chloroform:isoamyl alcohol solution (25:24:1) and phase lock gel tubes for phase separation. The DNA was precipitated for 4 hrs at RT by addition of 1/10 volume 3 M KAc (75 µl), 2.5 volumes of 100 % ethanol (2 ml) and 40 µg of glycogen. The precipitated DNA was pelleted for 30 min at 13000 rpm at RT, washed once with 70 % ethanol and resuspended in 40 µl of DNase-free water. Concentration was determined using the Qubit® dsDNA HS Assay Kit and samples stored at -20°C.

2.2.17 Quantitative PCR on 3C samples

For quantification of the proximity ligation efficiency, 0.5 - 1 ng DNA template was used for real-time PCR employing a C1000 Touch Thermal Cycler with a CFX384™ Real_time System. 3 µl of DNA samples, adjusted to equal concentration were mixed with 5 µl of DyNAmo Flash SYBR Green qPCR 2x MasterMix and primers at final concentrations of 0.5 µM to reach a final volume of 10 µl. The cycling profile was as follows: 10 min 95°C; 40 cycles of 15 sec 95°C, 1 min 60°C; 5 min 95°C; 1 min 65°C; melting curve with a gradient from 65°C to 97°C with 0.5°C/cycle and a cycle length of 5 sec.

2.2.18 Statistical analysis

The significance of the relationship between looping defective mutants and telomere length maintenance mutants was assessed using the Fisher's Exact test function as provided by R version 2.15.3 (http://www.r-project.org/). For the evaluation of expected overlap sizes, 10000 randomized gene sets of 112 genes were generated and their overlap with the TLM mutant data evaluated using inbuilt R functions.

3 Results

3.1 Detecting telomere loops with 3C in yeast

The scope of this project was to better understand the mechanisms underlying telomere looping in the budding yeast *S. cerevisiae*. Whereas human and mouse t-loops can be visualized via electron- and super resolution-microscopy (Griffith et al. 1999; Doksani et al. 2013), the short length and base composition of yeast telomeres prevent such approaches. Genetic and chromatin immunoprecipitation (ChIP)-based experiments have been employed so far to study telomere looping in yeast (Strahl-Bolsinger et al. 1997; de Bruin et al. 2000; de Bruin et al. 2001; Poschke et al. 2012). However, both methods are indirect and unsatisfactory in terms of analyzing the dynamic regulation of loop structures.

We therefore set out to establish an assay based on chromosome conformation capturing, or 3C, to detect and quantify the interaction of telomeres and their respective subtelomere (Figure 9).

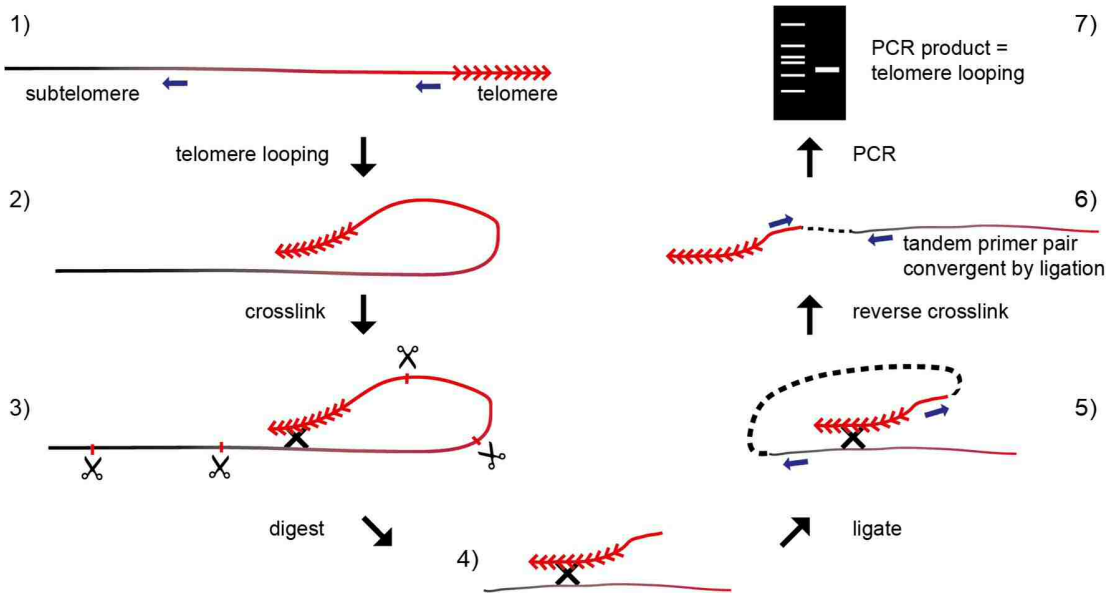

Figure 9: Scheme of telomeric 3C
The telomere and its subtelomere (1) form a telomere loop, bringing the telomeric sequences in close proximity to the subtelomeric DNA (2). This conformation gets crosslinked and stabilized by the addition of formaldehyde (3). The subtelomere gets digested by a restriction enzyme, resulting in a telomeric fragment interconnected with a subtelomeric fragment (4). Under very dilute conditions T4 ligase is employed to ligate the interconnected fragments (5). After decrosslinking at high temperature and in the presence of proteinase K a DNA stretch consisting of telomeric and subtelomeric DNA remains (6). The existence of this specific fragment can be detected by primer pairs that were in tandem orientation on the native genomic DNA (1) but that are now convergent upon ligation (6). The resulting amplicon allows quantification of the interaction frequency between the telomere and its subtelomere (7).

3.1.1 Telomeric 3C using DpnII

We could successfully reproduce a 3C signal at the *BLM10* locus (data not shown), which had previously been shown to form a gene loop (Singh and Hampsey 2007). We next scanned the *S. cerevisiae* subtelomeres for suitable restriction enzymes that would cut the subtelomeric DNA close to the telomeric tract and at a set of distal positions. We first identified DpnII as the restriction enzyme that would cut subtelomere 1L according to our prerequisites. DpnII digests the 1L subtelomere as indicated in Figure 10A, very close to the telomeric repeats as well as at further sites more distal to the telomere (additional sites more upstream are not shown). Upon formaldehyde treatment to crosslink and hence stabilize the three-dimensional chromatin conformation, we purified the DNA and digested it with DpnII (Also see section 2.2.16). After ligation in very dilute conditions and decrosslinking of the protein-DNA connections we used primer pairs that covered the expected ligation sites on telomere 1L to quantify the ligation frequency (Figure 10). As shown in Figure 10A these primers are in tandem configuration on native genomic DNA. Only upon digestion and ligation in the right configuration they can exponentially amplify a product. Other ligation products that will also arise, can only be covered by one primer, leading to linear amplification.

We could successfully amplify the expected DNA fragments (Figure 10B), which were verified by sequencing (data not shown). The signal that represents proximity ligation and hence telomere looping was obtained with all three primer sets consisting of P2, P3 or P4 together with the anchor primer P1 (Figure 10B). No product was obtained when we omitted any one of the steps outlined in Figure 9**,** indicating that the PCR product is crosslinking-, digestion- and ligation-dependent (Figure 10B). The loading control was an intergenic region on chromosome V that does not contain a DpnII restriction site and is therefore unaffected by digestion and ligation.

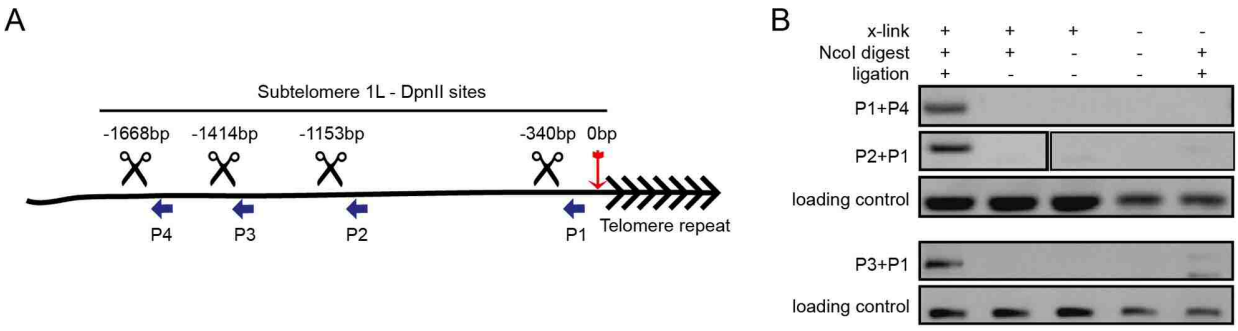

Figure 10: 3C at telomere 1L using DpnII
A) Scheme of DpnII restriction sites at telomere and subtelomere 1L and primers used for 3C. B) Agarose gel to analyze 1L telomere looping assessed via 3C. Crosslinked DNA was digested with DpnII. Different primer pairs are shown. Amplification is dependent on crosslinking, digest and ligation. The very right lane shows the random ligation control. Loading control is an intergenic region on chromosome V that does not contain DpnII sites.

3.1.2 Telomeric 3C using NcoI

Since DpnII cuts very frequently in the 1L subtelomere, several additional ligation products that arise due to incomplete restriction can be expected. In some cases our primer pairs also amplified larger products and several additional higher running bands appeared on the gel (data not shown). This fact impeded quantitative measurements employing qPCR and only allowed semi-quantitative densitometric in-gel analysis. We therefore set out to improve our setup and returned to the restriction enzyme selection. NcoI was chosen to fit our needs for quantitative measurements, with only few cutting sites in the 1L subtelomere (Figure 11A). Additional amplification products could thereby be eliminated. This setup, with primers flanking the NcoI restriction sites and P5 as the anchor primer (Figure 11A), allowed us not only to use in-gel quantification (Figure 11B+C) but also qPCR to detect the interaction frequency (Figure 11D). The PCR amplification product in Figure 11B was also digestion- and ligation-dependent. Most importantly, random ligation of non-crosslinked, NcoI digested genomic DNA did not lead to strong amplification of the expected telomeric 3C-product, even with high excess of template DNA (Figure 11C). These results demonstrate the applicability of telomeric 3C using NcoI for digestion. We went on and tested if qPCR was also feasible using the same primer pairs. Figure 11D shows that only samples that passed through the whole 3C process gave a proper Ct-value (cycle threshold when exponential increase in fluorescence is reached). The melting curve showed that only one amplification product was formed (data not shown). As a loading control the intergenic region on telomere V was quantified in the same qPCR analysis (Figure 11E). As

expected, similar Ct-values were obtained for the loading control, confirming equal amounts of DNA used in the reaction. To ensure that the signal we obtained was telomere specific we also used the anchor primer P5 with primers flanking NcoI restriction sites more distal to the telomere (Figure 11A; P7+P8+P9). We could detect a gradual decrease in amplification with increasing distance to the telomere, indicating that the signal was specific to telomere 1L (Figure 11F). Our results prompted us to use telomeric 3C for further analysis of telomere looping in *S. cerevisiae*.

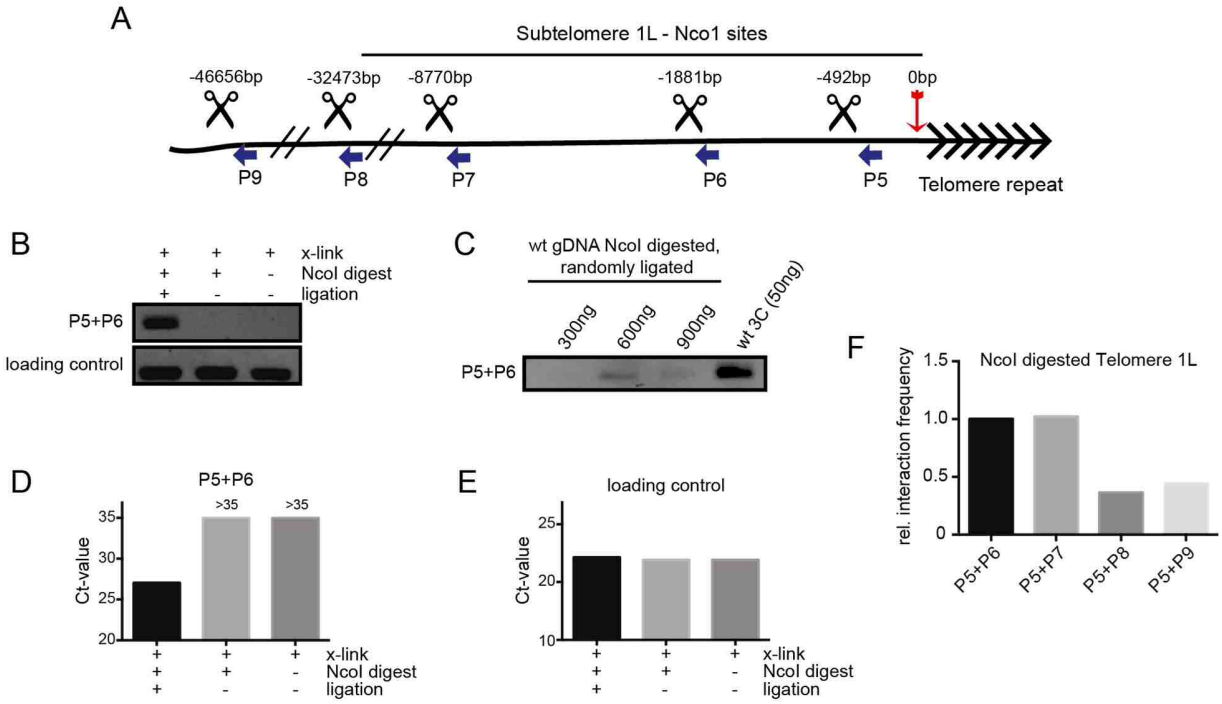

Figure 11: 3C at telomere 1L using NcoI
A) Scheme of NcoI restriction sites at telomere and subtelomere 1L and primers (P5-P9) used for 3C. B) DNA amplification with tandem primer pair P5 and P6 as a readout for proximity ligation and telomere looping and its dependency on digestion and ligation. As loading control an intergenic region on chromosome V was amplified using convergent primers (oRS39 and oRS40). C) Comparison of wild type 3C DNA cut with NcoI and the random ligation control of non-crosslinked, NcoI digested and randomly ligated wild type genomic DNA in excess. Amounts of DNA used are indicated. D) Ct-values of real-time PCR and their dependency on the indicated 3C-steps. Crosslinked DNA was digested with NcoI and amplification was performed with primers P5 and P6. E) Ct-values of real-time PCR of the same samples as in (D). Primers (oRS39 and oRS40) amplify an intergenic region as an internal loading control. F) Relative interaction frequency (3C product normalized by loading control) of telomere 1L with its subtelomere with increasing distance from the telomeric repeats. Digest by NcoI and assessed via gel quantification of RedSafe™ signal. D+E+F) n=1

3.1.3 Telomeric 3C can confirm published looping defects

In both a genetic assay as well as by ChIP based methods it has been demonstrated that the Sir2 histone deacetylase complex (HDAC) is required for telomere looping in yeast, likely by creating a favorable local chromatin state at telomeres (de Bruin et al. 2001; Poschke et al. 2012). Sin3, a

component of Rpd3 HDACs, was additionally demonstrated to be looping defective (Poschke et al. 2012).

As a first application and at the same time to verify telomeric 3C we wanted to confirm the telomere looping defect of these mutants. We chose *sin3* and *sir2* mutants and performed 3C employing DpnII followed by densitometric analysis (Figure 12A+B) and NcoI followed by densitometric analysis (Figure 12C+D) as well as qRT-PCR (Figure 12E).

With all three setups we could detect a reduced interaction frequency in the mutants compared to wild type, indicating a looping defect at telomere 1L in the absence of these HDACs (Figure 12B+D+E). Importantly, the analysis of the *BLM10* locus using published protocols (Singh and Hampsey 2007) showed no defect in gene looping in either mutant (Figure 12F+G), indicating that the lower signal in Figure 12A-E was telomere specific and no artefact of our sample preparation. Taken together, our direct approach is able to detect telomere looping and verify the defective fold-back formation in reported telomere looping mutants.

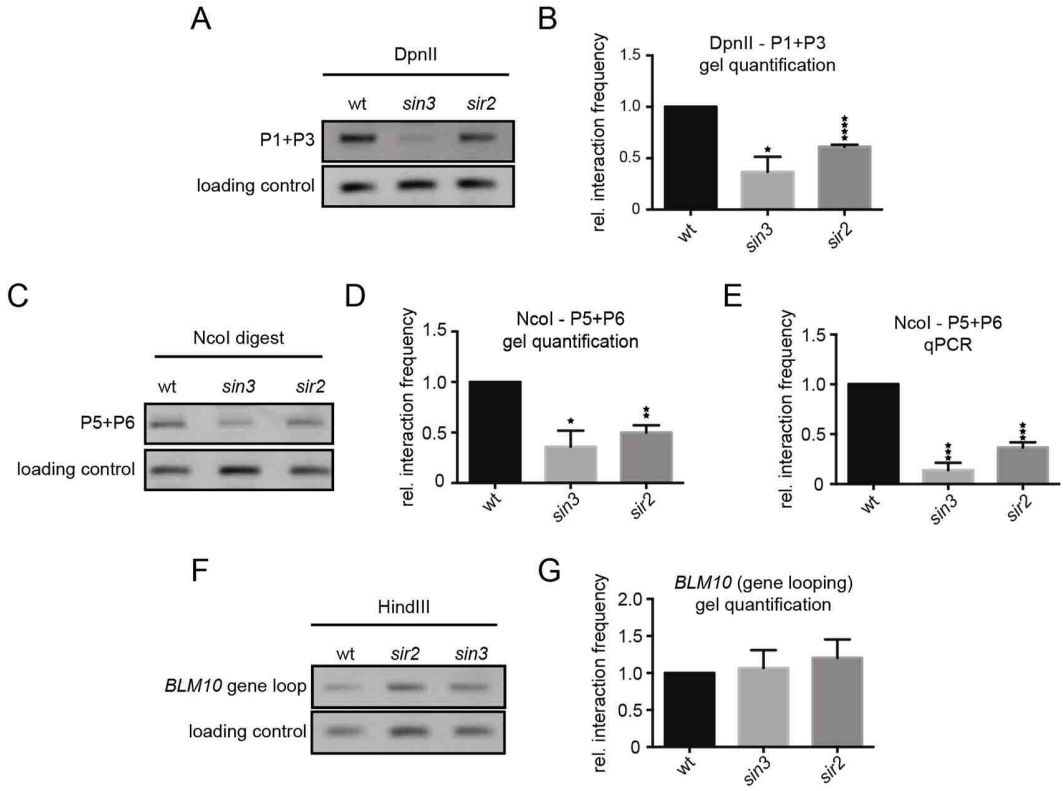

Figure 12: 3C verifies telomere looping defect in reported looping defective mutants
A) Amplification of ligation products upon crosslink and DpnII digest by primers P1 and P3 as readout for telomere looping. B) Relative interaction frequency or telomere looping at telomere 1L in *sin3* and *sir2* mutants compared to wild type using DpnII to digest DNA and primers P1 and P3 for PCR analysis. Assessed by quantification of

RedSafe incorporation in A) C) Amplification of ligation products upon crosslink and NcoI digest by primers P5 and P6 as readout for telomere looping. D) Relative interaction frequency or telomere looping at telomere 1L in *sin3* and *sir2* mutants compared to wild type using NcoI to digest DNA and primers P5 and P6 for PCR analysis. Assessed by quantification of RedSafe incorporation in C) E) Relative interaction frequency or telomere looping at telomere 1L in *sin3* and *sir2* mutants compared to wild type using NcoI to digest DNA and primers P5 and P6 for qPCR analysis. F) Amplification of ligation products upon crosslink and HindIII digest by primers oRS23 and oRS24 as readout for gene looping. G) Relative interaction frequency or gene looping at *BLM10* in *sin3* and *sir2* mutants compared to wild type using HindIII to digest DNA and primers oRS23 and oRS24 for PCR analysis. Assessed by quantification of RedSafe incorporation in F).
(Loading control represents the amplification of an intergenic region on chromosome V amplified by primers oRS39 and oRS40. Error bars represent the standard error of mean of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by student's t-test)

3.2 A relationship between telomere length regulation and telomere looping in *S. cerevisiae*

Using a genetic readout (see 1.4.2) Poschke and Dees et al. (2012) screened the viable yeast deletion collection (4,921 strains) for mutants that were defective in forming a fold-back structure at their telomeres. They identified 112 non-essential genes that were required, to various extents, for telomere fold-back formation/maintenance (Poschke et al. 2012). To determine if there was a relationship between telomere length and telomere structure we bioinformatically compared the 112 looping-defective (LD) mutants to genes that had previously been reported to be involved in telomere length maintenance (TLM), harboring either longer or shorter telomeres than wild type cells (Askree et al. 2004; Gatbonton et al. 2006; Ungar et al. 2009; Ben-Shitrit et al. 2012). We generated 10,000 independent lists of random 112 gene combinations derived from the previously screened deletion collection and determined the extent of overlap that could be expected from chance alone with the LD mutants (Figure 13). Whereas the TLM and LD mutants had 20 mutants in common, the 10,000 randomly generated lists most frequently resulted in an expected 7-gene overlap. The analysis therefore revealed a significant overlap ($p = 0.000019$) between LD mutants and all (long and short) TLM mutants (Figure 13A+B). When the TLM mutants were sub-divided into long and short telomere sub-categories, a significant overlap with LD mutants only occurred with short TLM mutants (Figure 13C), whereas the overlap with long-telomere mutants, although existent, was not statistically significant (Figure 13D). The most frequently expected overlap due to chance alone between a set of 112 deletion mutants and short TLM mutants is 3 - 4, whereas we observed 15 (Figure 13C). The actual overlap between LD and long TLM mutants was 5 mutants, and therefore almost not different to what could be observed by chance (Figure 13D). These data establish a statistically significant link between telomere length and telomere looping.

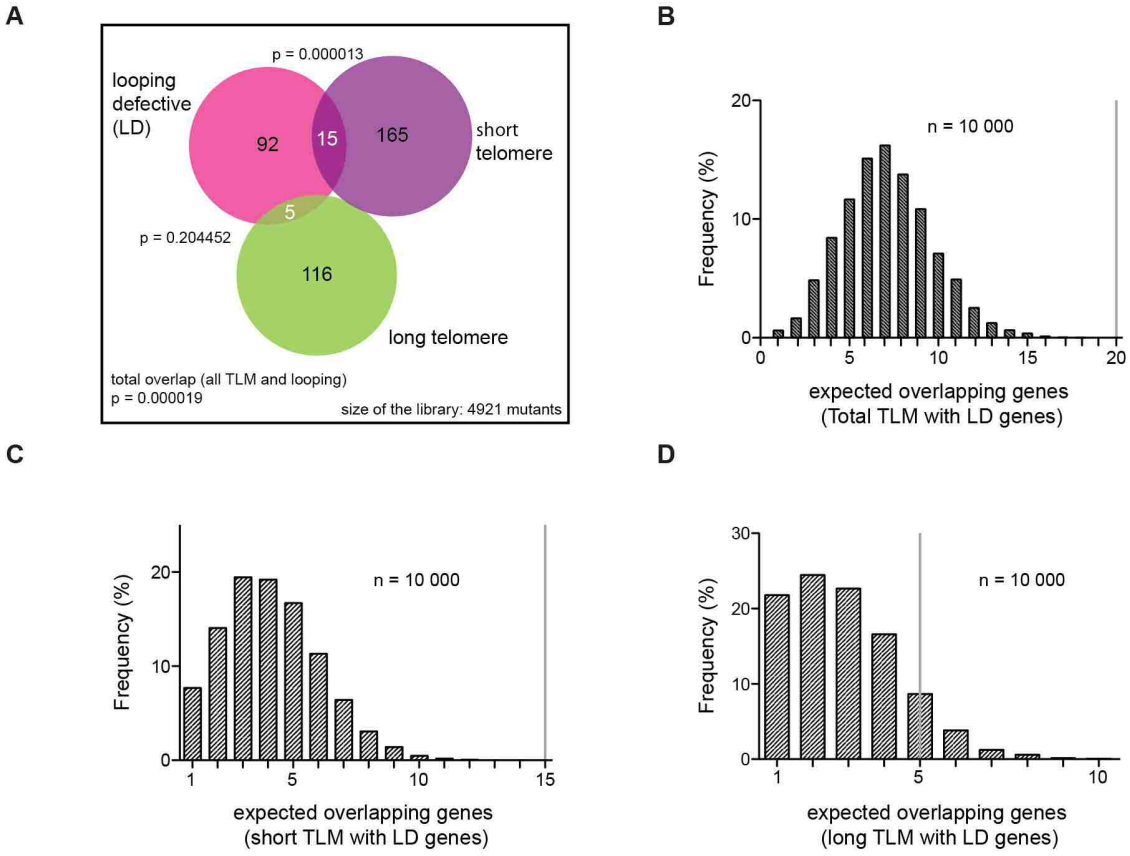

Figure 13: A relationship between telomere length and telomere looping.
(A) Venn diagram depicting the overlap of looping defective (LD) mutants with short- and long-telomere mutants, respectively. The statistical significance of the individual overlaps is shown as p-value from a Fisher's exact test for the respective mutant sets. Overall 4,921 deletion strains were tested for looping defects as well as for telomere length deviations from the wild -type length. (B, C and D) The expected overlap size between TLM mutants and a set of 112 deletion mutants was evaluated from random sampling (n = 10,000). The intersections of the random gene sets and all TLM mutants (B), only short- (C) and only long-telomere mutants (D) were calculated. A gray vertical line indicates the actual location of the measured intersection between LD mutants and TLM mutants.

3.2.1 Telomere looping is not required for bulk telomere length regulation

One interpretation of the results shown in Figure 13 is that telomere looping is essential for the proper establishment of telomere length. However, since many of the identified LD mutants have not been reported to have telomere length phenotypes (Figure 13A), combined with the fact that 5 LD mutants have long telomeres according to the TLM list, this would suggest that telomere length is only minimally influenced in the absence of a fold-back structure. In order to better understand the relationship between telomere length and the fold-back structure we extracted genomic DNA from all LD mutants and measured telomere length with respect to wild type using telomere PCR at different telomeres (Figure 14). We assessed the length of natural Y' and 6R telomeres as well as telomere 7L that harbored the genetic reporter construct used to screen for

looping defective mutants (Poschke et al. 2012). Although some of the LD mutants had short telomeres and some had long telomeres, the length of the majority of LD mutant telomeres did not deviate much from wild type telomere length (Figure 14). We observed that the LD mutants had a couple of long and short telomere phenotypes but without any clear trend in either direction (Figure 14). Only in the case of telomere 1L a slight shift towards short telomeres could be detected compared to wild type cells (Figure 14C). Nonetheless, there were also LD mutants with wild type length 1L telomeres as well as 1L telomeres that were longer than wild type (Figure 14C, see Supplemental table 2 for telomere length data).

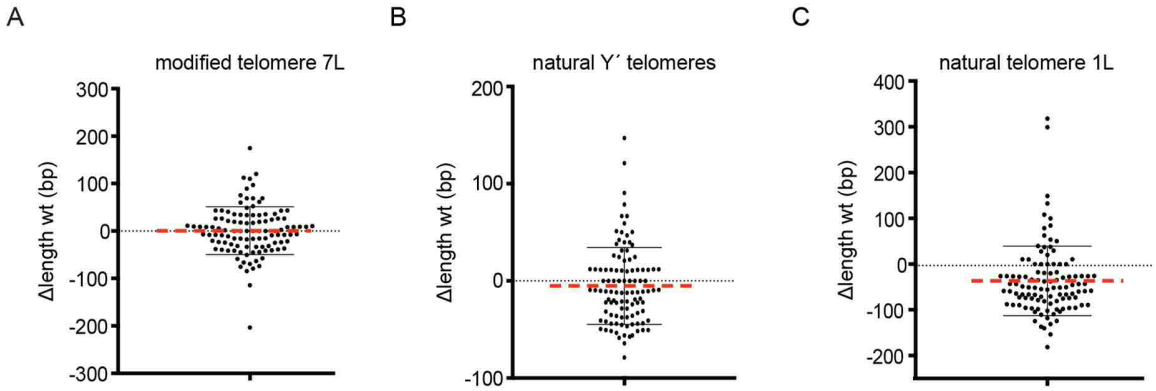

Figure 14:Telomere length of looping defective mutants
Quantification of telomere PCR performed on all LD mutants at indicated telomeres compared to wild type. The black dotted line denotes wild type length whereas the red dotted line denotes the mean of the plotted values. Raw data for telomere length can be found in Supplemental table 2 (Appendix).

Together, these data reveal that defects in telomere looping have very minor effects on telomere length homeostasis at natural telomeres.

3.2.2 Shortened telomeres are looping defective

As an alternative interpretation, the observed relationship between telomere length and looping could also infer the inverse: that telomere length has an influence on telomere loop establishment or maintenance, whereby wild type telomere length may be essential to establish a fold-back structure at chromosome ends. To assess the effect of telomere shortening on looping we deleted the catalytic subunit of telomerase (*EST2*). To start with wild type length telomeres we used *EST2*/*est2* heterozygous diploid cells that were sporulated and dissected. The grown colonies (representing ~25 generations) were passaged once more to get to ~50 generations and then inoculated for further experiments. As expected, telomere PCR showed that telomeres shortened in the absence of telomerase activity (Figure 15A): 25 population doublings yielded 1L

telomeres around 225 bp shorter than wild type, whereas 50 population doublings shortened 1L telomeres by approximately 311 bp. When assessing telomere looping via 3C, we detected that the loop structure was lost in both cases of telomerase negative cells with short telomeres (Figure 15B+C). This result implies that short telomeres are defective for telomere looping.

We also analyzed the relationship between telomere looping and long telomeres. For this purpose we lengthened telomeres in a wild type context by introducing a Cdc13-Est2 fusion protein (Evans and Lundblad 1999), which promotes constitutive telomerase recruitment to telomeres. We exogenously expressed the fusion from a plasmid under the control of a constitutive promoter for 50 generations. As expected, we observed telomere elongation in cells harboring the fusion construct when analyzing telomere length by telomere PCR (Figure 15D). However, we did not detect a telomere looping defect at long telomeres compared to wild type as assessed by 3C (Figure 15E), suggesting that long telomeres are capable of forming a loop.

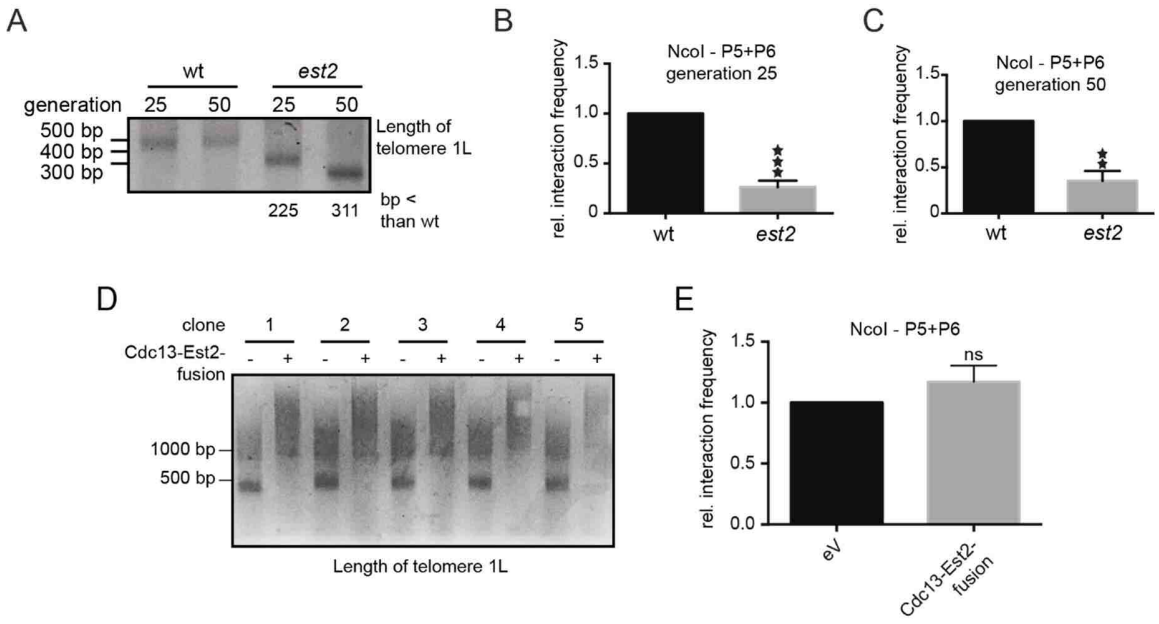

Figure 15: Telomere looping depends on telomere length

A) Representative telomere PCR to assess telomere length at telomere 1L in *est2* mutants compared to wild type dependent on generation number. B+C) Relative interaction frequency of telomere 1L and its subtelomere in *est2* mutants, compared to wild type after 25 (B) and 50 population doublings (C). Quantified by real-time PCR using primers P5 and P6 on NcoI digested DNA as readout for telomere looping. D) Telomere PCR to assess length of telomere 1L in wild type cells containing an empty plasmid or expressing a Cdc13-Est2-fusion to elongate telomeres. Numbers 1-5 represent the independent biological replicates that were used. E) Relative interaction frequency of telomere 1L and its subtelomere in wild type cells containing an empty plasmid or expressing and Cdc13-Est2-fusion. Quantified by real-time PCR using primers P5 and P6 on NcoI digested DNA as readout for telomere looping. (Error bar represents the standard error of mean of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by student's t-test)

If short telomeres were not able to form a fold-back structure, why were so many mutants harboring short telomeres according to the TLM list not represented in the genetic screen for LD mutants? To investigate this circumstance we chose four mutants that have a telomere length defect but are not represented in the list of LD mutants (Figure 16A+B). Whereas 1L telomeres in *upf1*, *ku70* and *rad50* mutants are almost 200 bp shorter than wild type, telomere 1L is only 90 bp shorter in a *sum1* mutant (Figure 16A). The 3C analysis revealed that *upf1*, *ku70* and *rad50* mutants with very short telomeres show a telomere looping defect (Figure 16B).

In contrast we detected a wild type looping signal in the *sum1* mutant with only slightly shortened telomeres, suggesting that a certain threshold may exist below which telomere looping is not possible anymore. Therefore, the absence of many mutants harboring very short telomeres from the LD list is likely because they were missed or cut out due to controls from the large-scale screen.

There is evidence that apart from telomere elongation, telomerase also carries out other non-canonical functions, such as action as a transcription factor (Parkinson et al. 2008). Additionally, the telomerase component Est2 has been shown to bind to the telomere even when telomerase is not active (Fisher et al. 2004). We thus wondered if the mere absence of telomerase or Est2 could be the cause for a looping defect, rather than the shortened telomeres. In an *EST2* deletion background we therefore exogenously expressed the catalytically dead telomerase subunit *est2*D670A from a plasmid (pSE358-*est2*D670A) under the control of a constitutive promoter. (Lingner et al. 1997; Xia et al. 2000; Chang et al. 2009). The cells were additionally transformed with a second vector containing a *URA* selection that was either empty or encoded a Cdc13-Est2 fusion to overelongate telomeres for 50 generations. Before 3C analysis of telomere looping the cells were streaked out on 5-FOA containing plates to select for cells that had lost the plasmid. The resulting cells were telomerase negative, expressing a catalytically dead *est2* mutant and harboring either short or elongated telomeres (Figure 16C). As a control we employed an *est2* mutant that exogenously expressed *EST2* and therefore maintained wild type telomere length. The 3C analysis of telomere looping showed that cells with short telomeres and an inactive telomerase were still looping defective, with the signal reduced by approximately 50 % when compared to cells with wild type telomere length (+*EST2*; Figure 16D). Cells with an inactive telomerase harboring elongated telomeres, however, were still able to form a fold-back structure

(Figure 16D). Together this shows that an inactive telomerase protein that might still be able to mediate protein-protein interactions is not able to reestablish telomere looping, reinforcing the short length of the telomeres as the cause for the looping defect.

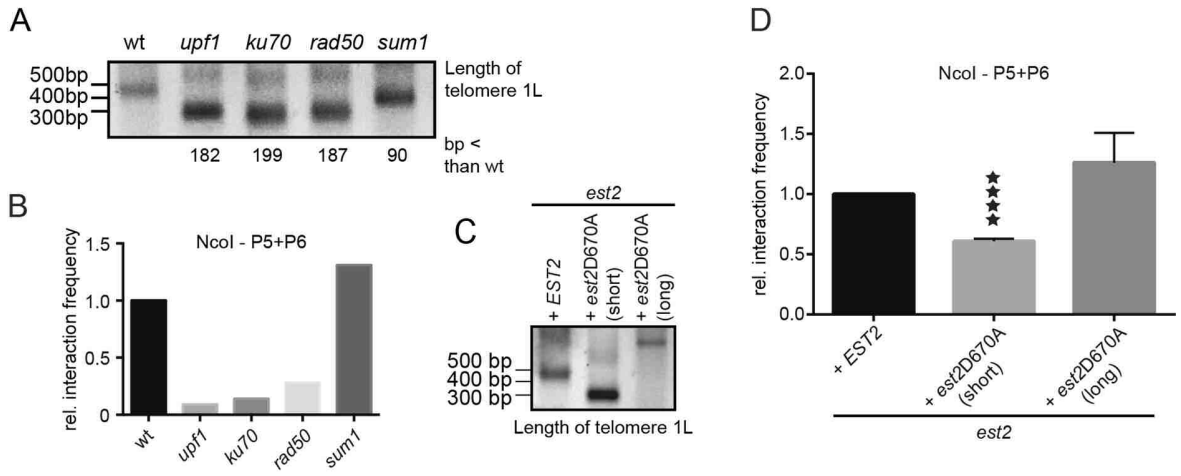

Figure 16: Telomere looping is independent of telomerase
A) Telomere PCR to assess length of telomere 1L in indicated mutants compared to wild type. B) Relative interaction frequency of telomere 1L and its subtelomere in indicated mutants compared to wild type. Quantified by real-time PCR using primers P5 and P6 on NcoI digested DNA as readout for telomere looping. C) Telomere PCR to assess length of telomere 1L in *est2* mutants exogenously expressing Est2 or a catalytically dead telomerase mutant *est2*D670A with short or overelongated telomeres compared to wild type D) 1L telomere looping in *est2* mutants exogenously expressing Est2 or a catalytically dead telomerase mutant *est2*D670A with short or overelongated telomeres compared to wild type. Quantified by real-time PCR using primers P5 and P6 on NcoI digested DNA as readout for telomere looping. (Error bar represents the standard error of mean of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by student's t-test)

It has been shown that in contrast to wild type telomeres that are replicated in late S phase, short telomeres are replicated in early S phase (Bianchi and Shore 2007). To rule out the possibility that an altered replication timing is the cause for the detected drop in the relative interaction frequency we employed an *est2tel1* mutant with short telomeres (Figure 17A) and a wild type cell cycle distribution (Figure 17B). The additional *tel1* mutation has been shown to rescue the early replication phenotype in the presence of short telomeres (Sridhar et al. 2014). 3C analysis showed that an *est2tel1* mutant had a similar looping defect as the *est2* single mutant (Figure 17C) ruling out an effect of replication timing on our telomere looping results.

In summary, these data suggest that very short telomeres compared to wild type length do not allow the chromosome end to fold back onto itself.

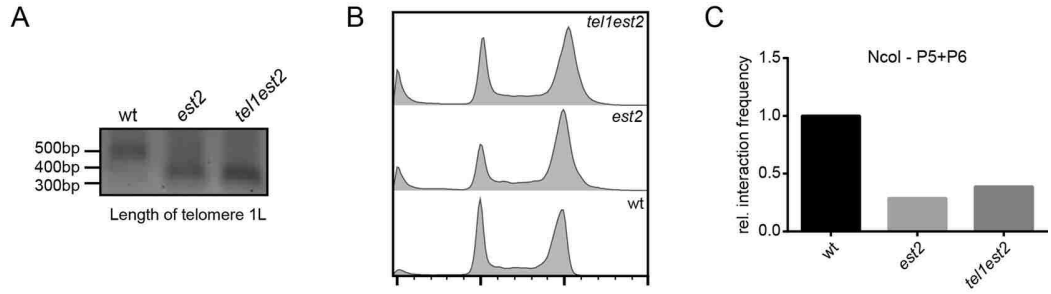

Figure 17: Early replication is not the cause for a looping defect.
A) Telomere PCR to assess length of telomere 1L in *est2* and *tel1est2* mutants compared to wild type. B) Cell cycle analysis of wild type, *est2* and *tel1est2* mutants following flow cytometry of DNA content. C) Relative interaction frequency of telomere 1L and its subtelomere in *est2* and *tel1est2* mutants compared to wild type. Quantified by real-time PCR using primers P5 and P6 on NcoI digested DNA as readout for telomere looping. n=1

3.3 Regulation and Maintenance of telomere looping in *S. cerevisiae*

To clarify the regulation and maintenance of telomere looping structures in yeast we made use of different mutants that have been implicated in different types of DNA looping including chromatin and gene loops.

3.3.1 Telomere loop formation is independent of homologous recombination

Since human t-loops are likely stabilized through a strand invasion event, a homologous recombination intermediate, we used 3C at yeast telomere 1L to test whether the homologous recombination machinery was required for yeast telomere looping. We made use of complete rad51 and rad52 deletion mutants, which renders cells recombination defective in budding yeast. Freshly dissected *rad51* and *rad52* mutants (derived from heterozygous diploids) showed wild type telomere length (Figure 18A) and wild type cell cycle distribution (Figure 18B). Furthermore, the HR mutants were still looping proficient (Figure 18C), indicating that telomere loop structures in *S. cerevisiae* are not dependent on homologous recombination.

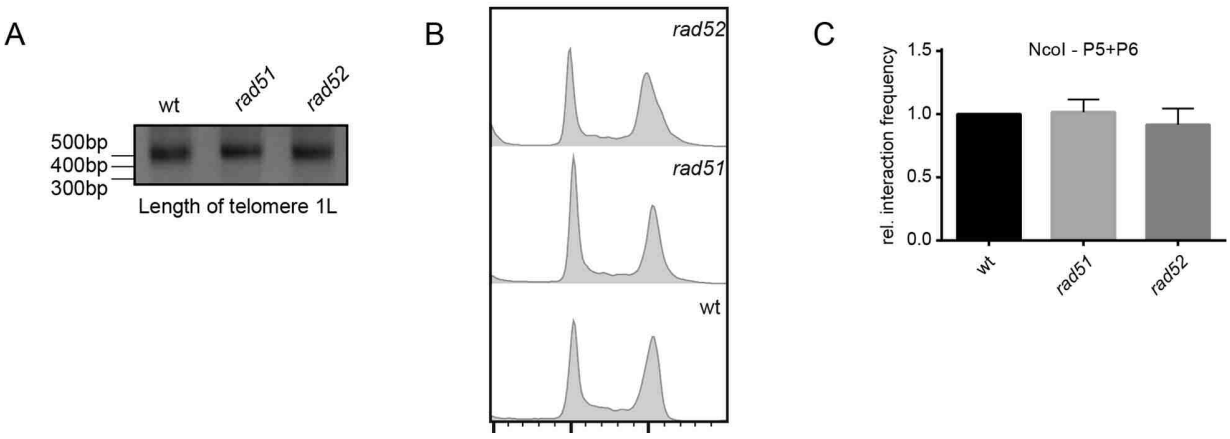

Figure 18: Telomere looping in *S. cerevisiae* is independent of homologous recombination
A) Telomere PCR to assess telomere length in HR mutants compared to wild type. B) Cell cycle analysis of HR mutants compared to wild type. C) Relative interaction frequency or telomere looping in HR mutants compared to wild type. Quantified by real-time PCR using primers P5 and P6 on NcoI digested DNA as readout for telomere looping. (Error bars represent the standard error of mean of three independent experiments)

3.3.2 Telomere loop formation requires the transcription machinery

3.3.2.1 Transcription

It has previously been shown that gene looping is dependent on transcription (Ansari and Hampsey 2005). To test whether telomere looping may be transcription dependent, we tagged the Rpb1 subunit of RNA polymerase II with an auxin inducible degron (AID) in a strain expressing the F-box protein Afb2 (Morawska and Ulrich 2013), and as expected saw a significant reduction in protein levels upon auxin addition for 2 hrs (Figure 19A). Importantly, we noticed that the telomere looping signal as measured by 3C was also significantly decreased upon auxin induction in the AID tagged Rpb1 strain, but not in untagged control cells (Figure 19E). The auxin-induced degradation of Rpb1 over 2 hours did not result in telomere length changes (Figure 19D), nor did it cause cell death (Figure 19B) or large changes in the cell cycle distribution, ruling out these factors as potential causes for loop disruption.

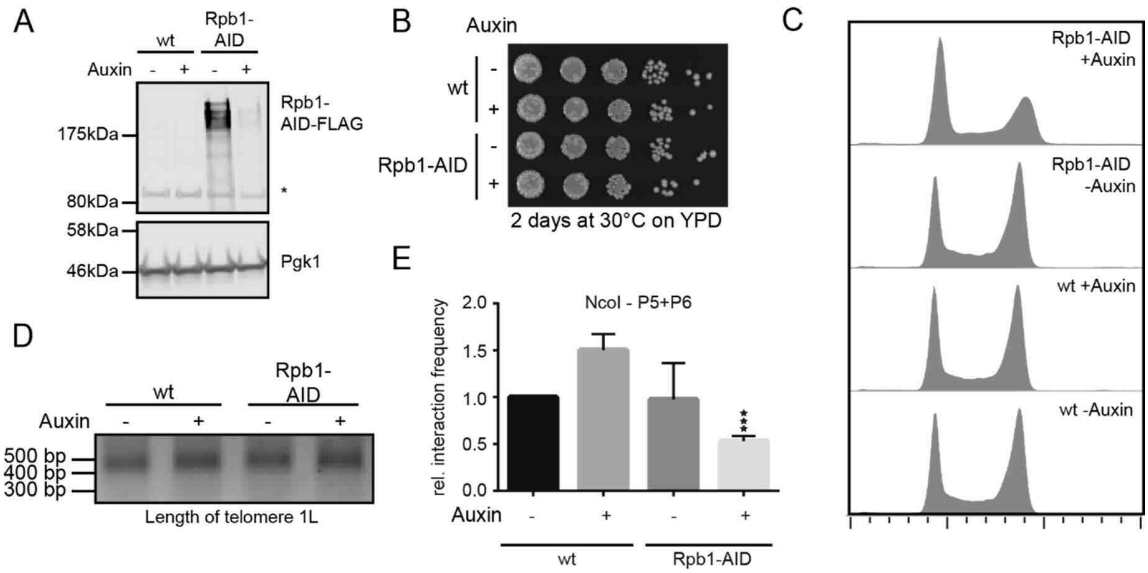

Figure 19: Telomere looping depends on transcription
A) Cells expressing the F-box protein Afb2 in a wild type background (wt) and a mutant expressing rpb1-AID-FLAG (auxin inducible degron). Auxin (1 M) was added to the media of exponentially growing cells for 2 hours using the indicated genotypes and proteins were extracted to determine levels of Rpb1 fused to AID-Flag. Western blotting was performed with anti-FLAG and anti-PGK1 antibodies as indicated. (* indicates an unspecific signal) B) After 2 hours in the presence of 1 M auxin, the indicated strains were spotted as 10-fold serial dilutions on YPD plates and incubated for 2 days at 30°C to assess viability. C) Cell cycle analysis of indicated strains in absence and presence of 1 M auxin for 2 hours. D) Telomere PCR to assess length of telomere 1L in indicated strains in absence and presence of 1 M auxin for 2 hours. E) The cells from A were subsequently crosslinked with formaldehyde and processed for quantitative 3C as previously described. Quantification was performed by real-time PCR using primers P5 and P6 on NcoI digested DNA as readout for telomere looping. (wt = Cells expressing the F-box protein Afb2 in a wild type background. Error bar represents the standard error of mean of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by student's t-test)

Another way to demonstrate the transcriptional dependency of looping is to employ a mutated version of the *SUA7* gene (*sua7-1*) that is defective in gene looping (Singh and Hampsey 2007). *sua7-1* encodes for a mutant form of transcription factor IIB (TFIIB) that confers a downstream shift in transcription start site selection (Sun and Hampsey 1996); also, the interaction with components of the Cleavage and Polyadenylation Factor (CPF) at the terminator is affected (Medler et al. 2011). However, transcription itself and RNA levels are reported to be unchanged in this mutant (Pinto et al. 1992; Cho and Buratowski 1999). Consistently, cell cycle distribution was almost unaffected (Figure 20B) and telomeres showed wild type length (Figure 20A). Our assay also detected a telomere looping defect for *sua7-1* (Figure 20C), which together with the results on Rpb1-AID suggests that transcription and the transcription machinery are involved in telomere loop formation.

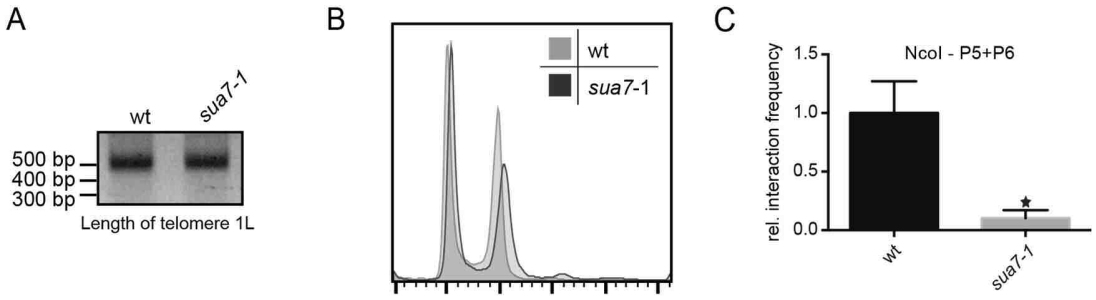

Figure 20: Telomere looping depends on transcription factor II B, Sua7.
A) Telomere PCR to assess length of telomere 1L in sua7-1 mutants compared to wild type. B) Cell cycle analysis of wild type and sua7-1 following flow cytometry of DNA content. C) Relative interaction frequency of telomere 1L and its subtelomere in sua7-1 mutants compared to wild type. Quantified by real-time PCR using primers P5 and P6 on NcoI digested DNA as readout for telomere looping. (Error bar represents the standard error of mean of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by student's t-test)

3.3.2.2 Mediator

The Mediator complex is a large multiprotein complex that acts on almost every step during transcription and also affects basal transcript levels (also see section 1.2.2). Several subunits of the complex are essential and we assessed telomere looping in a *hrs1* (*med3*) mutant, a non-essential subunit of mediator. The mutant showed slight telomere shortening (Figure 21A) and an almost wild type cell cycle distribution (Figure 21B). Strikingly, we detected a strong reduction in telomere looping (Figure 21C), thus also putting the mediator complex into the looping pathway. It is unlikely that the slight shortening of *hrs1* telomere 1L accounts for the looping defect detected (Figure 21A). Interestingly, two other subunits of the mediator complex, namely Med1 and Med5, have been found in the genetic screen for LD mutants (Poschke et al. 2012). Importantly, all the essential subunits could not be covered by the screen.

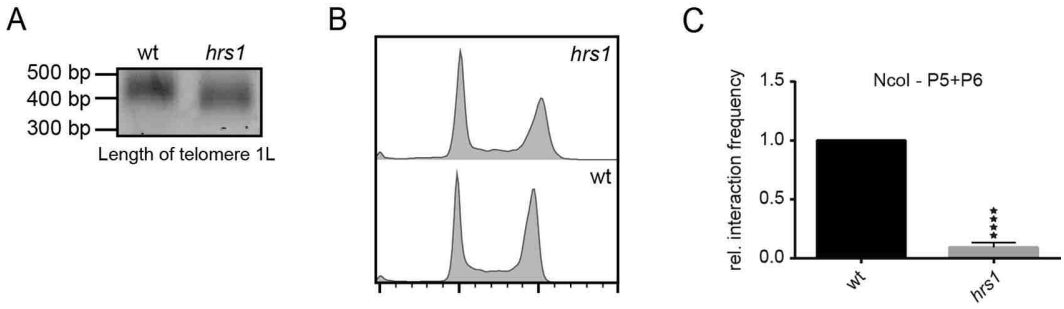

Figure 21: Telomere looping depends on the mediator complex
A) Telomere PCR to assess length of telomere 1L in *hrs1* mutants compared to wild type. B) Cell cycle analysis of wild type and *hrs1* following flow cytometry of DNA content. C) Relative interaction frequency of telomere 1L and its subtelomere in *hrs1* mutants compared to wild type. Quantified by real-time PCR using primers P5 and P6 on NcoI digested DNA as readout for telomere looping. (Error bar represents the standard error of mean of four independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by student's t-test)

3.3.2.3 RNA

Mediator exerts its initially identified function of transcription enhancement or suppression via the formation of chromatin loops. Even the involvement in certain gene loops has been demonstrated. An interesting aspect was identified for a class of activating ncRNAs (ncRNA-a): they can affect transcription of distant genes by mediating a chromatin loop that brings enhancer regions and the promoter in close proximity. Interestingly, this loop depends on both the presence of the RNA species and on the mediator complex located at the promoter region of the regulated gene (Lai et al. 2013).

We wondered if RNA might also be involved in telomere looping and depleted bulk RNA by RNase A treatment of *S. cerevisiae* spheroplasts, whose cell wall was depleted to allow penetration by RNase A. After 30 minutes total RNA was substantially depleted as can be seen in a non-denaturing agarose gel (Figure 22A). The time course in Figure 22B shows that the RNA degradation did not occur during RNA preparation, since there was still a substantial amount of RNA in non-spheroplasted cells that decreases over time with RNase A treatment, which is shown in a denaturing gel. When we assessed telomere looping with our telomeric 3C setup, we detected a loss of telomere looping (Figure 22C), indicating that the presence of RNA is a prerequisite for the looped telomere structure.

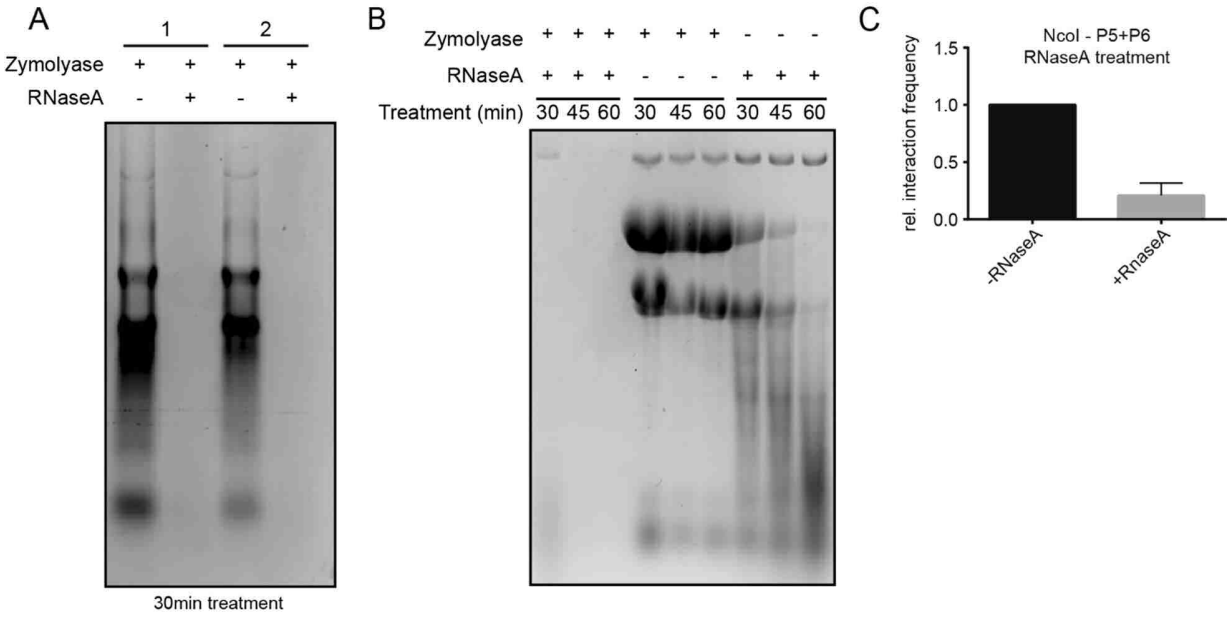

Figure 22: Telomere looping depends on RNA
A) Spheroplasted cells were treated *in vivo* with RNase A for 30 minutes. RNA was extracted using the hot phenol method and separated on formaldehyde containing agarose gel. 1 and 2 represent the independent biological replicates that were used in this experiment. B) Formaldehyde containing agarose gel showing a time course of

bulk RNA depletion by RNase A. RNase A degrades RNA even in cells that have not been treated with zymolyase. The gradual degradation as a function of incubation time shows that the depletion is not an artefact of the RNA preparation. C) Relative interaction frequency of telomere 1L and its subtelomere in RNase A treated spheroplasts from A compared to wild type and assessed by real-time PCR using primers P5 and P6 as readout for telomere looping as described above. (Error bar represents the standard error of mean of two independent experiments)

3.3.3 Cell cycle regulation of telomere looping

We also wondered whether telomere looping is regulated in a cell cycle dependent manner, potentially to allow passage of the replication machinery in S phase or HR dependent processes to facilitate recombination. We pursued two different strategies to address this question.

3.3.3.1 Telomere looping is lost upon permanent cell cycle arrest

The first strategy was to permanently arrest *bar1* cells in different cell cycle phases by employing α-factor (arrest in G1 phase), hydroxyurea (arrest in S phase) and nocodazole (arrest in G2/M). After a permanent arrest of 150 minutes without washing out the additives we took samples for cytometric analysis of DNA content, western blot and 3C (Figure 23A). The DNA content showed that cell cycle arrests occurred as expected (Figure 23B), and a western blot with an antibody against Sic1, which is a marker protein for G1 phase, demonstrated that hydroxyurea arrested cells were not stuck in G1 but in S phase of the cell cycle (Figure 23C). When we performed 3C to measure the interaction frequency we detected a consistent looping defect for all arrested populations independent of the cell cycle phase, indicating that the loop was dissolved upon prolonged arrest.

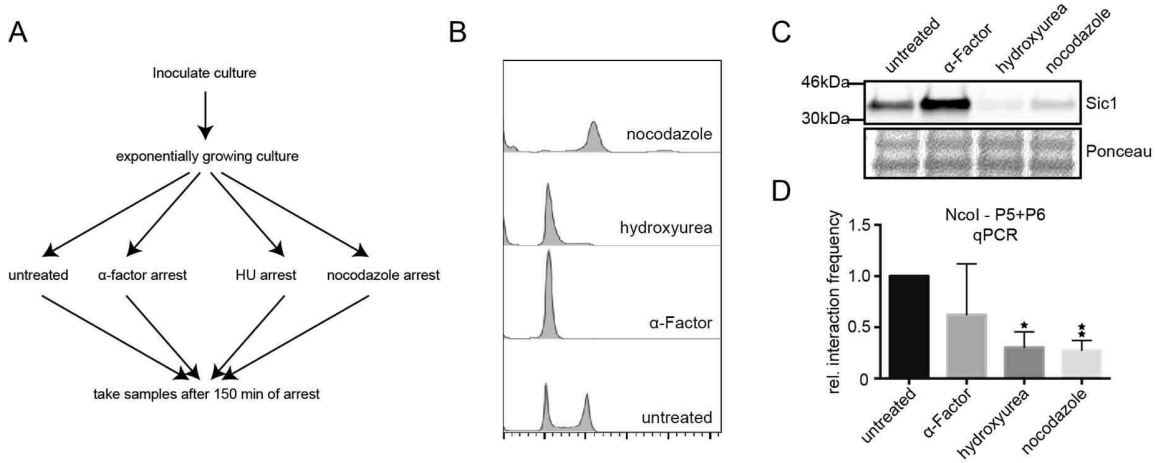

Figure 23: Permanent cell cycle arrest prevents telomere looping
A) Experimental outline. B) Cell cycle analysis of wild type and arrested cells. C) Western Blot. After drug treatment proteins were extracted to assess levels of Sic1 in arrested cells. Ponceau S staining is shown as loading control. D) Relative interaction frequency or telomere looping in arrested cultures compared to wild type in a 3C assay using NcoI to digest DNA and primers P5 and P6 for qPCR analysis. (Error bars represent the standard error of mean of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by student's t-test)

3.3.3.2 Cell cycle regulation of the telomere loop

For the second strategy we arrested the cells for 150 minutes with α-factor, followed by release into the cell cycle. α-factor was readded after 45-60 minutes to ensure that cells only cycled once and accumulated again in G1 phase. We took samples every 15 minutes and performed 3C; the DNA content was measured at the same time points to follow progression through the cell cycle (Figure 24A). The 3C results obtained in two independent experiments showed an increased looping signal 30 min after release in experiment 1 and 15 min after release in experiment 2. According to the DNA content, this corresponds to early to mid S phase in experiment 1 and early S phase in experiment 2 (Figure 24B+C). The following drop occurred during late S phase, when telomeres get replicated. After replication the signal might increase again (Figure 24B+C). The DNA analysis of the two experiments showed that cells were not exactly synchronous, necessitating to display both separately (Figure 24A).

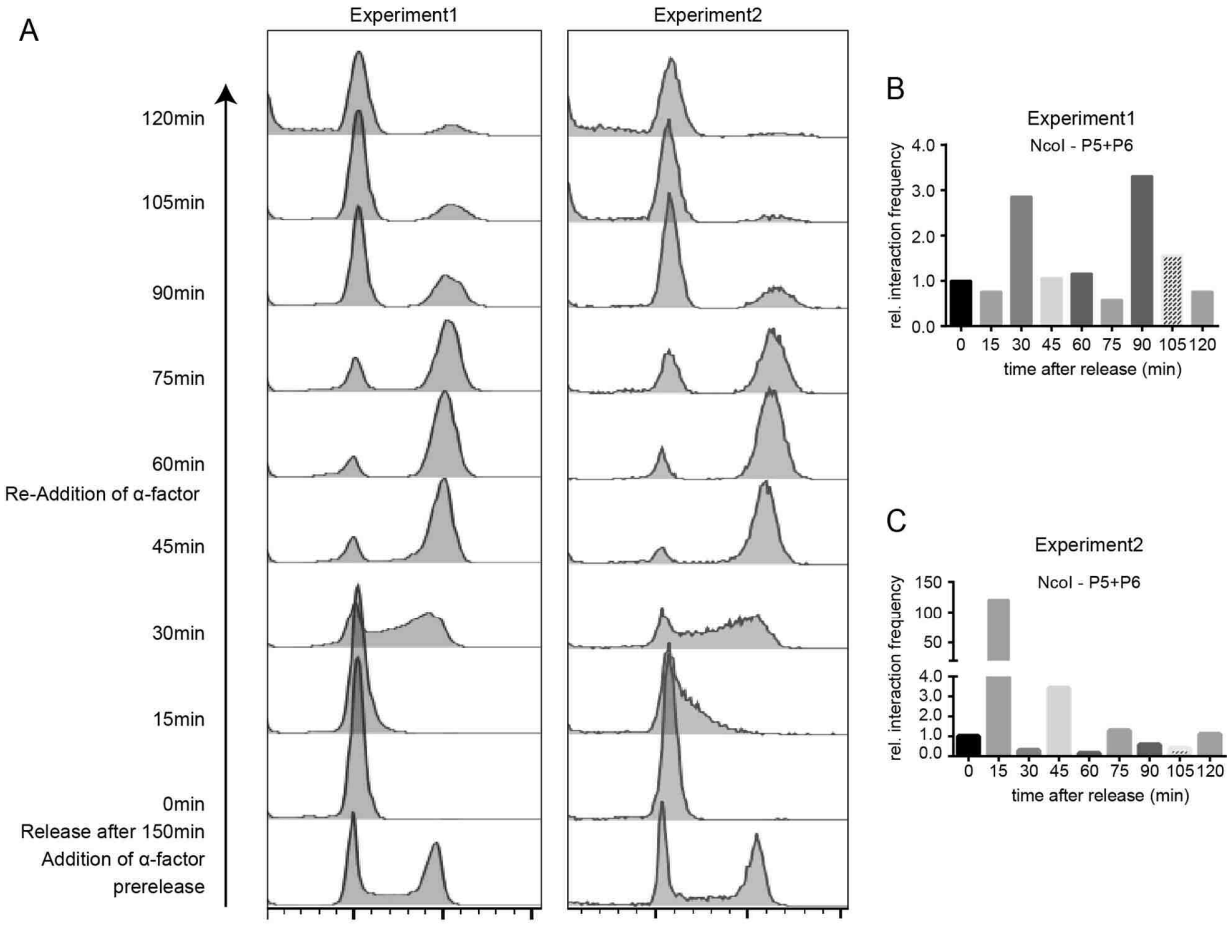

Figure 24: Cell cycle regulation of telomere looping

A) Cell cycle analysis of synchronized cells upon release into cell cycle after α-factor arrest following flow cytometry of DNA content. Left panel shows the experimental design. Two separate experiments are shown. B+C) Relative interaction frequency of telomere 1L and its subtelomere in synchronized cells during cell cycle. Quantification was performed by real-time PCR using primers P5 and P6 on NcoI digested DNA as readout for telomere looping. Two separate experiments are shown.

3.3.4 Telomere looping is independent of G4-Quadruplexes

Further, we looked at the involvement of G4-quadruplexes in yeast telomere looping (Tang et al. 2008). These structures consist of planar G-squares, containing four guanine bases each that stack on top of each other. They have been found at the 3' overhang of telomeres (Tang et al. 2008) and are also formed by TERRA (Biffi et al. 2012). The Pif1 helicase was shown to undo quadruplex-structures (Paeschke et al. 2011) and we reasoned that its overexpression could reveal the involvement of quadruplexes in telomere looping. Exogenous overexpression of Pif1 via a galactose inducible promoter did not affect 1L telomere length (Figure 25A) and did not affect the cell cycle distribution (Figure 25B). Importantly, we could not detect a reduction in telomere looping as assessed by 3C (Figure 25C), suggesting that G4-quadruplexes are required for telomere loop formation/maintenance.

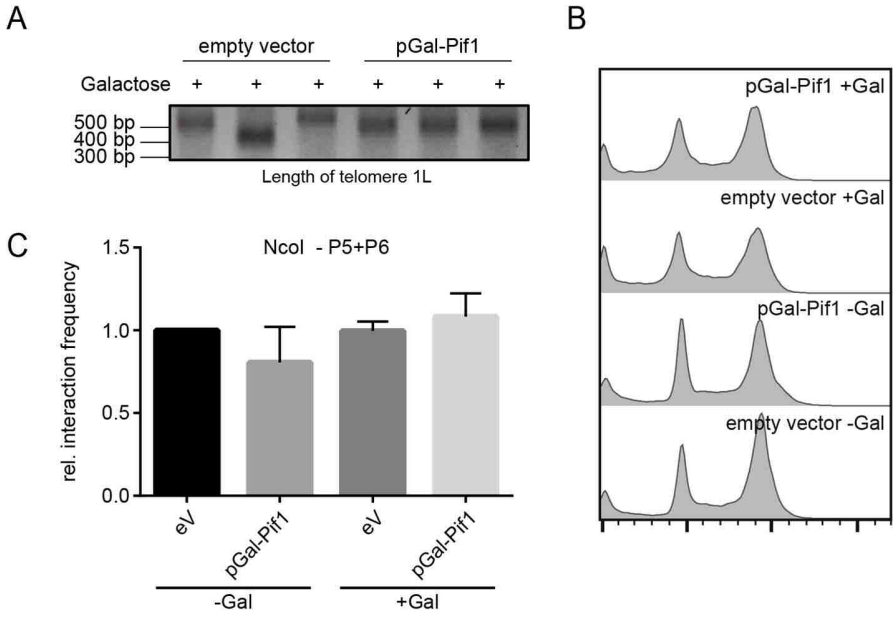

Figure 25: Telomere looping is not regulated by G4-quadruplexes
A) Telomere PCR to assess telomere length in wild type cells containing an empty vector or harboring Pif1 under the control of a galactose inducible promoter under inducible conditions after 3 hours of galactose induction (2 %). B) Cell cycle analysis of indicated strains after 3 hours of galactose induction (2 %). C) Relative interaction frequency or telomere looping in arrested cultures compared to wild type in a 3C assay using NcoI to digest DNA and primers P5 and P6 for qPCR analysis. (Error bars represent the standard error of mean of three independent experiments) D) Relative interaction frequency or telomere looping in arrested cultures compared to wild type in

a 3C assay using NcoI to digest DNA and primers P5 and P6 for qPCR analysis. (Error bars represent the standard error of mean of two independent experiments)

3.4 On the functions of telomere looping in *S. cerevisiae*

Even though our focus was on telomere loop regulation, we also wondered about possible functions of telomere fold-back structures in *S. cerevisiae*. One of the functions of gene- and chromatin looping is the regulation of transcript levels, and since the telomere is transcribed into the telomeric transcript TERRA, we asked the question if telomere looping might be in charge of regulating TERRA levels at the telomere.

3.4.1 TERRA levels are not regulated by telomere looping

We measured TERRA levels in different looping defective mutants compared to wild type by qRT-PCR. Telomere PCR was used to measure telomere length and to confirm shortening of the short telomere mutants (Figure 26A). We employed primer pairs specifically binding certain subtelomeric regions to measure TERRA levels derived from defined telomeres. When comparing mutant 1L TERRA levels with wild type levels we could see a strong increase in TERRA in *sin3* and *sir2* mutants, a slight increase in *rad50* mutants, and a decrease in *upf1* mutants (Figure 26B). 1L TERRA levels in the *ku70* mutant were similar to wild type cells (Figure 26B). When we measured TERRA levels of different telomeres in a *sua7-1* mutant compared to its wild type we could detect an increase of TERRA for telomeres 1L and 15L and a decrease for telomeres 6R and 6Y' (Figure 26C). All together, we did not detect a homogeneous regulation of TERRA in looping defective mutants, indicating that telomere looping does not regulate TERRA level.

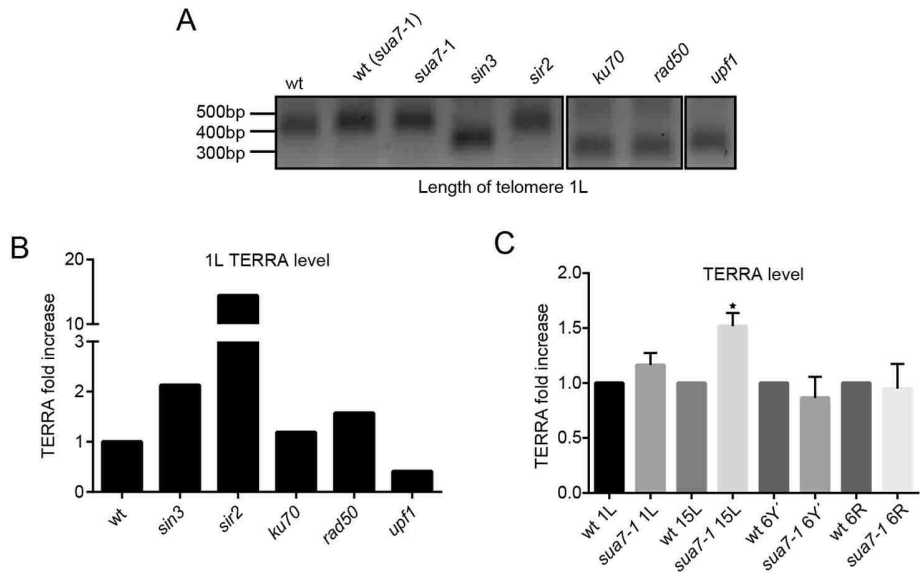

Figure 26: Telomere loops do not regulate TERRA levels
A) Telomere PCR to assess length of telomere 1L in looping defective mutants compared to wild type. *sua7-1* is run next to the wild type of its own background. B) Relative TERRA level of telomere 1L in the telomere looping defective mutants compared to wild type assessed by qRT-PCR.C) Relative TERRA level of different telomeres in a sua7-1 mutant compared to wild type assessed by qRT-PCR. (Error bars represent the standard error of mean of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by student's t-test)

4 Discussion

4.1 What has been known before this study

The presence of fold-back structures at linear chromosome ends appears to be conserved throughout all eukaryotes tested so far. Telomere loops have been demonstrated to occur within the telomeric tract (t-loops) in human and mouse cells. Yeast telomeres more likely fold back into the subtelomeric sequences. The structures are likely involved in chromosome end protection. Furthermore, mammalian telomeres form long range interactions that reach sequences up to several megabase pairs away from the telomere. Such long range loops have been implicated in the regulation of distant genes, for example as a transcriptional response to telomere shortening (Robin et al. 2014). Publications concerning telomere looping are rare and have focused on few main topics so far. Among them are, in particular, the identification of telomere loops in different species, the introduction of new microscopic technologies to visualize looped structures and the involvement of TRF2 and its interaction partners in loop formation. TRF2, a component of the shelterin complex at mammalian telomeres, is required for t-loop formation as well as long-range telomere fold-back structures. However, apart from TRF2 in human cells and the chromatin environment that is influenced by histone deacetylases (HDACs) in yeast, the requirements necessary for telomere loop formation have remained obscure, largely due to the technical difficulties associated with telomere loop detection. *In vitro* data furthermore suggests that homologous recombination is necessary to form the mammalian t-loop. However, the genome wide screen of the *S. cerevisiae* viable deletion collection performed in our lab indicates the involvement of many more pathways in telomere loop formation.

4.2 3C – a new tool to understand telomere looping

Investigating telomere loop structures so far involved laborious and time consuming microscopic techniques or indirect methods based on genetic readouts or chromatin immunoprecipitation (ChIP) based approaches (see section 1.4). While microscopy is not feasible for yeast telomeres due to unmet size and sequence prerequisites, the alternatives to date are indirect and not satisfying. Our approach uses the chromosome conformation capturing (3C) technique to directly quantify the interaction frequency of a telomere and its subtelomere (see section 1.4.4). The mere presence of a PCR looping signal does not necessarily mean that a looped conformation

exists, since the detected ligation product will also occur by chance, even though less abundant. However, the dependency of the signal on all steps of the 3C protocol, together with the fact that the signal declines with increasing distance and that mutants and conditions exist where the signal is considerably reduced, strongly indicate that yeast telomeres form fold-back structures (Figure 10 and Figure 11). Importantly, the signal is strongly diminished when genomic DNA is digested and ligated without prior crosslinking (Figure 10B and Figure 11C). This result shows that the signal we observe does not occur just by chance and is the first direct evidence for telomere looping in yeast.

According to our results at telomere 1L, with looping signals slightly declining with increasing distance from the telomeric tract, we believe that the telomere does not have a specific touch-down point in the subtelomere that is represented by a certain binding motive. Rather, a whole stretch of DNA where the telomere gets in proximity to the subtelomere, maybe by protein-protein or RNA-protein interactions, could be responsible for loop formation/maintenance. Consistently, in ChIP assays, the telomere repeat binding protein Rap1 could be detected at several subtelomeric positions at different distances from the telomere (Strahl-Bolsinger et al. 1997).

In this study we have utilized 3C, adapted for the natural telomere 1L in budding yeast, to investigate the prerequisites and mechanisms behind telomere loop formation. We could demonstrate that the interaction of telomeres with internal subtelomeric positions occurs in a transcription dependent manner. Both depletion of RNA Pol II as well as a mutated transcription factor II B (TFIIB, *sua7-1*) abolished telomere looping (Figure 27). In addition, the transcription activating mediator complex seems to be required and depletion of bulk RNA by RNase A treatment resulted in a looping defect (Figure 27). Moreover, as a physiologically more relevant scenario, telomeres must harbor a critical repeat length in order to achieve efficient loop formation (Figure 27). In other words, when telomeres shorten due to the end replication problem, they adopt an open conformation. If the looped conformation hides the telomeric overhang, then an open loop might allow access to telomere maintenance mechanisms like telomerase or the recombination machinery to elongate short telomeres. According to our and others' data, telomere loops are unlikely to regulate telomeric transcription. However, we cannot exclude that looping is involved in a telomere position effect (TPE) to affect subtelomeric gene

expression (Figure 27). In summary, we could extend the understanding of telomere loop formation and shed light on the regulation and maintenance of the fold-back structure. Resolving the precise functions of the loop, however, will need further analysis.

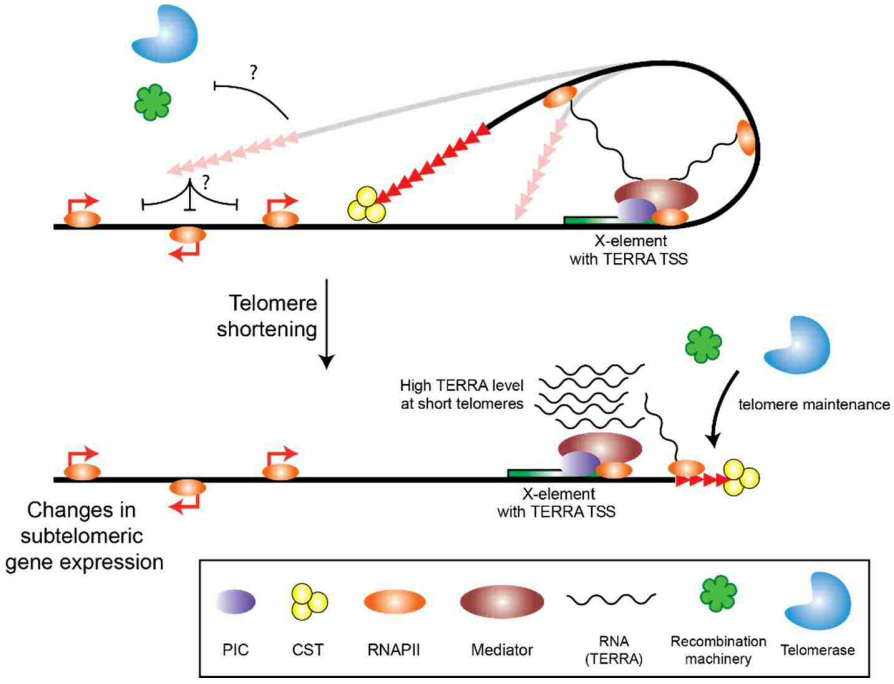

Figure 27: Current Model for telomere looping
Telomere looping depends on transcription, the mediator complex and an RNA species. The size of the loop is dynamic. Short telomeres are looping defective and have high TERRA levels. Due to the open structure telomere maintenance machineries might gain access to the telomere for telomere elongation. Subtelomeric gene expression is regulated by the loop.

Our approach focuses on telomere 1L and even though sequence similarities between the telomeres might allow primer binding at other positions, sequencing confirmed our approach. For the same reason we cannot make assumptions on general telomere looping (i.e. at all telomeres). Nevertheless, telomere 1L as an X-only telomere (Figure 1) might allow us to draw conclusions of looping at other X-only telomeres. If telomere looping at Y' telomeres is regulated similarly remains unclear at the moment and comparison of looping at different telomeres has not been addressed so far. However, it is possible that fold-back structures are indeed differentially regulated. For instance Y' elements, which are present at only half of the telomeres, are not silenced, and also the peripheral localization seems to differ depending on the specific telomere composition. Additionally, TERRA regulation is distinct at different telomeres. Rap1-binding proteins Rif1 and Rif2 are mainly responsible for TERRA repression at Y' telomeres,

whereas the Sir2/3/4 complex, together with the Rif proteins, regulates TERRA at X-only telomeres (Iglesias et al. 2011).

Another limitation of our approach is the lack of information on the proportion of looped telomeres. We analyze a whole population of cells and only the comparison of mutants or conditions allows us to make any conclusions, the total number of loops cannot be determined. Microscopic approaches enable the examiner to count telomere loops or to calculate ratios of looped and non-looped telomeres. Doing so, a proportion of up to 40 % of telomeres have been counted to be in a t-loop conformation in immortalized mouse embryonic fibroblasts (Doksani et al. 2013). However, these experiments probably underestimate the real numbers. Different factors are responsible for this. First, if psoralen does not crosslink every D-loop, t-loops might be resolved and counted as linear telomeres. Second, the occurrence of DNA breaks during the telomeric repeat isolation can alter the results. If a looped telomeric repeat breaks within the t-loop, it would not be counted as a t-loop. If a looped telomeric repeat breaks within the non-looped tract, it will be counted as one looped and one non-looped telomere. Finally, if a linear telomere tract breaks, it will be counted as two non-looped telomeres. The third reason for underestimating the t-loop proportion is a potential cut-off for very small loops or other elimination criteria. With the methods available so far, an unambiguous determination of the proportion of looped telomeres is not possible. The conserved character of telomere loops, however, indicates an important function, which makes it highly likely that all telomeres are in a looped conformation at least during a certain cell cycle phase.

4.3 Short telomeres adopt an open conformation

The genetic screen to identify telomere looping defective (LD) mutants identified 112 genes implicated in a fold-back structure (Poschke et al. 2012). The bioinformatical comparison of LD and telomere length maintenance (TLM) mutants pointed towards a relationship between telomere length and telomere looping. We considered the two inverse possibilities that looping helps to maintain telomere length or that a certain length is necessary for a loop to form. If the first consideration was true, then the telomere length in LD mutants should be considerably altered, which we found not to be the case (Figure 14). Even if some of the hits obtained in the screen might represent false positive results, there should still be a trend towards elongated or shortened telomeres if telomere looping defects affect telomere length. However, we detected

shortened, elongated as well as unaffected telomeres in the LD mutant list compared to wild type telomere length (Figure 14), but the mean did not deviate greatly from wild type.

The other potential relationship would mean that telomeres can only form a fold-back structure if telomere length is similar to wild type. The bioinformatical analysis showed in particular a significant enrichment of short telomere mutants in the list of LD mutants, which indicated a connection between telomere shortening and loop deficiency. Consistently, our 3C analysis revealed a looping defect in a telomerase negative strain with very short telomeres (Figure 15), indicating that very short telomeres are in an open, non-looped conformation.

We realized that if our assumption of looping defective short telomeres was true one would expect more short TLM mutants in the list of LD mutants. While the list of short TLM mutants contains 180 mutants, the overlap was only 15. We expect that this is due to the fact that many mutants with short telomeres are missed in the genome-wide screen, for example due to control or cut-off levels. This is supported by 3C analysis of four mutants that were published to contain short telomeres but did not overlap with the LD list. Whereas we detected a looping defect at very short telomeres, a telomere approximately 90 base pairs (bp) shorter than wild type was still looping proficient (Figure 16). Our data indicate that short telomere mutants below a certain length threshold of at least >90 bp shorter than wild type are looping deficient. If this is true, then many short telomere mutants of the TLM list that have only slightly shortened telomeres are looping proficient and would not appear in the genome-wide screen, which would explain the small overlap between TLM and LD lists.

Our result means that a shortening telomere, as it naturally happens due to the end replication problem, adopts an open conformation when length drops below a certain value. This length dependent change in telomere conformation is consistent with changes in TPE-OLD upon telomere shortening (Robin et al. 2014). Even before a DNA damage response, they observed that short telomeres lost interaction with genes far in the subtelomere. On the one hand, the open structure we detected at short telomeres might ensure telomerase accessibility to telomeres, which is supported by data showing that telomerase acts predominantly on the shortest telomeres (Teixeira et al. 2004). On the other hand, these results suggest that telomerase-negative senescing cells are also in an open conformation. Telomerase negative cells in humans and yeast face telomere shortening until at one point telomere elongation via homology directed

recombination takes place to produce human ALT cells or yeast survivors. A telomere loop structure might inhibit such events and hence would have to be opened to allow recombination. Therefore, short telomeres might lose their looped conformation to additionally allow telomerase-independent telomere length maintenance (Tomaska et al. 2009), for example by giving access to DNA repair enzymes. Consistently, short telomeres have been shown to be more prone to exonuclease-mediated resection (Fallet et al. 2014). Another interpretation is based on the early replication phenotype of short telomeres (Bianchi and Shore 2007). Telomerase acts only after passage of the replication machinery and it has been speculated that the early passage ensures that telomerase can predominantly act on short telomeres for a prolonged period of time (Bianchi and Shore 2007). Since telomere loops have been suggested to present an obstacle for the replication machinery, their resolution at short telomeres might promote this early replication. Importantly, we could rule out that the early replication phenotype observed for short telomeres (Bianchi and Shore 2007) mediated the detected looping defect. Late replicating *tel1est2* mutants (Sridhar et al. 2014) still showed a length dependent reduction in telomere looping (Figure 17).

In a recent study it was shown that short telomeres get extensively SUMOylated to promote survivor formation (Churikov et al. 2016). Telomeric 3C in cells lacking the yeast SUMO E3 ligases Siz1 and Siz2 could help to determine if this SUMOylation might negatively affect telomere looping. Whereas one can speculate that telomere loops are reestablished upon telomerase mediated elongation of telomeres, it has not been addressed so far what happens to telomere loops upon telomerase-independent elongation in yeast survivors. Studies in human ALT cells, however, indicate that these cells also contain t-loops (Cesare and Griffith 2004).

Apart from telomerase and recombination-mediated telomere maintenance, the telomere loop itself has been discussed to mediate telomere elongation. It was speculated that the invading 3' overhang of mammalian t-loops gets elongated within the loop itself. Such a homology directed event would not even require loop opening (Tomaska et al. 2009) but stands in contrast to short telomeres that are looping defective.

In contrast to short telomeres, elongated telomeres are looping proficient in our 3C assay (Figure 15). This result is consistent with the bioinformatical comparison that did not detect a significant enrichment of TLM mutants with long telomeres within the list of LD mutants (see Figure 13A+D).

In addition, elongated telomeres in *K. lactis* were also able to form a t-loop (Cesare et al. 2008) and mammalian t-loops were detected in different cell lines with naturally distinct wild type telomere length.

If the yeast telomere loop is in place to regulate a telomere position effect, then the open conformation upon telomere shortening might mediate a transcriptional response for approaching senescence. This is further reinforced by studies on telomerase negative cells harboring short telomeres that show an altered expression profile of subtelomeric genes (Nautiyal et al. 2002), upregulated ncRNAs (Niederer et al. 2016) and changes in TPE-OLD (Robin et al. 2014). Additionally, shortening of mammalian telomeres was shown to alter long range telomere looping to elicit a gene expression response (Robin et al. 2014). For a more detailed discussion of the functions of the loop see section 4.5.

It remains an open question with regards to what makes short telomeres undo the loop. Yeast telomeres reach a length of 250 -350 bp. The ideal size of a protein-free DNA loop seems to be around 400 bp (Shimada and Yamakawa 1984; Amouyal 2014) but DNA binding proteins can further reduce this length requirement (Perez et al. 2014). Taz1 of *S. pombe* could promote *in vitro* t-loop formation of a model telomere of 518 bp (Tomaska et al. 2004). However, shortening of budding yeast telomeres might generate tension within the telomere loop due to the small size. This tension could then affect the DNA conformation and hence DNA binding proteins. It is believed that telomere looping in yeast occurs by forming a fold-back into the subtelomere and with an expected telomere loop size of at least 1000 - 2000 bp in yeast (Strahl-Bolsinger et al. 1997; Poschke et al. 2012) it is unlikely that physical restraints control telomere looping. We therefore propose a regulation of telomere looping that relies on some sort of protein counting mechanism. The shortening depletes telomeric binding sites and hence levels of telomere binding proteins are reduced. Alternatively, upon shortening the ratio of two proteins changes, facilitating breakup of the loop. In yeast, one candidate for a protein counting mechanism to keep the loop intact is the Sir2/3/4 HDAC complex, whose depletion is accompanied by loss of heterochromatin marks, which is also seen at short telomeres. However, the depletion of Sir proteins could not phenocopy the expression response upon telomere shortening (Nautiyal et al. 2002), suggesting that other factors may play a contributing role, for example Rif1 and Rif2.

In contrast to yeast, mammalian telomeres are rather long and t-loops have been observed in strains where wild type telomere length can greatly differ between strains. Furthermore, different t-loop sizes have been observed within the same cell line (Doksani et al. 2013) and sizes differ from species to species (de Lange 2004). This indicates that also the mammalian t-loop is not regulated by physical means or bending-mediated tension. Especially the long range interactions observed to be responsible for TPE-OLD support this hypothesis. Long before telomeres are critically short the interaction with distant loci changes (Robin et al. 2014).

Consistent with a protein counting mechanism, mammalian cells with short telomeres are supposed to contain reduced amounts of TRF2, which could lead to deprotection and loop opening at the telomere (Feuerhahn et al. 2015). Interestingly, the D-loop promoting activity of TRF2 in an *in vitro* assay was shown to be concentration dependent with a Gaussian distribution; the highest activity was obtained with an intermediate TRF2 concentration (Bower and Griffith 2014).

4.4 Starting to understand the mechanisms

4.4.1 Homologous recombination is not involved in yeast telomere looping

t-loops in human cells are formed by strand invasion of the 3' overhang into the duplex DNA of the telomere. This D-loop depends on TRF2 (Stansel et al. 2002). By wrapping 90 bp around its TRFH domain, TRF2 primes DNA topology to facilitate the strand invasion reaction (Benarroch-Popivker et al. 2016). Taz1, the TRF2 homolog in *S. pombe*, is also able to mediate t-loop formation of a model telomere template in an overhang-dependent manner, suggesting a homology-directed mechanism (Tomaska et al. 2004). One of the hypotheses concerning telomere loop formation postulates that t-loops represent a recombination intermediate and hence depend on proteins of the HR machinery. *In vitro* assays with mammalian cell extracts showed that both Rad51 and Rad52 as well as TRF1 and TRF2 are necessary for D-loop formation, a step required for t-loop assembly (Verdun and Karlseder 2006). Furthermore, abolishing HR in non-dividing cells results in telomere repair (Badie et al. 2010). By promoting t-loop formation, HR might therefore protect the telomere in a replication-independent manner by facilitating homology search and opening of the dsDNA (Bower and Griffith 2014). Data obtained in *K. lactis* mutants with overelongated telomeres also show that t-loop formation in these cells is dependent on the HR protein Rad52 (Cesare et al. 2008). In contrast to these results another *in*

vitro assay using purified proteins showed that TRF2 is able to inhibit the D-loop promoting activity of Rad51 on a telomeric model DNA (Bower and Griffith 2014). The study showed that both Rad51 and TRF2 alone can promote a D-loop. Preincubation of a telomeric template with TRF2, however, inhibits the ability of Rad51 to promote telomeric D-loop formation, suggesting that *in vivo* Rad51 does not facilitate t-loop formation and that TRF2 inhibits HR at the telomere. Interestingly, a DNA binding defective TRF2 mutant was shown to promote Rad51-mediated D-loop formation, indicating that domains of TRF2 can have different effects on Rad51 activity, potentially explaining how TRF2 can promote HR at non-telomeric sequences (Bower and Griffith 2014). TRF1, on the other hand, promotes Rad51-mediated telomeric D-loop formation.

A D-loop, probably formed by homologous sequences, seems to be a prerequisite for t-loops, and *E. coli* single-stranded DNA binding protein (SSB) could be bound to the t-loop junction *in vitro* (Griffith et al. 1999). How exactly D-loop formation at the junction is regulated *in vivo* remains unclear. *In vitro* data are contradictory, but other protein factors might be necessary for D-loop regulation and shelterin might orchestrate HR mediated t-loop formation by both its promoting and inhibiting effects.

Telomere loops in budding yeast are supposed to represent a fold-back structure in which the telomere folds back onto the subtelomeric region since telomere binding proteins could coimmunoprecipitate subtelomeric sequences (Strahl-Bolsinger et al. 1997; Poschke et al. 2012). Even though telomere homology search in the subtelomere might be difficult, the subtelomere does contain telomere-like sequences that would potentially allow DNA base pairing in a D-loop as described for human cells (Griffith et al. 1999). However, the short length of 10 - 15 bp of yeast telomere overhangs might not allow its formation. In *in vitro* studies with *S. pombe* it was shown that Taz1 was not able to remodel telomeric DNA into loops in the presence of a 14 bp overhang (Tomaska et al. 2004). Furthermore, a TRF-like protein to promote t-loops has not been identified yet in the budding yeast genome. To answer the question if HR is involved in yeast telomere loop formation, we took mutants of the HR machinery (*rad51* and *rad52*) to control for their role in looping, since no TRF-like protein has been identified in budding yeast so far. As expected, we did not see an effect on loop formation. This indicates that a D-loop or homology-directed base pairing is not involved in yeast telomere looping. We cannot exclude the involvement of other proteins that might mediate homology-directed stabilization of the loop, but nevertheless we

believe that telomere loops in *S. cerevisiae* do not depend on HR but are rather stabilized by protein interactions.

4.4.2 Only a transcribed telomere is forming a fold-back

Gene looping, a widespread type of chromatin folding, depends on transcription (Ansari and Hampsey 2005). Interestingly, the genetic screen of the viable yeast deletion collection to determine telomere looping defective mutants identified multiple components of the transcriptional machinery. These included RNA polymerase II factors (Rbp9, Ctk1), mediator subunits (Med1, Nut1), as well as the histone methyltransferase Set2, which promotes transcription elongation (Poschke et al. 2012). It is important to remember that the screen only included viable deletion mutants, meaning that the most prominent and essential components of the transcription machinery had not been covered. Rpb9 is a subunit of RNA Pol II (Cramer 2002) and required for start site selection at promoters (Hull et al. 1995), similar to transcription factor II B (Sua7). Ctk1 is the catalytic subunit of the C-terminal domain kinase (CTDK-1) that regulates the C-terminal domain (CTD) of RNA Pol II. Ctk1 phosphorylates serine 2 of the CTD and thereby promotes transcription elongation (Cho et al. 2001). Set2 was shown to associate with the RNA Pol II CTD and Ctk1 where it also supports transcription elongation (Schaft et al. 2003).

Studies on the transcription dependency of gene loops usually employ the temperature sensitive (ts) allele *rpb1-1* of the largest subunit Rpb1 of RNA Pol II (Nonet et al. 1987). This allele ceases mRNA synthesis at the non-permissive temperature of 37°C. At the telomere, however, the elevated temperature decreased the looping signal already in the wild type (data not shown). This could be due to limitations of our method, but an effect of high temperature on telomere looping cannot be excluded. Consistently, mammalian TRF2 levels at telomeres are reduced upon heat shock (Petrova et al. 2014) and *S. cerevisiae* telomeres shorten during growth at elevated temperatures (Paschini et al. 2012; Millet et al. 2015). Furthermore, heat shock in immortalized mouse embryonic fibroblasts (MEF) provoked a reversible increase in TERRA levels (Schoeftner and Blasco 2008). According to these results, it does not seem feasible to use a temperature shift when looking at telomere structures. To circumvent usage of a ts allele, we employed an auxin inducible degron to deplete Rpb1. After 2 hrs of auxin addition, Rpb1 was largely depleted, however, a faint signal was still detectable. Telomeric 3C could detect a reduction of telomere looping by 50 % (Figure 19), whereas remaining looping might be due to residual Rpb1. We cannot

exclude indirect effects of our approach, since transcription inhibition by Rpb1 knock down might deplete other proteins that are required for telomere looping. However, Christiano et al. (2014) determined a median protein half-live of 8.8 hrs in budding yeast (Christiano et al. 2014). We also searched their published list of protein half-lives of a large subset of budding yeast proteins for interesting candidates with half-lives shorter than 2 hrs (Christiano et al. 2014). We only identified the RNA Pol II CTD kinase Ctk1 with a half-live of 0.2 hrs. Since Ctk1 is implicated in transcription itself, we do not expect an additional influence on the outcome of our Rpb1-depletion experiment. Further, the half-live of proteins that might be involved in telomere looping are over 6 hrs (Sin3: 6.4 hrs; Sir2: 6.4 hrs; Sir3: 9.9 hrs; Sua7: 7.1 hrs; Hrs1: 6.8 hrs; Ku70: 7.2 hrs; Ku80: 9.3 hrs; Rad50: 7.7 hrs; Upf1: 9.1 hrs; Est1: 9.8 hrs). We therefore think that it is unlikely that the observed looping defect upon Rpb1 depletion is due to indirect effects on protein levels. Our results rather point towards a transcriptional regulation of yeast telomere looping structures, just like it has been shown for gene loops (Ansari and Hampsey 2005). Although it has not been proven so far, it seems likely that the transcription of TERRA is the transcriptional step required for loop formation (see below for further details). It also remains an open question if constant transcription is required or if an initial round of transcription is sufficient to form a telomere loop, as it has been proposed for gene loops (Ansari and Hampsey 2005).

We could furthermore show that cells with a defective transcription preinitiation machinery (*sua7-1*) are telomere looping defective. Sua7 encodes for transcription factor II B, and cells harboring the allele *sua7-1* contain an E62K mutation within its B finger domain (Pinto et al. 1994). As a result, they are defective in RNA Pol II transcription start site selection and cold sensitive (Sun and Hampsey 1996), but show wild type transcript levels (Pinto et al. 1992; Cho and Buratowski 1999). Interestingly, Sua7 has been shown to functionally interact with the RNA Pol II subunit Rpb9 (Sun et al. 1996) that was reported to be telomere looping defective according to the genetic screen described above (Poschke et al. 2012). The mutant *sua7-1* can still interact with RNA Pol II and the TATA box binding protein (TBP) similar to wild type and effectively assembles the preinitiation complex (PIC) (Sun and Hampsey 1996). Its binding affinity for the promotor region as well as the recruitment of general transcription factors is also unaffected. However, *sua7-1* is unable to interact with terminator bound factors, resulting in a loss of gene looping (Medler et al. 2011; Mukundan and Ansari 2013). This is not surprising, since it represents the connection of

promoter and terminator bound factors. In a telomere loop context, however, one has to ask the question if the telomeric sequences even contain these terminator bound factors. The telomeric sequence does not allow for a canonical terminator sequence and it remains to be determined if factors of the CPF or CFI associate with the telomeric repeats in yeast. In addition, gene looping requires an intact polyadenylation signal, which is also missing in the telomeric repeats.

Since TERRA, as the telomeric transcript, has been shown to contain telomeric repeats ranging from 100 – 400 bp independent of the telomere length (Porro et al. 2010), it seems that termination of the transcript is rather a stochastic event independent of the telomere sequence. The canonical termination machinery is probably not involved, although yeast TERRA is polyadenylated. The status of human TERRA modifications further supports the hypothesis of an alternative termination pathway. Human TERRA exists as a polyadenylated and a non-polyadenylated, chromatin associated population. Polyadenylation therefore probably happens in the nucleus, post- and not cotranscriptionally. Interestingly, the poly- and non-polyadenylated fractions terminate in different sequences, indicating different termination processes (Porro et al. 2010). Since the canonical termination machinery is missing, we speculate that Sua7, probably located at the TERRA promoter, might interact with a distinct telomere binding protein to promote telomere looping.

The involvement of transcription and the transcription machinery in telomere looping is further supported by the telomere looping defect of a non-essential mediator mutant. The multiprotein mediator complex is a dynamic complex that consists of up to over 25 subunits. Many different roles during transcription initiation, transcription elongation and termination have been assigned to the complex and 10 subunits appear in the TLM list, affecting telomere length positively and negatively (Peng and Zhou 2012). Mediator in fact has been shown to bind in the subtelomere 1000 – 2000 bp away from the telomeric repeats and bordering the X elements. Within the X elements mediator seems to be absent (Zhu et al. 2011). The distance of mediator to the telomeric repeats might represent the suggested touch-down point or area of the telomere loop in yeast (Poschke et al. 2012). The genetic screen to detect telomere looping defective mutants in yeast, only covering the non-essential components of mediator, identified the middle module components Med1 and Med5 (Nut1) as necessary for telomere looping (Poschke et al. 2012). Both *med1* and *med5* mutants have been shown to have slightly shortened telomeres and unaffected

TPE at a modified telomere depleted of X and Y' elements (Peng and Zhou 2012). *MED5* deletion furthermore led to shorter replicative life span in yeast and to a depletion of mediator from the subtelomeric regions flanking X elements (Zhu et al. 2011). Interestingly, in this study *med5* was shown to provoke desilencing of a subtelomere that contained the natural X element (Zhu et al. 2011).

In the genetic screen to test for looping defective mutants, *med1* and *med5* mutants were able to grow on 5-FOA containing plates, which on the one hand indicates a telomere looping defect and on the other hand argues against a silencing defect in these mediator mutants (Poschke et al. 2012). We chose a mutant of the non-essential mediator component 3 (Med3/Pgd1), also called Hrs1, for telomeric 3C analysis. Hrs1 is part of the tail module of mediator together with Med15/Gal11, Med2 and Med16/Sin4 (Gonzalez et al. 2014). Hrs1 is required for transcription activation by the tail module, which is also required for the recruitment of the core mediator complex and correct PIC assembly. Yeast Hrs1 has been shown to be regulated via phosphorylation by the mediator kinase module Cdk8, resulting in Hrs1 degradation and loss of gene activation (Gonzalez et al. 2014). Hrs1 has also been implicated in telomere biology, since *hrs1* mutants show reduced telomeric transcript levels (Yu et al. 2014). The slight shortening of telomere length that we detected supports data published previously (Peng and Zhou 2012). In telomeric 3C we detected a strong looping defect of this mediator mutant (Figure 21). Since Cdk8-mediated turnover of Hrs1 prevents mediator recruitment at least at some promoters, a deletion is probably having the same effect on PIC formation (Gonzalez et al. 2014). Together with the reduced telomeric transcription, the data supports both the requirement for transcription and the promoter-bound complexes to form a fold-back at the telomere. However, we have to take into account that mutating Hrs1 could lead to depletion of mediator from the telomere. Since mediator also plays a role in forming a boundary between heterochromatin mediated by the HDAC Sir2 and euchromatin mediated by the histone acetyltransferase (HAT) Sas2, its absence could result in a misbalance between the two. Shifting the ratio towards higher Sas2 levels can increase histone acetylation at the telomere, opening the telomeric chromatin for higher transcription rates and desilencing. *med5* might have a similar effect, but as described earlier, the effect of *med5* on silencing is controversial (Zhu et al. 2011; Peng and Zhou 2012). The looping defect of mediator mutants could therefore also be a result of reduced Sir2 level at the telomere

or reduced silencing in general. However, reduced TERRA levels in a *hrs1* mutant are in contrast to this finding (Yu et al. 2014). Taken together, our results draw striking parallels between canonical gene looping and telomere looping in that both are strongly dependent on an intact transcription apparatus.

Telomeres are transcribed into the long non-coding RNA (lncRNA) TERRA. It has been shown that ncRNAs can be involved in mammalian chromatin loop formation, where the RNA expressed at a distant enhancer locus is required for the regulation of the target gene by mediating a chromatin loop (Li et al. 2013). Importantly, in one report it was shown that the ncRNA-a associates with the promoter-bound mediator complex to form a looped conformation (Lai et al. 2013). Both depletion of mediator components (MED1 of the middle module and MED12 of the kinase module and probably others) as well as depletion of the ncRNA by RNA interference abolished the chromatin loop. Interestingly, siRNA against the ncRNA also depleted mediator from the promoter region of the target gene. It would be interesting to analyze mediator localization to the telomere upon TERRA depletion or at a non-transcribed telomere. Our data on *hrs1* and transcription prompted us to ask if TERRA or another RNA species might be involved in telomere looping.

Since yeast does not express the components necessary for RNA interference, it is difficult to deplete specifically one certain RNA species. We tried to introduce a terminator sequence downstream of the TERRA transcriptional start site, but technical difficulties have impeded the generation of such a TERRA-less telomere so far. A doxycycline responsive TERRA transcription cassette has been published before (Pfeiffer and Lingner 2012), but TERRA levels were only reduced upon doxycycline addition and not completely depleted. Furthermore, such a cassette largely alters the subtelomeric sequence, with unknown effects on telomere looping. One problem that arises by these approaches would be that we affect TERRA levels only indirectly via the transcription. Even though a telomere loop defect would strengthen the transcription dependency directly at the telomere, it would not necessarily confirm a TERRA dependency. Directly depleting the RNA would therefore be the method of choice. To get a first insight into RNA involvement in telomere looping we depleted bulk RNA *in vivo* by the addition of RNase A to zymolyase-treated cells. These spheroplasts are missing the cell wall and can easily take up the enzyme; consequently, RNA was hardly detectable after 30 minutes (see Figure 22A+B). Our

telomeric 3C revealed a looping defect upon RNA depletion, indicating that RNA is indeed necessary for telomere looping. We cannot distinguish between TERRA and other RNA species and we cannot rule out indirect effects due to depletion of telomerase RNA or a reduction in protein levels. We speculate, however, that 30 minutes are not enough to exert a major effect on protein levels. So far it is not known if mediator, a known RNA interacting protein, also associates with TERRA molecules, but studies on the TERRA interactome could not identify mediator components (Lopez de Silanes et al. 2010; Scheibe et al. 2013). Mediator, however, can promote chromatin loop formation by an interaction with lncRNA molecules in mammals (Lai et al. 2013). Loss of yeast telomere looping upon RNA depletion or in a mediator mutant therefore opens the interesting possibility that telomere fold-back structures are similarly regulated. Interestingly, also human TRF2, required for t-loop formation, has been shown to bind telomeric DNA and TERRA simultaneously (Biffi et al. 2012).

It is important to carefully interpret the looping data upon RNA depletion. RNase A treatment affects all RNA molecules and a mediator mutation can also affect different pathways and chromatin looping in an RNA-independent fashion. Mediator's CDK8 histone kinase activity could also directly affect chromatin architecture, for example by phosphorylation of histone H3 (Meyer et al. 2008).

TERRA might be necessary for telomere looping, but if so, it does not seem to be sufficient, as short telomeres are highly transcribed and also telomere looping-defective *sir2* mutants express high levels of TERRA (Iglesias et al. 2011). However, a multifunctional nature of TERRA is highly likely (see 1.1.5). At short and non-looped telomeres TERRA seems to exert functions that promote recombination, probably by the formation of RNA:DNA hybrids (Balk et al. 2013). However, transcription also seems to be an important prerequisite for telomere looping. Since highly induced telomeric transcription in yeast is able to shorten telomeres, it might be a question of transcription rates or transcript levels to switch between functions at the telomere. Even if it is not TERRA, it will still be interesting to determine the identity of the loop-promoting RNA. Assessing its transcriptional regulation might furthermore help to understand the temporal regulation of the telomere loop itself.

Since our results indicate that telomere looping is dependent on mediator and RNA, it might also be interesting to analyze the involvement of cohesin. Cohesin, with its ring structure and role in

mammalian chromatin looping together with mediator or CTCF, is predestined to be studied in telomere looping as well (Kagey et al. 2010; Shibayama et al. 2014). Cohesin also localizes to mammalian telomeres (Deng et al. 2012) and inactivation of ATRX, a chromatin remodeler in human cells, led to reduction of cohesin at the telomere, which correlated with reduced TERRA levels (Eid et al. 2015). If there is a direct connection between telomere looping and cohesin has not yet been determined.

4.4.3 Temporal telomere loop regulation

Another interesting aspect would be the understanding of the temporal regulation of telomere looping. The strand-invasion at the junction of human t-loops could be an intermediate structure of HR-mediated DNA repair and represent an obstacle for the cellular replication machinery. Consistently, it has been shown that TRF2 recruits the helicase RTEL1 to telomeres in S phase. RTEL1 is able to resolve D-loops and might be responsible to undo t-loops as well (Vannier et al. 2012). RTEL1 recruitment might therefore allow replication or elongation by telomerase. Loss of interaction between TRF2 and RTEL1 or deletion of RTEL1 leads to telomere length heterogeneity and telomere loss (Vannier et al. 2013; Sarek et al. 2015), showing that loops also inherit a deleterious effect if not properly resolved. In addition, the yeast CST complex that caps and protects the terminal end of telomeres is only required during S phase (Vodenicharov et al. 2010). If telomere loops also protect the telomere, two interpretations are conceivable: 1. The CST complex is necessary in S phase because a telomere loop is not established at that time, or 2. In S phase, telomere loops and the CST complex are parallel pathways to protect the telomere, as only the combination of uncapping and a looping mutant had an effect on viability (Poschke et al. 2012). We have detected a looping defect at very short telomeres and interestingly, it has also been shown that eroded telomeres recruit the CST complex (Khadaroo et al. 2009), maybe as a backup mechanism for protection. In budding yeast the loop is probably not hiding the free 3’ end by strand invasion and thus telomere binding proteins could be additionally required for telomere protection, supporting the second interpretation. Interestingly, mammalian nuclear extracts from synchronized mammalian cells in S/G2 phase have the highest D-loop formation activity, in agreement with the highest activity of HR (Verdun and Karlseder 2006). Maybe telomere loops have to be resolved when replication reaches the chromosome ends but then needs to be reestablished upon passage of the replication machinery to protect the free end.

The telomere loop might regulate telomeric transcription (also see section 4.5.2). TERRA has been shown to be cell cycle regulated with a peak in G1, followed by a strong reduction in S phase (Porro et al. 2010).

To obtain a first idea of telomere loop cell cycle regulation we arrested yeast cells in different cell cycle phases, namely S phase, G1 and G2/M phases. For all arrested populations we detected a loss of telomere looping, arguing that a process during the cell cycle is necessary for loop formation or maintenance. Interestingly, a prolonged mitotic arrest in mammalian cells leads to dissociation of TRF2 from the telomere and induction of a DDR (Hayashi et al. 2012). Since TRF2 is thought to be the main mediator of t-loops, it is possible that its dissociation leads to an open conformation at the telomere.

We also tried to investigate the temporal regulation of telomere looping in cycling cells upon release from α-factor arrest. Importantly, we noted that after the release we detected absolute telomere looping values at all time points that were many fold lower than in pre-arrested cells. This means that something must have happened to the cells during the arrest that reduced all subsequent looping signals. This is consistent with the permanently arrested cells that also showed a reduced signal compared to a cycling wild type that has never been arrested before. However, in one of the experiments (experiment 2) the absolute value after 15 min, which corresponds to early/mid S-phase, is similar to pre-arrested cells. This could be an artefact, but it could also be an indication that only during this short period of the cell cycle telomere looping is present or up-regulated. In the other experiment (experiment 1) we did not see the same effect, but the cell cycle analysis showed that cells had not yet progressed into S phase. In other words, after 15 minutes, cells of experiment 2 had already progressed further into the cell cycle than cells of experiment 1. Hence, the 15 minute samples of both experiments did not represent the very same phase, which could explain the different looping signals at both 15 minute time points. It is possible that the windows between different samples in one experiment were too long, and as a consequence we might have missed the increased looping in experiment 1. Either shorter windows should be chosen or, as another solution, growth at lower temperatures could slow down the cell cycle. In any case, the looping pattern of the cell cycle in these two experiments shows a reduction in telomere looping especially during late S phase, which would correlate with the passage of the replication machinery, since telomeres are replicated late. At the same cell

cycle phase TERRA levels are reduced in HeLa cells (Porro et al. 2010), which would argue that a telomere loop positively affects TERRA (see more on TERRA regulation in 4.5.2).

Furthermore, our data suggests that looping is upregulated in early S phase when early replication at other chromatin loci takes place, which would indicate a very narrow window of telomere loop formation. Whether telomere looping is then again upregulated after replication is difficult to determine with our results. Further experiments will be necessary to allow a more detailed analysis of telomere loop cell cycle regulation. Since we consistently see a drop in looping upon cell cycle arrest, applying another method for the synchronization of *S. cerevisiae* might be required. An option that could avoid the encountered problems might be elutriation, a method based on centrifugation to separate particles according to their size, mass and shape distribution (Futcher 1999; Amon 2002). Since all these aspects of a cycling yeast cell can be attributed to specific cell cycle phases, it is possible to separate and isolate cells in the same phase and highly synchronously without the need of an arrest and release experiment. Perturbations of cell growth and division are thereby avoided. If an arrest per se reduces telomere looping, we can expect higher and more accurate interaction values with this method. The necessity of high culture volumes and a potential stress response are, however, clear drawbacks of this method.

4.4.4 (Temporo-) Spatial telomere loop regulation

It has been shown that chromosomes, and especially telomeres, are spatially organized within the yeast nucleus (Taddei and Gasser 2012). This compartmentalization could help regulate nuclear processes including gene transcription, silencing and repair (Schober et al. 2009). Whereas centromeres in interphase nuclei are attached to the spindle pole body (SPB), telomeres are clustered at the nuclear periphery in G1 and early S phase (Gotta et al. 1996; Hediger et al. 2002; Bystricky et al. 2004; Bystricky et al. 2005). Telomere localization at the nuclear periphery contributes to the establishment of silent heterochromatin at yeast telomeres (Taddei and Gasser 2012) and is regulated by at least two redundant pathways (Schober et al. 2009). One pathway requires Sir4 that localizes heterochromatin to the nuclear envelope through an interaction with the nuclear envelope protein Esc1 in S phase (Hediger et al. 2002; Taddei et al. 2004). Sir 4 also interacts with the SUN-domain protein Mps3 at the nuclear envelope (Taddei and Gasser 2012). The second pathway involves yKu80 that interacts directly with an unknown nuclear envelope factor during G1, as well as indirectly via telomerase binding to Mps3 to mediate anchorage of

telomeres to the periphery in S phase cells (Schober et al. 2009; Taddei and Gasser 2012). Interestingly, the telomere associated sequences (X and Y' elements) influence the relative importance of the two pathways (Hediger et al. 2002).

Importantly, peripheral localization is not static. Replicating telomeres are released from the periphery in late S phase (Ebrahimi and Donaldson 2008) and short telomeres have been shown to move to the nuclear pore complex (NPC) to colocalize with Rad52, a protein of the homology-directed repair pathway (Khadaroo et al. 2009). It would be interesting to analyze if the temporal telomere looping pattern correlates with the localization of telomeres in the nucleus.

Gene loops are tethered to the nuclear periphery in a looping dependent manner and when the localization is abolished, gene loops lose transcriptional memory (Tan-Wong et al. 2009). Since memory gene loops (MGL) were shown to require localization to the nuclear periphery to maintain the looped structure, it might be possible that telomere loops also correlate with the nuclear localization. Different scenarios could be envisioned: In the first scenario the telomere loop might be present at the nuclear envelope, consistent with MGLs. The loop could be in place to ensure the localization or the heterochromatic character of the telomere with low TERRA expression. During S phase telomeres get released for replication. The loop opens to allow passage of the replication machinery, also explaining why the telomere protecting CST complex is required during this stage (Vodenicharov et al. 2010). Since short telomeres are looping defective and early replicating, the telomere loop could also somehow tether the telomeres to the periphery. Loss of looping might release telomeres and allow replication.

In the second scenario a telomere loop at the periphery might not be necessary because the localization to the nuclear envelope protects the telomere per se from repair pathways and recombination. Consistently, increased chromatin mobility promoted non-homologous end-joining in mouse cells (Dimitrova et al. 2008). Only during replication in S phase, when the chromosome is released from the periphery, looping could be required to protect the telomere similar to the CST complex (Vodenicharov et al. 2010). Consistent with this hypothesis, unscheduled release from the periphery causes unprotected and hyperrecombinant telomeres (Schober et al. 2009).

The third scenario is independent of the others and is only relevant when telomeres get very short in a telomerase negative background. It has been demonstrated that extensive SUMOylation of

telomere binding proteins at eroded telomeres recruits the SUMO-targeted ubiquitin ligase (STUbL) Slx5-Slx8. This STUbL relocalizes eroded telomeres to the nuclear pore complex (NPC) to promote type II survivor formation (Churikov et al. 2016). In addition, TERRA is upregulated at short telomeres (Cusanelli et al. 2013) and we detected a looping defect upon extensive telomere shortening. However, whether loss of looping leads to relocalization of the telomere or vice versa needs to be further analyzed. The same holds true for TERRA expression, where the observed increase could be caused by the missing loop or by the relocalization itself.

Since the telomere localization can be addressed to precise cell cycle phases (see above), the temporal analysis of telomere looping could also help to get a first idea on spatial regulation. Again, our data is probably not precise enough, requiring further and more detailed repetitions. If the increase in looping we see approximately in S phase is true, this would argue that looping is required when telomeres are released from the periphery, consistent with the requirement for the CST complex. Another option to study the relationship between telomere localization and looping would be the analysis of anchoring-defective mutants. Analyzing the mutant *mps3Δ75-150* defective in telomere anchoring to the nuclear envelope (Bupp et al. 2007) would allow to investigate the role of the nuclear envelope in telomere looping. The same holds true for *sir4* or *siz1* and *siz2* SUMO E3 ligase mutants that lack telomere SUMOylation, accompanied by a loss of peripheral localization (Ferreira et al. 2011). Cells lacking the myosin-like proteins 1 and 2 (Mlp1 and Mlp2) and *ulp1ΔN* or *slx8* mutants are deficient in localization of short telomeres to the NPC (Churikov et al. 2016) and could help to decipher the role of the nuclear pore in telomere looping. In the inverse experiment one could tether telomeres to the NPC and then investigate telomere looping (Churikov et al. 2016).

4.4.5 More on telomere loop regulation

Another structure that can be formed by the telomere is the G4-quadruplex, formed by G-rich repeats, either by the single-stranded overhang or by dsDNA (Tang et al. 2008). The G4-quadruplex on the one hand was speculated to protect the telomere and regulate telomerase, since a quadruplex stabilizing molecule was able to inhibit telomerase (Shin-ya et al. 2001). On the other hand it has been shown that G4-quadruplexes can form at the junctions of a DNA loop *in vitro* (Xu et al. 2007; Xu et al. 2008). We reasoned that also the fold-back structure at the budding yeast telomere might be stabilized by quadruplex formation where the single-stranded

overhang would pair with homologous regions in the subtelomere to form the quadruplex stacks. The overexpression of Pif1, a helicase that was shown to undo quadruplex-structures (Paeschke et al. 2011) without an effect on telomere length or the cell cycle, did also not effect telomere looping (Figure 25). This result indicates that quadruplex formation at the telomere is not involved in telomere loop formation in *S. cerevisiae*. Since G-rich telomeric sequences meet the prerequisites for quadruplex-formation, they might represent an additional structure with additional functions at the telomere that coexists with telomere loops.

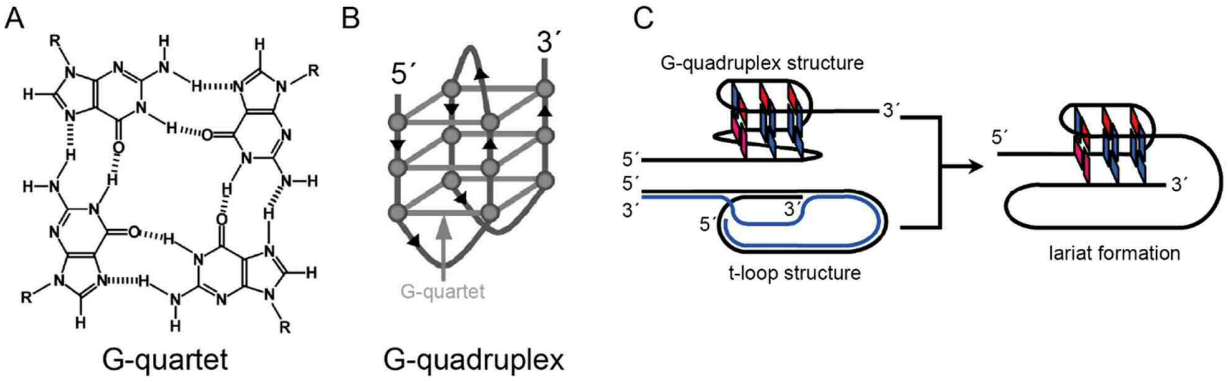

Figure 28: Structure of G4-quadruplex
A+B) Adapted from (Tang et al. 2008) A) Guanines form one stack of a G4-quadruplex by hydrogen bonding. B) A G-rich sequence can fold back to form a G4-quadruplex consisting of several guanine stacks. C) Adapted from (Xu et al. 2008) graphical abstract. A t-loop might be a combination of 3' strand invasion and a G4 quadruplex at the junction of the loop.

4.5 Why do telomeres loop?

4.5.1 Telomere protection

Telomere looping might be in place to protect the telomere from resection, unscheduled activity of the repair machinery, from telomerase or end-to-end fusions. However, it is likely that telomere end protection is maintained by redundant mechanisms that include both telomere binding proteins and the telomere loop structure. The consequence is that the absence of one pathway can be compensated by the other. A corresponding model that has been described before (Cesare and Karlseder 2012; Luke-Glaser et al. 2012) proposes three states of telomeres. The first state is the fully protected state that is characterized by a telomere loop and ample telomere binding proteins. The second state is an intermediate with reduced binding proteins or reduced protective looping structures. Importantly, even though intermediate-state telomeres exert a DDR, they still repress end-to-end fusions. The third state is represented by an unprotected telomere that lacks both proteins and the telomere loop and is prone to fusion

events. A recent study by the Giraud-Panis group supports this model. They introduced a TRF2 separation of function allele, TRF2 topless, that is deficient in DNA wrapping around itself. This mutant was shown to be t-loop defective, while TRF2 remained within the shelterin complex at the telomere. The reduced levels of t-loops correlated with ATM checkpoint activation. However, they were still protected from chromosome end-to-end fusions. Only the additional depletion of TRF2-bound RAP1 caused an increase in fusion events, while deletion of RAP1 alone had no effect on fusion rates (Benarroch-Popivker et al. 2016). Also in our experiments we could not detect an increase in fusion events in looping defective yeast mutants (data not shown). The depletion of POT1, the ssDNA binding shelterin component, leads to ATR activation without loss of telomere looping (Doksani et al. 2013), indicating that t-loops are in place to protect the telomere specifically against ATM activation. Consistently, mutant TRF2 proteins lacking the capability to promote strand invasion were also shown to elicit ATM activation (Okamoto et al. 2013). It was proposed that t-loops protect the telomere against end-to-end fusions by hiding the free DNA end, but RAP1 seems to represent an additional backup pathway that prevents fusion events. Hence, TRF2 protects the telomere from NHEJ by two distinct pathways involving RAP1 recruitment and t-loop formation, where t-loops might have evolved as the first means of telomere protection (de Lange 2015). In yeast, the combination of a telomere looping defective mutant (*sin3*) with a temperature sensitive allele of the CST complex (*cdc13-1*) supports the idea of parallel pathways for telomere protection. Only at the non-permissive temperature, when telomeres were supposed to be both non-looped and uncapped, cells faced premature senescence, cell cycle arrest and an increase in Exo1-mediated ssDNA at the telomere, indicative of extensive resection (Poschke et al. 2012).

4.5.2 Transcription regulation and telomere position effect

Our results show strong parallels between gene and telomere looping and it will be interesting to determine whether, like gene loops and chromatin loops, telomere loops have an influence on gene transcription (Figure 27). There are a few indications to suggest that this may be the case. Long range telomere loops involved in TPE-OLD in human cells were shown to regulate gene expression of distant genes (Robin et al. 2014). Furthermore, short telomeres elicit a signature gene expression profile in senescent cells, including an upregulation of subtelomeric genes (Nautiyal et al. 2002). Finally, mediator is generally involved in transcription regulation of genes,

which is oftentimes DNA looping-dependent. One can speculate that the gene expression profile in senescent cells is due to the inability of short telomeres to form a telomere loop, which would be consistent with the data obtained for TPE-OLD. However, the relocalization of short telomeres to the nuclear pore complex (Khadaroo et al. 2009) as well as the upregulated TERRA levels at short telomeres might also mediate the effects on gene transcription. In human cancer cells, upregulated TERRA levels were shown to repress innate immune genes, which was sequence independent but required G4-quadruplex formation of the RNA (Hirashima and Seimiya 2015). But apart from TERRA, if it was true that the effects of short telomeres on a subset of different genes was directly mediated by the loss of telomere looping, two different explanations could account for this: 1. the telomere forms a very dynamic loop that can approach different genomic loci (Figure 29A). This could explain why, when looking at a population of cells, one telomere can be found close to different genes (Robin et al. 2014). Further, long human genes were shown to form dynamic gene loops, indicating that a gene loop is not a static entity (Larkin et al. 2012). Or 2. the telomere is part of a multigene complex or topologically associated domain (TAD; Figure 29B). Such a complex represents a looping hotspot that can include several chromatin loops, whose bases associate in one domain (Fanucchi et al. 2013). In such a conformation the telomere could affect several genes simultaneously (Figure 29B). The hierarchical formation observed for a multigene complex (Fanucchi et al. 2013) could even allow sequential resolution and temporally coordinated elimination of specific genes of the complex. Ongoing shortening of a telomere could therefore regulate a set of genes according to the telomere length status. Results obtained with chromosome conformation capturing shows that a telomere interacts with more than one locus far in the subtelomere (Robin et al. 2014; Robin et al. 2015). However, to exclude that this is the result of looking at a population of cells, one would need to look at this at a single cell level. Interestingly, some genes were up- and some down-regulated, indicating that a telomere loop can have distinct effects on transcription, depending on the gene setting.

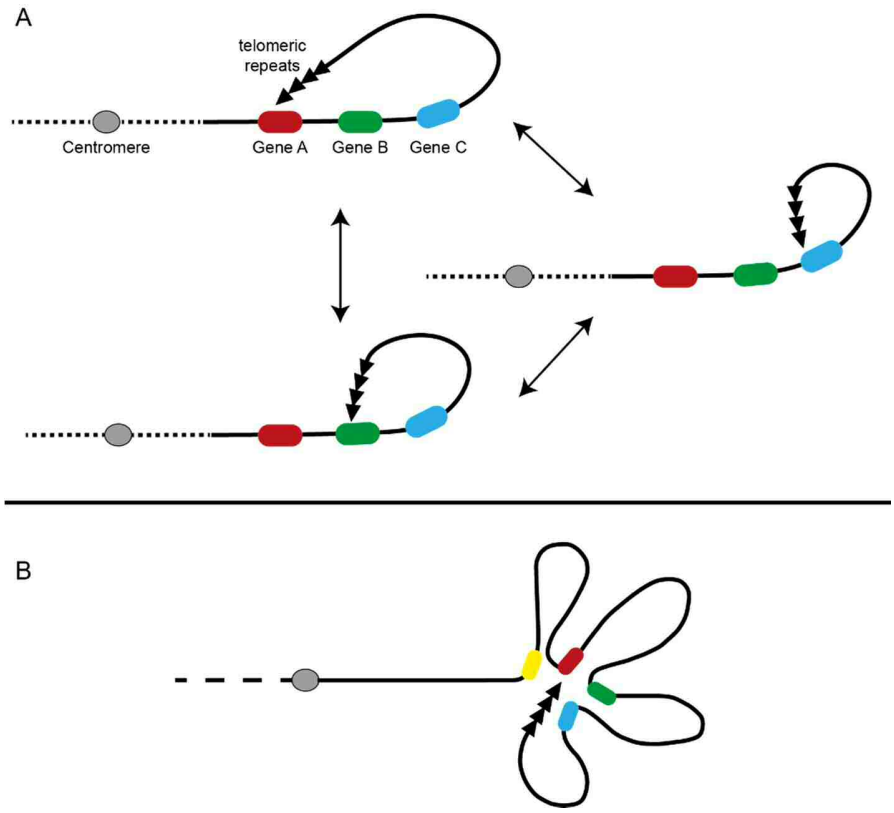

Figure 29: Potential telomere conformations
A) The telomere in a dynamic loop that forms between the telomeric repeats and one gene on the chromosome. This conformation is dynamic and the specific gene may be different in different cells or under different conditions.
B) The telomere in a multigene loop. The telomeric repeats get into close proximity of several genes at the same time.

Whereas telomere shortening affects the transcription of distant genes in TPE-OLD, telomere looping structures might also affect the chromatin state and silencing in the vicinity of the telomere. Induced transcription at the yeast telomere was shown to abolish TPE (de Bruin et al. 2000). This effect correlated with a loss of Rap1 signals in the subtelomere, while Sir3 levels declined only slowly. It was speculated that a loss of telomere looping might be the cause for this observation, yet forced telomere transcription is a non-natural situation that has been shown to also lead to telomere shortening (Maicher et al. 2012).

Functions of the telomeric transcript TERRA are still under heavy debate, as is its regulation. Telomere loops might be a means of the cell to control transcription not only of subtelomeric genes but also of the telomeric transcript itself. As induced TERRA transcription leads to telomere shortening (Maicher et al. 2012), it is likely that excessive transcription must be prevented by the cell. In contrast, TERRA in RNA:DNA hybrids at short telomeres promotes elongation by HR

mediated mechanisms (Balk et al. 2013). Telomere looping should therefore be required to prevent TERRA transcription under normal conditions, and loop opening as a result of telomere shortening could allow for the necessary increase in TERRA transcription. High TERRA levels at short telomeres in a human and yeast background (Cusanelli et al. 2013) as well as in a looping defective *sir2* mutant (Iglesias et al. 2011) are in support of a negative effect of telomere looping on TERRA transcription. At long telomeres that are looping proficient in our assay (Figure 15), TERRA levels are probably low, which would also fit to the hypothesis, although in this context different results on TERRA levels have been published (Arnoult et al. 2012; Smirnova et al. 2013; Van Beneden et al. 2013). A connection of looping and TERRA transcription is also illustrated by CTCF and cohesin that bind to the human TERRA promoter region. Depletion of CTCF, an insulator involved in chromatin looping, reduced TERRA levels as well as cohesin- and RNA Pol II-association with the promoter (Deng et al. 2012). Measuring TERRA levels in all our supposed telomere looping defective strains does not support this idea. In fact, TERRA levels do not seem to be regulated by telomere looping, as we detect increased, unaffected and even decreased levels in looping defective mutants (Figure 26). We cannot exclude that the mutants affect TERRA levels independent of telomere looping, since Sua7 is implicated in transcription and others in silencing and telomere localization (Sin3, Sir2 and Ku70). Final conclusions are hence difficult to draw.

An interesting observation is the involvement of general transcription factors (TFIIB and TFIID) and transcriptional activators in recruiting polyadenylation factors to gene terminators (Dantonel et al. 1997; Wang et al. 2010; Nagaike et al. 2011), providing a link between the promoter and terminator. Consistently, *sua7-1* and *med18* show a transcription termination defect that correlates with defective looping at a subset of genes (Mukundan and Ansari 2013). Since canonical terminator sequences are missing in the telomere, telomere looping could promote TERRA termination at the telomere and it would be interesting to analyze TERRA length or TERRA posttranscriptional modifications in a telomere looping defective mutant.

Another discussed function of gene loops is the generation of transcriptional bursts to increase transcriptional noise and to allow better adaptation to changing environments (Hebenstreit 2013). Telomere loops could have the same function on telomere transcription. However, transcriptional bursts are thought to occur when loops are closed; a conformation that allows fast

recycling of the RNA polymerase from the terminator to the promoter. The fact that telomere looping mutants like *sir2* show high levels of TERRA contradicts this hypothesis (Figure 26B).
Interestingly, TERRA is not the only transcript of the telomere. There is also a telomeric antisense transcript that was termed ARRET, identified in *S. cerevisiae* (Luke et al. 2008) and human cells (Azzalin et al. 2007). In *S. pombe*, a whole telomere transcriptome could be identified (Bah et al. 2012). Since gene loops have been shown to confer directionality at bidirectional promoters (Tan-Wong et al. 2012), telomere loops could be a means to regulate the telomeric transcriptome. Unfortunately, the other transcripts are even less abundant than TERRA and thus analyzing their levels is difficult and has not been addressed so far.

4.6 Concluding remarks and future perspectives

Most of the studies on telomere looping have been performed in mammalian cells, where one has to distinguish between the t-loop that involves only the telomeric repeats and long range interactions that seem to be responsible for telomere position effects on genes up to megabase pairs away from the telomere. The identity of the telomere loop we observe with our approach and whether it belongs to one of these two mammalian classes is not yet clear.
This work has established a new way to directly look at telomere looping in yeast. It has uncovered novel aspect of yeast telomere loop formation, unravelling mechanisms how fold-back structures at the telomere are generated and how they might be maintained. We could identify key regulatory factors and show that loop formation in yeast is telomere length dependent and forms in a transcription dependent manner. It will be interesting to analyze if the telomeric transcript TERRA is involved in telomere looping, which might also help to decipher the role of this ncRNA. However, important for this will be the generation of a TERRA less telomere, whose generation has proven to be difficult so far.
We furthermore show that the telomere loop in yeast is maintained independent of Rad51 and Rad52, which is in contrast to *in vitro* data obtained for mammalian t-loops. However, it does not exclude the possibility that sequence homologies in the subtelomere are used for loop formation by other telomere binding proteins, as for example Rap1. Whereas in mammalian cells data indicate that TRF2 is directly involved in t-loop formation, no yeast telomere binding proteins have been identified so far that would act in a similar way and 3C at the telomere might help to identify these proteins. Apart from further insights into telomere loop regulation and

maintenance, it will also be important to determine its function. So far only few studies have investigated possible roles of a fold-back. This could be due to the lack of tools to specifically block loop formation but also due to a lack of associated phenotypes. If telomere loop structures evolved as the first means to protect the telomere, maybe the telomere binding proteins followed, providing an alternative mechanism for chromosome end protection and taking over if looping is disturbed.

Mammalian t-loops and yeast telomere fold-back structures clearly represent two distinct structures, however their regulation might be similar and yeast could give us hints on important pathways required for t-loop formation. For instance, it will be interesting to investigate whether t-loops also require transcription for their formation

With our studies we will have more opportunities to understand the functional relevance of telomere looping in terms of fundamental processes such as replicative senescence and the role of 3D genome architecture in the regulation of gene expression.

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6 Table of Figures

Figure 1: Natural yeast telomeres and their associated repetitive sequences 18
Figure 2: Telomere binding proteins 21
Figure 3: Telomere looping 22
Figure 4: The mediator complex 34
Figure 5: Gene looping 38
Figure 6: Different methods to visualize telomeric fold-back structures. 42
Figure 7: Scheme of construct2. 43
Figure 8: Chromosome conformation capturing (3C) and its variants 45
Figure 9: Scheme of telomeric 3C 65
Figure 10: 3C at telomere 1L using DpnII 67
Figure 11: 3C at telomere 1L using NcoI 68
Figure 12: 3C verifies telomere looping defect in reported looping defective mutants 69
Figure 13: A relationship between telomere length and telomere looping. 71
Figure 14:Telomere length of looping defective mutants 72
Figure 15: Telomere looping depends on telomere length 73
Figure 16: Telomere looping is independent of telomerase 75
Figure 17: Early replication is not the cause for a looping defect. 76
Figure 18: Telomere looping in *S. cerevisiae* is independent of homologous recombination 77
Figure 19: Telomere looping depends on transcription 78
Figure 20: Telomere looping depends on transcription factor II B, Sua7. 79
Figure 21: Telomere looping depends on the mediator complex 79
Figure 22: Telomere looping depends on RNA 80
Figure 23: Permanent cell cycle arrest prevents telomere looping 81
Figure 24: Cell cycle regulation of telomere looping 82
Figure 25: Telomere looping is not regulated by G4-quadruplexes 83
Figure 26: Telomere loops do not regulate TERRA levels 85
Figure 27: Current Model for telomere looping 88
Figure 28: Structure of G4-quadruplex 106
Figure 29: Potential telomere conformations 109

7 Abbreviations

Abbreviation	
µg	Microgram
µl	Microliter
µm	Micrometer
3C	Chromosome Conformation Capturing
3D	Three-dimensional
3D-FISH	Three-dimensional fluorescence in situ hybridization
FOA	5-fluoro-orotic-acid
aa	Amino acid
ALT	Alternative lengthening of telomeres
bp	Base pairs
CF1	Cleavage factor 1
ChIP	Chromatin Immunoprecipitation
CPF	Cleavage and polyadenylation factor
CTCF	CCCTC-binding factor
CTD	C-terminal domain
dd	Double distilled
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide
ds	Double-stranded
DSB	Double-strand break
eRNA	Enhancer RNA
FISH	Fluorescence in situ hybridisation
FSC	Forward Scatter
g	Gram
Gal	Galactose
Gal4	GALactose metabolism; yeast gene
HDAC	Histone deacetylase
HF	High Fidelity
His	Histidine
HIV	Human immunodeficiency virus
hr	hour
HR	Homologous recombination
hrs	hours
HYG	Hygromycin
KAN	Kanamycin
K. lactis	*Kluyveromyces lactis*
Kb	Kilobase
L	Liter

Abbreviation	
LD	Looping defective
Leu	Leucine
LiAc	Lithium acetate
lncRNA	Long non-coding RNA
Lys	Lysine
M	Molar
Mb	Megabase pairs
MEF	Mouse embryonic fibroblasts
mg	Milligram
MGL	Memory gene loops
min	Minute
ml	Milliliter
mM	Micromolar
mRNA	Messenger RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAT	Nourseothricin
ncRNA	Non-coding RNA
ncRNA-a	Activating non-coding RNA
ng	Nanogram
NHEJ	Non-homologous end joining
nM	Nanomolar
Noc	Nocodazole
NPC	Nuclear pore complex
nt	Nucleotide
OD	Optical density
ON	Over night
P. sativum	Pisum sativum
PCR	Polymerase chain reaction
PD	Population doubling
PIC	Preinitiation complex
Pol	Polymerase
Raff	Raffinose
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RPA	Replication protein A
rpm	Rounds per minute
RT	Room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SD	Synthetic dropout
sec	seconds
Ser	Serine
SPB	Spindle pole body

Abbreviation	
ss	Single-stranded
SSC	Side Scatter
STORM	Stochastic optical reconstruction microscopy
STR	Subtelomeric repeated element
TAD	Topologically associated domain
TAP	Tandem affinity purification
TAS	Telomere associated sequences
TERRA	Telomeric repeat-containing RNA
TF	Transcription factor
TLM	Telomere length maintenance
t-loop	Telomere loop
TPE	Telomere position effect
TPE-OLD	Telomere position effect over long distances
T. brucei	Trypanosoma brucei
UAS	Upstream activating sequence
Ura	Uracil
UV	Ultra violet
V	Volt
wt	Wild type
YPD	Yeast extract peptone dextrose
α	Anti

8 Appendix

Supplemental table 1 – Yeast strain list of looping defective mutants (Poschke et al. 2012)

Code	Name	Genotype
YMD129	cbs2Δ	*MAT*a *his3 leu2 met15 ura3 cbs2::KAN*
YMD235	guf1Δ	*MAT*a *his3 leu2 met15 ura3 guf1::KAN gal80::NAT 7L::con2-URA3*
YMD237	ber1Δ	*MAT*a *his3 leu2 met15 ura3 ber1::KAN gal80::NAT 7L::con2-URA3*
YMD239	sin3Δ	*MAT*a *his3 leu2 met15 ura3 sin3::KAN gal80::NAT 7L::con2-URA3*
YMD241	hda3Δ	*MAT*a *his3 leu2 met15 ura3 hda3::KAN gal80::NAT 7L::con2-URA3*
YMD243	hda2Δ	*MAT*a *his3 leu2 met15 ura3 hda2::KAN gal80::NAT 7L::con2-URA3*
YMD245	tal1Δ	*MAT*a *his3 leu2 met15 ura3 tal::KAN gal80::NAT 7L::con2-URA3*
YMD247	oca2Δ	*MAT*a *his3 leu2 met15 ura3 oca2::KAN gal80::NAT 7L::con2-URA3*
YMD249	trk1Δ	*MAT*a *his3 leu2 met15 ura3 trk1::KAN gal80::NAT 7L::con2-URA3*
YMD251	amd1Δ	*MAT*a *his3 leu2 met15 ura3 amd1::KAN gal80::NAT 7L::con2-URA3*
YMD253	med1Δ	*MAT*a *his3 leu2 met15 ura3 med1::KAN gal80::NAT 7L::con2-URA3*
YMD255	rif1Δ	*MAT*a *his3 leu2 met15 ura3 rif1::KAN gal80::NAT 7L::con2-URA3*
YMD257	ppm1Δ	*MAT*a *his3 leu2 met15 ura3 ppm1::KAN gal80::NAT 7L::con2-URA3*
YMD259	set2Δ	*MAT*a *his3 leu2 met15 ura3 set2::KAN gal80::NAT 7L::con2-URA3*
YMD261	rtg1Δ	*MAT*a *his3 leu2 met15 ura3 rtg1::KAN gal80::NAT 7L::con2-URA3*
YMD263	spe1Δ	*MAT*a *his3 leu2 met15 ura3 spe1::KAN gal80::NAT 7L::con2-URA3*
YMD265	ptc6Δ	*MAT*a *his3 leu2 met15 ura3 ptc6::KAN gal80::NAT 7L::con2-URA3*
YMD267	slm5Δ	*MAT*a *his3 leu2 met15 ura3 slm5::KAN gal80::NAT 7L::con2-URA3*
YMD269	YJR119CΔ	*MAT*a *his3 leu2 met15 ura3* YJR119C*::KAN gal80::NAT 7L::con2-URA3*
YMD271	bud31Δ	*MAT*a *his3 leu2 met15 ura3 bud31::KAN gal80::NAT 7L::con2-URA3*
YMD273	pub1Δ	*MAT*a *his3 leu2 met15 ura3 pub1::KAN gal80::NAT 7L::con2-URA3*
YMD275	rav1Δ	*MAT*a *his3 leu2 met15 ura3 rav1::KAN gal80::NAT 7L::con2-URA3*
YMD277	inp53Δ	*MAT*a *his3 leu2 met15 ura3 inp53::KAN gal80::NAT 7L::con2-URA3*
YMD279	rps24aΔ	*MAT*a *his3 leu2 met15 ura3 rps24a::KAN gal80::NAT 7L::con2-URA3*
YMD281	sif2Δ	*MAT*a *his3 leu2 met15 ura3 sif2::KAN gal80::NAT 7L::con2-URA3*
YMD283	rpl24bΔ	*MAT*a *his3 leu2 met15 ura3 rpl24b::KAN gal80::NAT 7L::con2-URA3*
YMD285	hda1Δ	*MAT*a *his3 leu2 met15 ura3 hda1::KAN gal80::NAT 7L::con2-URA3*
YMD287	npc2Δ	*MAT*a *his3 leu2 met15 ura3 npc2::KAN gal80::NAT 7L::con2-URA3*
YMD289	gcr2Δ	*MAT*a *his3 leu2 met15 ura3 gcr2::KAN gal80::NAT 7L::con2-URA3*
YMD291	sap30Δ	*MAT*a *his3 leu2 met15 ura3 sap30::KAN gal80::NAT 7L::con2-URA3*

Code	Name	Genotype
YMD293	rtg3Δ	*MAT*a *his3 leu2 met15 ura3 rtg3::KAN gal80::NAT 7L::con2-URA3*
YMD295	ubp3Δ	*MAT*a *his3 leu2 met15 ura3 ubp3::KAN gal80::NAT 7L::con2-URA3*
YMD297	swr1Δ	*MAT*a *his3 leu2 met15 ura3 swr1::KAN gal80::NAT 7L::con2-URA3*
YMD299	pho23Δ	*MAT*a *his3 leu2 met15 ura3 pho23::KAN gal80::NAT 7L::con2-URA3*
YMD301	rim21Δ	*MAT*a *his3 leu2 met15 ura3 rim21::KAN gal80::NAT 7L::con2-URA3*
YMD303	tir3Δ	*MAT*a *his3 leu2 met15 ura3 tir3::KAN gal80::NAT 7L::con2-URA3*
YMD305	YDL073WΔ	*MAT*a *his3 leu2 met15 ura3* YDL073W*::KAN gal80::NAT 7L::con2-URA3*
YMD307	rrd1Δ	*MAT*a *his3 leu2 met15 ura3 rrd1::KAN gal80::NAT 7L::con2-URA3*
YMD309	ctk1Δ	*MAT*a *his3 leu2 met15 ura3 ctk1::KAN gal80::NAT 7L::con2-URA3*
YMD311	nut1Δ	*MAT*a *his3 leu2 met15 ura3 nut1::KAN gal80::NAT 7L::con2-URA3*
YMD313	leo1Δ	*MAT*a *his3 leu2 met15 ura3 leo1::KAN gal80::NAT 7L::con2-URA3*
YMD315	mrt4Δ	*MAT*a *his3 leu2 met15 ura3 mrt4::KAN gal80::NAT 7L::con2-URA3*
YMD317	tps2Δ	*MAT*a *his3 leu2 met15 ura3 tps2::KAN gal80::NAT 7L::con2-URA3*
YMD319	sec28Δ	*MAT*a *his3 leu2 met15 ura3 sec28::KAN gal80::NAT 7L::con2-URA3*
YMD321	rpb9Δ	*MAT*a *his3 leu2 met15 ura3 rpb9::KAN gal80::NAT 7L::con2-URA3*
YMD323	msc1Δ	*MAT*a *his3 leu2 met15 ura3 msc1::KAN gal80::NAT 7L::con2-URA3*
YMD325	mks1Δ	*MAT*a *his3 leu2 met15 ura3 mks1::KAN gal80::NAT 7L::con2-URA3*
YMD327	ubp1Δ	*MAT*a *his3 leu2 met15 ura3 ubp1::KAN gal80::NAT 7L::con2-URA3*
YMD329	pih11Δ	*MAT*a *his3 leu2 met15 ura3 pih11::KAN gal80::NAT 7L::con2-URA3*
YMD331	htd2Δ	*MAT*a *his3 leu2 met15 ura3 htd2::KAN gal80::NAT 7L::con2-URA3*
YMD333	lsm1Δ	*MAT*a *his3 leu2 met15 ura3 lsm1::KAN gal80::NAT 7L::con2-URA3*
YMD335	siw14Δ	*MAT*a *his3 leu2 met15 ura3 siw14::KAN gal80::NAT 7L::con2-URA3*
YMD337	sur4Δ	*MAT*a *his3 leu2 met15 ura3 sur4::KAN gal80::NAT 7L::con2-URA3*
YMD339	ssf1Δ	*MAT*a *his3 leu2 met15 ura3 ssf1::KAN gal80::NAT 7L::con2-URA3*
YMD341	gcn2Δ	*MAT*a *his3 leu2 met15 ura3 gcn2::KAN gal80::NAT 7L::con2-URA3*
YMD343	fkh2Δ	*MAT*a *his3 leu2 met15 ura3 fkh2::KAN gal80::NAT 7L::con2-URA3*
YMD345	tkl1Δ	*MAT*a *his3 leu2 met15 ura3 tkl1::KAN gal80::NAT 7L::con2-URA3*
YMD347	rpl27aΔ	*MAT*a *his3 leu2 met15 ura3 rpl27a::KAN gal80::NAT 7L::con2-URA3*
YMD349	tpm2Δ	*MAT*a *his3 leu2 met15 ura3 tpm2::KAN gal80::NAT 7L::con2-URA3*
YMD351	rsa3Δ	*MAT*a *his3 leu2 met15 ura3 rsa3::KAN gal80::NAT 7L::con2-URA3*
YMD353	kgd1Δ	*MAT*a *his3 leu2 met15 ura3 kgd1::KAN gal80::NAT 7L::con2-URA3*
YMD355	gep4Δ	*MAT*a *his3 leu2 met15 ura3 gep4::KAN gal80::NAT 7L::con2-URA3*
YMD357	nap1Δ	*MAT*a *his3 leu2 met15 ura3 nap1::KAN gal80::NAT 7L::con2-URA3*

Code	Name	Genotype
YMD359	mrpl9Δ	*MAT*a *his3 leu2 met15 ura3 mrpl9::KAN gal80::NAT 7L::con2-URA3*
YMD361	alo1Δ	*MAT*a *his3 leu2 met15 ura3 alo1::KAN gal80::NAT 7L::con2-URA3*
YMD363	stp2Δ	*MAT*a *his3 leu2 met15 ura3 stp2::KAN gal80::NAT 7L::con2-URA3*
YMD365	mep1Δ	*MAT*a *his3 leu2 met15 ura3 mep1::KAN gal80::NAT 7L::con2-URA3*
YMD367	aco2Δ	*MAT*a *his3 leu2 met15 ura3 aco2::KAN gal80::NAT 7L::con2-URA3*
YMD369	YIL055CΔ	*MAT*a *his3 leu2 met15 ura3 YIL055C::KAN gal80::NAT 7L::con2-URA3*
YMD371	hit1Δ	*MAT*a *his3 leu2 met15 ura3 hit1::KAN gal80::NAT 7L::con2-URA3*
YMD373	mal13Δ	*MAT*a *his3 leu2 met15 ura3 mal13::KAN gal80::NAT 7L::con2-URA3*
YMD375	oca1Δ	*MAT*a *his3 leu2 met15 ura3 oca1::KAN gal80::NAT 7L::con2-URA3*
YMD377	vtc3Δ	*MAT*a *his3 leu2 met15 ura3 vtc3::KAN gal80::NAT 7L::con2-URA3*
YMD379	pep8Δ	*MAT*a *his3 leu2 met15 ura3 pep8::KAN gal80::NAT 7L::con2-URA3*
YMD383	pcp1Δ	*MAT*a *his3 leu2 met15 ura3 pcp1::KAN gal80::NAT 7L::con2-URA3*
YMD385	fmp49Δ	*MAT*a *his3 leu2 met15 ura3 fmp49::KAN gal80::NAT 7L::con2-URA3*
YMD387	ubr1Δ	*MAT*a *his3 leu2 met15 ura3 ubr1::KAN gal80::NAT 7L::con2-URA3*
YMD389	urm1Δ	*MAT*a *his3 leu2 met15 ura3 urm1::KAN gal80::NAT 7L::con2-URA3*
YMD391	vps28Δ	*MAT*a *his3 leu2 met15 ura3 vps28::KAN gal80::NAT 7L::con2-URA3*
YMD393	bud25Δ	*MAT*a *his3 leu2 met15 ura3 bud25::KAN gal80::NAT 7L::con2-URA3*
YMD395	pet130Δ	*MAT*a *his3 leu2 met15 ura3 pet130::KAN gal80::NAT 7L::con2-URA3*
YMD397	spe3Δ	*MAT*a *his3 leu2 met15 ura3 spe3::KAN gal80::NAT 7L::con2-URA3*
YMD401	msb3Δ	*MAT*a *his3 leu2 met15 ura3 msb3::KAN gal80::NAT 7L::con2-URA3*
YMD403	msn4Δ	*MAT*a *his3 leu2 met15 ura3 msn4::KAN gal80::NAT 7L::con2-URA3*
YMD405	rco1Δ	*MAT*a *his3 leu2 met15 ura3 rco1::KAN gal80::NAT 7L::con2-URA3*
YMD409	yme1Δ	*MAT*a *his3 leu2 met15 ura3 yme1::KAN gal80::NAT 7L::con2-URA3*
YMD411	kre28Δ	*MAT*a *his3 leu2 met15 ura3 kre28::KAN gal80::NAT 7L::con2-URA3*
YMD413	igo2Δ	*MAT*a *his3 leu2 met15 ura3 igo2::KAN gal80::NAT 7L::con2-URA3*
YMD415	oca5Δ	*MAT*a *his3 leu2 met15 ura3 oca5::KAN gal80::NAT 7L::con2-URA3*
YMD417	chs6Δ	*MAT*a *his3 leu2 met15 ura3 chs6::KAN gal80::NAT 7L::con2-URA3*
YMD419	elp4Δ	*MAT*a *his3 leu2 met15 ura3 elp4::KAN gal80::NAT 7L::con2-URA3*
YMD421	asf1Δ	*MAT*a *his3 leu2 met15 ura3 asf1::KAN gal80::NAT 7L::con2-URA3*
YMD423	msr1Δ	*MAT*a *his3 leu2 met15 ura3 msr1::KAN gal80::NAT 7L::con2-URA3*
YMD425	puf6Δ	*MAT*a *his3 leu2 met15 ura3 puf6::KAN gal80::NAT 7L::con2-URA3*
YMD427	ldb16Δ	*MAT*a *his3 leu2 met15 ura3 ldb16::KAN gal80::NAT 7L::con2-URA3*
YMD429	cos10Δ	*MAT*a *his3 leu2 met15 ura3 cos10::KAN gal80::NAT 7L::con2-URA3*

Code	Name	Genotype
YMD431	eaf3Δ	*MAT*a *his3 leu2 met15 ura3 eaf3::KAN gal80::NAT 7L::con2-URA3*
YMD435	pin2Δ	*MAT*a *his3 leu2 met15 ura3 pin2::KAN gal80::NAT 7L::con2-URA3*
YMD437	mhr1Δ	*MAT*a *his3 leu2 met15 ura3 mhr1::KAN gal80::NAT 7L::con2-URA3*
YMD439	arp6Δ	*MAT*a *his3 leu2 met15 ura3 arp6::KAN gal80::NAT 7L::con2-URA3*
YMD441	pdx3Δ	*MAT*a *his3 leu2 met15 ura3 pdx3::KAN gal80::NAT 7L::con2-URA3*
YMD445	sur2Δ	*MAT*a *his3 leu2 met15 ura3 sur2::KAN gal80::NAT 7L::con2-URA3*
YMD447	oca6Δ	*MAT*a *his3 leu2 met15 ura3 oca6::KAN gal80::NAT 7L::con2-URA3*
YMD451	ski3Δ	*MAT*a *his3 leu2 met15 ura3 ski3::KAN gal80::NAT 7L::con2-URA3*
YMD455	srp40Δ	*MAT*a *his3 leu2 met15 ura3 srp40::KAN gal80::NAT 7L::con2-URA3*
YMD457	swi4Δ	*MAT*a *his3 leu2 met15 ura3 swi4::KAN gal80::NAT 7L::con2-URA3*
YMD459	tip41Δ	*MAT*a *his3 leu2 met15 ura3 tip41::KAN gal80::NAT 7L::con2-URA3*
YMD461	idh1Δ	*MAT*a *his3 leu2 met15 ura3 idh1::KAN gal80::NAT 7L::con2-URA3*
YMD611	lys14Δ	*MAT*a *his3 leu2 met15 ura3 lys14::KAN gal80::NAT 7L::con2-URA3*
YMD613	mvb12Δ	*MAT*a *his3 leu2 met15 ura3 mvb12::KAN gal80::NAT 7L::con2-URA3*
YMD670	ede1Δ	*MAT*a *his3 leu2 met15 ura3 ede1::KAN gal80::NAT 7L::con2-URA3*

Supplemental table 2: Telomere length of LD mutants measured by telomere PCR

		Telomere 1L			Telomere 7L			Y' Telomeres		
Gene	YMD	length 1L (bp)	related wt (bp)	Δlength (bp)	length 7L (bp)	related wt (bp)	Δlength (bp)	length Y' (bp)	related wt (bp)	Δlength (bp)
CBS2	129	443,15	486,18	-43,03						
GUF1	235	459,89	486,18	-26,29	480,43	488,25	-7,82	555,03	576,19	-21,16
BER1	237	485,63	512,17	-26,54	465,17	488,25	-23,08	544,74	576,19	-31,45
SIN3	239	413,98	512,17	-98,19	450,39	488,25	-37,86	524,73	576,19	-51,46
HDA3	241	494,32	512,17	-17,85	443,17	488,25	-45,08	555,03	576,19	-21,16
HDA2	243	468,7	512,17	-43,47	488,25	488,25	0	609,33	631,55	-22,22
TAL1	245	468,7	512,17	-43,47	496,2	488,25	7,95	587,9	631,55	-43,65
OCA2	247	460,46	512,17	-51,71	454,09	485,81	-31,72	642,97	631,55	11,42
TRK1	249	477,09	512,17	-35,08	528,59	485,81	42,78	718,43	651,64	66,79
AMD1	251	437,01	508,37	-71,36	477,68	485,81	-8,13	651,64	651,64	0
MED1	253	480,34	508,37	-28,03	446,49	485,81	-39,32	662,32	651,64	10,68
RIF1	255	453,85	508,37	-54,52						
PPM1	257	412,91	508,37	-95,46	495,31	477,24	18,07	641,12	651,64	-10,52
SET2	259	447,82	520,94	-73,12	554,24	554,24	0	553,11	581,57	-28,46
RTG1	261	483	520,94	-37,94	694,43	542,73	151,7	591,38	581,57	9,81
SPE1	263	653,61	520,94	132,67	469,68	485,81	-16,13	581,57	581,57	0
PTC6	265	820,06	520,94	299,12	575,14	485,81	89,33	742,18	651,64	90,54
SLM5	267	456,36	520,94	-64,58	454,09	485,81	-31,72	562,44	581,57	-19,13
YJR119C	269	431,2	520,94	-89,74	523,53	514,75	8,78	571,93	581,57	-9,64
BUD31	271	583,51	520,94	62,57	652,93	542,73	110,2	730,21	651,64	78,57
PUB1	273	412,91	508,37	-95,46	434,2	492,81	-58,61			

		Telomere 1L			Telomere 7L			Y' Telomeres		
Gene	**YMD**	**length 1L (bp)**	**related wt (bp)**	**Δlength (bp)**	**length 7L (bp)**	**related wt (bp)**	**Δlength (bp)**	**length Y' (bp)**	**related wt (bp)**	**Δlength (bp)**
RAV1	275	354,94	508,37	-153,43	485,52	485,52	0			
INP53	277	502,87	502,87	0	533,38	492,81	40,57			
RPS24A	279	428,7	502,87	-74,17	605,37	492,81	112,56	720,15	680,5	39,65
SIF2	281	403,8	502,87	-99,07	448,17	492,81	-44,64	643,04	680,5	-37,46
RPL24B	283	651,74	502,87	148,87	516,77	492,81	23,96	720,15	680,5	39,65
HDA1	285				477,45	492,81	-15,36	578,44	619,24	-40,8
NPC2	287	420,79	508,37	-87,58	639,65	542,73	96,92	685,91	619,24	66,67
GCR2	289				476,72	484,71	-7,99	588,38	619,24	-30,86
SAP30	291				520,88	542,73	-21,85	568,66	619,24	-50,58
RTG3	293	428,72	526,51	-97,79	546,84	485,52	61,32	706,69	680,5	26,19
UBP3	295	634,64	526,51	108,13	457,48	485,52	-28,04	680,5	680,5	0
SWR1	297	461,98	526,51	-64,53	553,99	542,73	11,26	578,44	619,24	-40,8
PHO23	299	436,8	526,51	-89,71	531,69	542,73	-11,04	631,01	680,5	-49,49
RIM21	301	526,51	526,51	0	495,24	485,52	9,72	536,43	592,47	-56,04
TIR3	303	470,69	526,51	-55,82	448,5	485,52	-37,02	568,66	619,24	-50,58
YDL073W	305	526,51	526,51	0	505,15	485,52	19,63	568,66	619,24	-50,58
RRD1	307				502,67	502,67	0	632,51	621,01	11,5
CTK1	309	514,17	474,43	39,74	427,46	502,67	-75,21	577,07	621,01	-43,94
NUT1	311	524,61	474,43	50,18	468,78	542,73	-73,95	621,01	621,01	0
LEO1	313	292,82	474,43	-181,61	458,4	542,73	-84,33	577,07	621,01	-43,94
MRT4	315	403,94	474,43	-70,49	501,36	542,73	-41,37	536,43	592,47	-56,04
TPS2	317	429,06	474,43	-45,37	717,4	542,73	174,67	604,36	592,47	11,89
SEC28	319	503,93	474,43	29,5	548,34	542,73	5,61	547,19	592,47	-45,28
RPB9	321	400,19	510,96	-110,77	436,21	502,67	-66,46	580,81	592,47	-11,66
MSC1	323	452,2	510,96	-58,76	512,96	502,67	10,29	604,36	592,47	11,89
MKS1	325	510,96	510,96	0	536,2	542,73	-6,53	604,36	592,47	11,89
UBP1	327	407,24	510,96	-103,72	479,4	542,73	-63,33	620,68	658,2	-37,52
PIH1	329	379,77	510,96	-131,19	428,62	542,73	-114,11	611,64	658,2	-46,56
HTD2	331	386,46	510,96	-124,5	520,68	477,24	43,44	598,64	621,01	-22,37
LSM1	333	484,89	510,96	-26,07	523,45	502,67	20,78	708,3	658,2	50,1
SIW14	335	468,89	495,09	-26,2	479,09	477,24	1,85	593,95	658,2	-64,25
SUR4	337	405,61	495,09	-89,48	445,14	502,67	-57,53	648,61	658,2	-9,59
SSF1	339	495,09	495,09	0	520,68	477,24	43,44	629,85	658,2	-28,35
GCN2	341	532,31	495,09	37,22	507,13	498,15	8,98	642,97	631,55	11,42
FKH2	343	444,08	495,09	-51,01	489,32	498,15	-8,83	639,16	658,2	-19,04
TKL1	345	405,61	495,09	-89,48	480,65	498,15	-17,5	549,66	603,01	-53,35
RPL27A	347	453,25	519,75	-66,5	489,32	498,15	-8,83	577,07	621,01	-43,94
TPM2	349	530,02	519,75	10,27	464,7	498,15	-33,45	593,78	603,01	-9,23
RSA3	351	453,25	519,75	-66,5	482,29	498,15	-15,86	603,01	603,01	0
KGD1	353	453,25	519,75	-66,5	491,33	498,15	-6,82	558,22	603,01	-44,79
GEP4	355	453,25	519,75	-66,5	519,49	498,15	21,34	584,68	603,01	-18,33
NAP1	357	573,15	519,75	53,4	500,54	498,15	2,39	566,9	603,01	-36,11
MRPL9	359	475,81	495,86	-20,05	464,7	498,15	-33,45	598,64	621,01	-22,37
ALO1	361	444,47	519,75	-75,28	473,41	498,15	-24,74	593,78	603,01	-9,23
STP2	363	506,21	495,86	10,35	482,29	498,15	-15,86	653,01	640,86	12,15
MEP1	365	447,23	495,86	-48,63	529,43	495,16	34,27	665,38	640,86	24,52
ACO2	367	506,21	495,86	10,35	486,94	495,16	-8,22	640,86	640,86	0
YIL055C	369	466,09	495,86	-29,77	538,37	495,16	43,21	677,99	640,86	37,13
HIT1	371	813,83	495,86	317,97	615,48	495,16	120,32	787,86	640,86	147
MAL13	373	495,86	495,86	0	495,16	495,16	0	628,95	640,86	-11,91
OCA1	375	377,25	495,09	-117,84	478,86	495,16	-16,3	583,45	640,86	-57,41
VTC3	377	481,1	507,7	-26,6	470,91	495,16	-24,25	592,46	604,39	-11,93

		Telomere 1L			Telomere 7L			Y' Telomeres		
Gene	**YMD**	**length 1L (bp)**	**related wt (bp)**	**Δlength (bp)**	**length 7L (bp)**	**related wt (bp)**	**Δlength (bp)**	**length Y' (bp)**	**related wt (bp)**	**Δlength (bp)**
PEP8	379	455,9	507,7	-51,8	438,9	480,3	-41,4	604,39	604,39	0
PCP1	383	472,55	507,7	-35,15	489,03	480,3	8,73	569,3	604,39	-35,09
FMP49	385	447,8	507,7	-59,9	446,88	480,3	-33,42	628,99	604,39	24,6
UBR1	387	607,44	507,7	99,74	516,21	480,3	35,91	604,39	604,39	0
URM1	389	586,03	507,7	78,33	438,9	480,3	-41,4	609,63	621,8	-12,17
VPS28	391	367,62	507,7	-140,08	401,07	480,3	-79,23	525,65	604,39	-78,74
BUD25	393	495,01	514,16	-19,15	446,88	480,3	-33,42	654,58	604,39	50,19
PET130	395	598,47	514,16	84,31	477,37	477,37	0	742,87	621,8	121,07
SPE3	397	534,05	514,16	19,89	552,71	477,37	75,34	659,79	621,8	37,99
MSB3	401	441,73	514,16	-72,43	526,36	477,37	48,99	619,6	619,6	0
MSN4	403	417,28	514,16	-96,88	477,37	477,37	0	577,97	619,6	-41,63
RCO1	405	476,57	514,16	-37,59	432,93	477,37	-44,44	608,92	619,6	-10,68
RIM101	407	433,42	514,16	-80,74	529,43	477,24	52,19	568,01	619,6	-51,59
YME1	409	437,09	510,86	-73,77	503,63	477,24	26,39			
KRE28	411	414,96	510,86	-95,9	503,63	477,24	26,39	642,97	631,55	11,42
IGO2	413	452,51	510,86	-58,35	503,53	477,24	26,29	609,63	621,8	-12,17
OCA5	415	373,98	510,86	-136,88	512,62	477,24	35,38	598,52	631,55	-33,03
CHS6	417	460,42	510,86	-50,44	485,85	477,24	8,61	642,97	631,55	11,42
ELP4	419	476,65	510,86	-34,21	512,62	477,24	35,38	583,1	594,27	-11,17
ASF1	421	429,59	510,86	-81,27	503,53	477,24	26,29	586	621,8	-35,8
MSR1	423	482,33	509,99	-27,66	494,61	477,24	17,37			
PUF6	425	423,48	509,99	-86,51	518	484,71	33,29	630,46	619,6	10,86
LDB16	427	385,89	509,99	-124,1	476,72	484,71	-7,99	583,1	594,27	-11,17
COS10	429	408,03	509,99	-101,96	553,57	484,71	68,86	672,96	621,8	51,16
EAF3	431	482,33	509,99	-27,66	501,07	484,71	16,36	587,08	576,19	10,89
PIN2	435	400,51	509,99	-109,48	544,45	484,71	59,74	605,65	594,27	11,38
MHR1	437	431,43	509,99	-78,56				641,13	594,27	46,86
ARP6	439	452,18	511,49	-59,31	485,85	477,24	8,61	598,17	576,19	21,98
PDX3	441	436,53	511,49	-74,96	485,08	554,24	-69,16	572,14	594,27	-22,13
SUR2	445	493,79	511,49	-17,7	503,91	554,24	-50,33	653,41	594,27	59,14
OCA6	447	485,17	511,49	-26,32	513,6	554,24	-40,64	572,14	594,27	-22,13
SKI3	451	406,85	511,49	-104,64	533,53	554,24	-20,71	597,7	621,8	-24,1
SRP40	455	468,39	511,49	-43,1	538,6	505,3	33,3	674,32	642,91	31,41
SWI4	457	444,29	511,49	-67,2	466,55	505,3	-38,75	584,41	642,91	-58,5
TIP41	459	513,98	486,18	27,8	574,1	505,3	68,8	622,79	642,91	-20,12
IDH1	461	523,6	486,18	37,42	538,6	505,3	33,3	663,69	642,91	20,78
LYS14	611	459,89	486,18	-26,29	574,1	505,3	68,8	632,77	642,91	-10,14
MVB12	613	435,01	486,18	-51,17	481,68	505,3	-23,62	674,32	642,91	31,41
EDE1	670	427,03	486,18	-59,15	350,96	554,24	-203,28	685,13	642,91	42,22

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