TELOMERE ARCHITECTURE AND MAINTENANCE IN

SCHIZOSACCHAROMYCES POMBE

BY

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Submitted to the graduate degree program in Molecular and Integrative Physiology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Telomeres are specialized nucleoprotein structures located at the ends of eukaryotic chromosomes that promote genomic integrity. Impaired telomere function has been implicated in cancer as well as several degenerative diseases such as dyskeratosis congenita. Telomeric DNA consists of tandem G-rich repeats with a single-stranded 3' overhang that can be extended by telomerase, a reverse transcriptase, which counteracts the shortening caused by incomplete replication or nucleolytic degradation at chromosome ends. Telomere binding proteins, specifically recruited by the telomeric DNA, provide essential functions for the protection and maintenance of telomeres. The telomere maintenance machinery of fission yeast S*chizosaccharomyces pombe*, a genetically tractable organism, shares fundamental similarities with that of humans, making it an ideal model system to study telomere biology. In fission yeast, the double-stranded telomeric repeats are directly bound by Taz1, which recruits Rap1. Pot1, aided by Tpz1, binds the single-stranded DNA and Poz1 forms a bridge between Taz1/Rap1 and Pot1/Tpz1. Different parts of this telomeric complex contribute differently to telomere length equilibrium. Deletion of Taz1, Rap1 or Poz1 results in substantial telomere elongation suggesting their negative roles in length regulation. In contrast, deletion of Tpz1 or Pot1 causes rapid telomere loss, suggesting that they play positive roles in telomere protection and telomerase recruitment and activation. The molecular details of the interactions between these proteins and their contributions to the maintenance of telomere length equilibrium is not well understood. By systematically dissecting the role of each component in the complex, we demonstrate that each

individual interaction within the Taz1-Rap1-Poz1-Tpz1 unit is critical for length regulation but can be replaced with a static linker, arguing against a necessity for dynamic regulation of the interactions. Furthermore, Rap1 and Poz1 function as interaction modules to provide a molecular bridge between Taz1 and Tpz1 which can be replaced by a short linker. Our results suggest that the architecture of the telomere complex, rather than the protein components *per se*, is critical for the maintenance of telomere length equilibrium. We established minimal complexes (mini-telosomes) for telomere length regulation. Further characterization of the mini-telosomes provides new insights into the separation of different telomere functions. In addition, we investigated the molecular details of the Poz1-Tpz1 interaction, which is at the interface of negative and positive regulators. The crystal structure was solved by the Thomä lab and together with my *in vivo* work demonstrates a conserved binding motif used at different parts of telomeric complexes in human and *S. pombe*. Furthermore, a zinc ion is bound at the interface of the Poz1-Tpz1 interaction and promotes the interaction as well as telomere length regulation. In addition, Poz1-Tpz1 forms a heterotetrameric arrangement, which may provide the basis for higher order structures.

Acknowledgements

I would like to dedicate my dissertation to my parents, Chuanjun Pan and Hongying Liu. Your sacrifices and dedication have made this day possible for me. Although I am here on the opposite side of the planet, I have never stopped receiving your unconditional love and support.

I cannot completely express my gratitude to everyone who has contributed to the past five years of my life. However, these following people deserve special recognition.

I am indebted to my mentor Peter. Peter, thank you for taking me as a graduate student. I have learned so much from you which I will continue benefiting from beyond this PhD. You have inspired me to be independent, creative and critical. Thank you for always having faith in me even at times when I doubted myself. I am lucky to have you as my mentor.

Special thanks to my committee members Shrikant Anant, Ron Conaway, Jennifer Gerton and Christophe Nicot for guiding me through this journey. You have provided valuable advice on my project and my career. I would also like to thank the Physiology department, especially my director, Michael Wolfe, for keeping me on track.

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I must thank everyone in the Baumann lab for your contributions to my research, from creating an awesome working environment, to technical support, to intellectually stimulating discussions. I would like to acknowledge Katie Hildebrand, Chen Li and Evan Janzen for their help with my project.

Looking back at this journey, I am surprised at how much I have grown during these years. Coming here alone from a different country and experiencing a different culture was a big shock initially. The beginning of my project was not very successful

either. Without the help and support of my friends, I would not have been able to get through those bad days and become stronger. Now I have learned to appreciate life more and realized there were so many joyful memories. Special thanks to my rotation supervisor and great friend, Aracely. You were there from the day I joined this lab and have always been supportive and encouraging. It is lucky to have a good friend but also a teacher one can learn from. Allie, Diego, Evan, Guan, Kristi, Li, Maggie, Rachel, Ram, without you guys, I would never have known that Kansas City is actually a fun place with many great cuisines and fun activities. You have made my life here smooth and enjoyable.

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Chapter I: Introduction

I.1: Introduction to telomeres

The genetic information of a cell is stored in the form of highly stable deoxyribonucleic acid polymers and packed in thread-like structures called chromosomes. The faithful replication and separation of chromosomes to daughter cells is crucial for living organisms. There are two forms of chromosomes: circular and linear. Most prokaryotic cells have circular chromosomes, whereas all eukaryotic cells have linear chromosomes to accommodate greater genomic complexity and to maintain heterozygosity during meiosis [1]. However, the cost of having linear chromosomes is the existence of two ends for each chromosome, which comes with two major challenges for cells: the end protection problem and the end replication problem.

I.1.1: The end protection problem

The faithful transmission of the genetic information is critical for cells. However, cells are continually exposed to a variety of mutagenic insults such as radiation, chemical toxins and reactive oxygen species that can damage DNA. One particularly harmful type of damage is the double strand break (DSB), which occurs when a nick in the sugar phosphate backbone arises in each of the two strands of duplex DNA. Even one DSB in the cell can lead to cell death if improperly repaired [2]. Consequently, cells have developed sophisticated repair pathways to effectively detect and repair DSBs. Two major repair pathways are homologous recombination (HR) which uses a homologous sequence as a template and thus is error-free; and non-homologous end-joining (NHEJ)

which joins the two broken ends directly and is error prone [3]. If natural chromosome ends are recognized and repaired as DSBs, the fusion of them will generate dicentric chromosomes and leads to mitotic catastrophe.

Pioneering studies led by Hermann Muller and Barbara McClintock in the 1930s-1940s first recognized critical differences between natural linear ends and broken DNA. By treating *Drosophila* cells with X-rays, Muller observed broken chromosomes are highly unstable and undergo fusions and chromosomal rearrangements. Surprisingly, in contrast to the break sites, natural chromosome termini are stable with no tendency to fuse. He postulated that the natural termini must have some special properties that serve as protective "caps" for chromosomes and named them "telomeres" from the Greek words "telos"(end) and "meros" (part) [4].

This concept was further advanced by McClintock's work in maize. She described the "breakage-fusion-bridge" cycles in which two centromeres of dicentric chromosomes are pulled to opposite poles creating broken ends which later fuse to produce new ring chromosomes that propagate the cycle. In contrast, the natural ends are stable, fusing neither with broken ends nor with other natural ends. She also demonstrated that in some early embryonic tissues, the broken ends could be "healed" and become permanently stable, presumably by acquisition of new telomeres [5, 6]. Their work formed the foundation of telomere biology

I.1.2: The end replication problem

In the early 1960s, Leonard Hayflick reported the limited dividing potential of human diploid cell lines in culture which is called the Hayflick limit [7]. Furthermore, cells from younger individuals could undergo more divisions before ceasing to divide than cells from older individuals, suggesting a cellular theory of aging [8].

After the structure of DNA and the mechanism of semi-conservative DNA replication was unraveled [9, 10], another problem with linear chromosome was realized. DNA polymerases require a 23'-hydroxyl (3'-OH) group for nucleotide addition which is provided by a polynucleotide primer (mostly RNA). All the DNA synthesis is carried out in the 5' to 3' direction. On the leading strand, DNA is synthesized until the end of the chromosome, producing a complete end. However, on the lagging strand, due to the 5' to 3' directional synthesis, DNA polymerases produce a series of short fragments, called Okazaki fragments, each starting with an RNA primer. The RNA primer is then removed and the resulting gap is further filled by extending a preceding Okazaki fragment. At the end of a linear chromosome, however, the very 5'-terminal gap cannot be filled due to the lack of a preceding 3'-OH, resulting in loss of genetic information from the daughter strand (Figure 1.1). This "problem of the terminal replication" was independently recognized by James Waston and Alexey Olovnikov [11-13]. Olovnikov further tied the end replication problem to the Hayflick limit observation and proposed that telomere shortening could act as an internal clock to determine the number of divisions cells could

experience before death. Both Waston and Olovnikov believed that there must be some special mechanism to prevent the shortening of the telomeres.

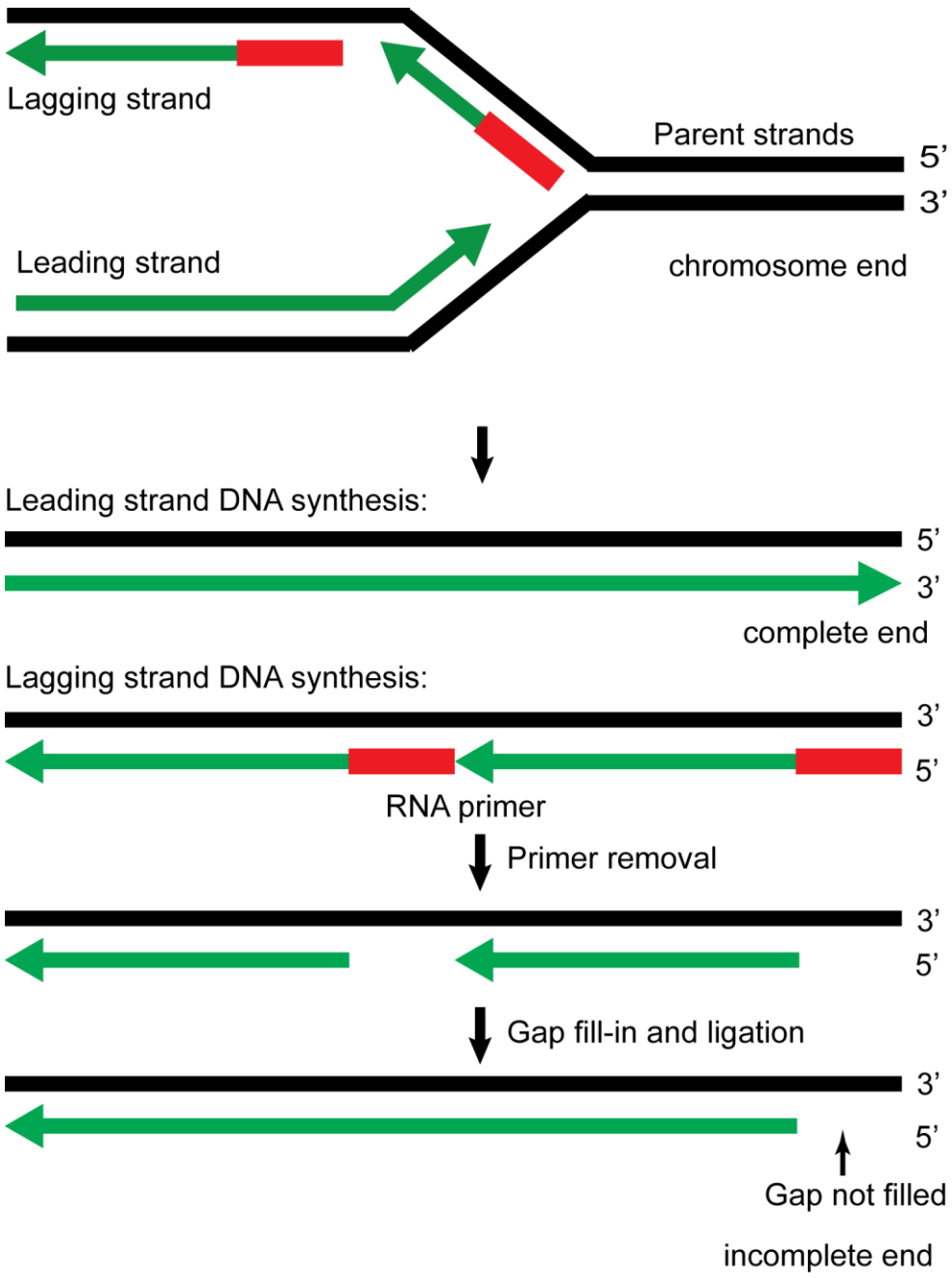


Figure 1.1: The end replication problem. DNA polymerase uses each of the parental strands as template for semi-conservative replication and requires a polynucleotide

primer (mostly RNA) to initiate synthesis. The primer is then removed and the resulting gap is filled by extending the preceding DNA fragment. While the synthesis of the leading strand is completed to the end of the chromosome, the very 5'-terminal gap of the lagging strand cannot be filled due to the lack of an upstream fragment, resulting in progressive shortening of DNA at each round of replication.

Various solutions to the end replication problem have been demonstrated or proposed. Some viruses with linear chromosomes undergo circularization (e.g. lambda phage) or concatemerization (e.g. T7) for replication [11, 14]. Phage Φ29 and adenoviruses utilize a protein that is covalently attached to the DNA to substitute for the 3'-OH group that should otherwise be provided by an oligonucleotide to prime strand synthesis [15-17]. Cavalier-Smith proposed a solution which is to have palindromic ends with two-fold rotational symmetric sequences which can fold back by pairing with itself and form a terminal loop that provides the 3'-OH. After DNA polymerase and ligase action, the closed loop can be nicked by an endonuclease to unfold [18]. This led to the idea that all telomeres in a given species share common sequences so that only one sequence-specific endonuclease is needed. Although this model is not used by eukaryotes to solve the end replication problem, the idea that telomeres in a given species share common sequences has proven to be largely true based on the identification and sequencing of the telomeres in a wide range of organisms. After decades of study, we now know eukaryotic cells have evolved conserved nucleoprotein structures to cap the chromosome ends to solve both the end protection problem and the end replication problem.

I.1.3: Telomeric DNA

The first telomeric DNA sequence was identified by Elizabeth Blackburn [19]. She utilized the ease of isolation of high copy number rDNA molecules (10^4 / nucleus) from the macronucleus of the ciliated protozoan *Tetrahymena thermophile* and found the sequences of all telomeres to be tandem repeats of C_4A_2/T_2G_4. Cloning the end sequences to a linearized plasmid resulted in stable maintenance of the plasmid in yeast while a linearized plasmid without the ciliate telomere sequences cannot be maintained [20]. Further characterization showed that not only the ends are maintained, they are further increased in size by the addition of sequences corresponding to the natural yeast telomeres, providing the first evidence of the conservation of telomere sequences and functions.

Further identification of telomere sequences in different species showed that although the exact sequence varies, almost all telomeric DNA, with the exception of *Drosophila*, consists of double-stranded short tandem G-rich repeats terminating in a single-stranded 3' overhang. The GGGTTA repeats are shared by a variety of species including all vertebrates, slime molds, some plants and fungi [21-23]. Budding yeast *Saccharomyces cerevisiae* has degenerative sequences comprised of TG_{1-3} repeats.

The length of telomeres varies greatly in different species. However, each organism maintains telomeres length within a characteristic range with a defined mean length that varies from as short as 36 bp in *Oxytricha*, to approximately 300bp in yeast, to

5-15 kb in human cells, to 30-150 kb in some inbred laboratory mouse strains [21, 24-26].

Fission yeast *Schizosaccharomyces pombe* chromosome organization and dynamics including centromeres, heterochromatin and the RNA interference (RNAi) pathway are similar to humans. Its telomere structures also share remarkable similarities to humans, making it a valuable model organism for various studies, especially for telomere research. Fission yeast telomeres consist of ~250 bp of degenerative repeats of $TTAC(A)(C)G_{2-8}$ with the most frequent one being TTACAGG [27, 28]. Additionally, the telomeric repeats are internally flanked by about 19 kb of loosely repetitive sequences, named telomere-associated sequences (TAS), found on four or five of the six chromosome ends depending on the strain background [27].

I.1.4 Telomerase, the solution to the end replication problem

After initial identification and characterization of telomere sequences, the hypothesis that an unknown enzyme is responsible for the *de novo* extension of the chromosome ends became appealing. This enzyme activity was first successfully identified by Carol Greider and Elizabeth Blackburn in *Tetrahymena* cell extract [29]. Named telomerase, this specialized reverse transcriptase is used by almost all eukaryotic species to counteract the shortening of telomeres caused by the end replication problem and nucleolytic degradation.

In place of the canonical short repetitive telomere sequences and telomerase, a few species utilize alternative solutions to the end replication and protection problem.

The most well characterized is the *Drosophila* telomeres that are comprised of arrays of retrotransposable elements maintained mainly by retrotransposition [30].

I.2: Telomeric proteins and their structures

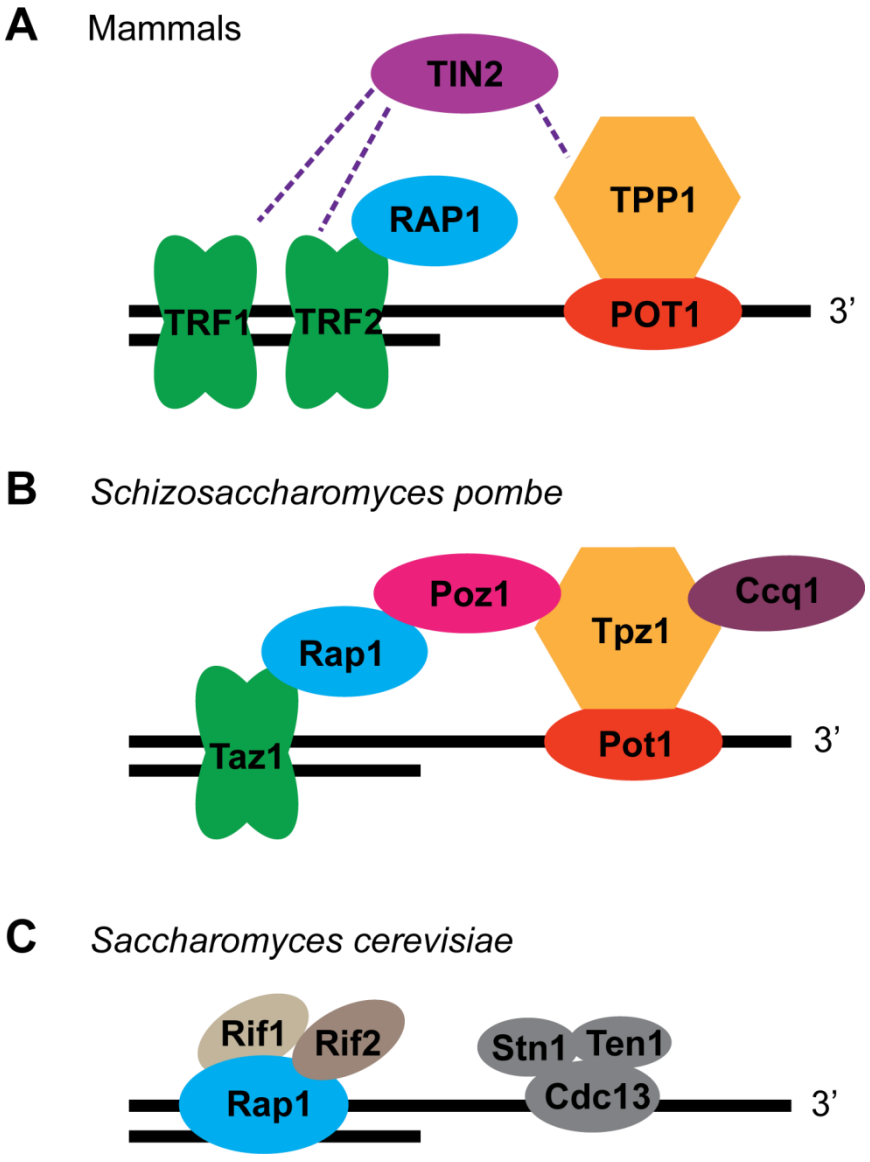


Figure 1.2: Telomere associated proteins across different species. **(A)** In mammals, the complex comprised of six telomere associated proteins is referred to as the shelterin complex. **(B)** in fission yeast *S. pombe.* **(C)** in budding yeast *S. cerevisiae.*

Both the double-stranded repeats and the single-stranded overhang of telomeres recruit specific proteins that are crucial for the integrity of the chromosome ends. They distinguish natural chromosome ends from DSBs and recruit and regulate telomerase to maintain length homeostasis. The telomere binding proteins that exist in both fission yeast *S. pombe* and humans are similar. However, some proteins are missing in budding yeast *S. cerevisiae* (Figure 1.2).

I.2.1: Taz1

Through a yeast one hybrid screen with telomere sequence as the bait, Cooper *et al* identified the first telomere binding protein in fission yeast: Taz1 (Figure 1.2B) [31]. Taz1 is the only known ortholog of human TRF1 and TRF2 [31-36]. Taz1 specifically binds double-stranded telomeric DNA repeats. Like its human counterparts, Taz1 protein has a helix-turn-helix motif that is homologous to the Myb DNA binding domain in the carboxy-terminal region (Figure 1.3) [31, 36]. However, different from some transcription factors that contains multiple Myb domains, Taz1/TRF1/TRF2 contains only one Myb domain and binds DNA as a homodimer [32, 34, 37]. Taz1 also has a centrally located sequence motif of about 200 amino acids (a.a.), called telomere repeat factor homology (TRFH) domain, which is unique to this gene family (Figure 1.3) [36]. In hTRF1 and hTRF2, this domain mediates homodimerization and interaction with other proteins [38, 39]. However, the function of TRFH in Taz1 has yet to be characterized. Both fission yeast Taz1 and human TRF2 have a Rap1 binding motif (RBM) that mediates interaction with Rap1 proteins [40]. A homolog of Taz1 in budding yeast has

not been identified. Instead, budding yeast uses Rap1 to directly bind the double-stranded telomeric repeats [41].

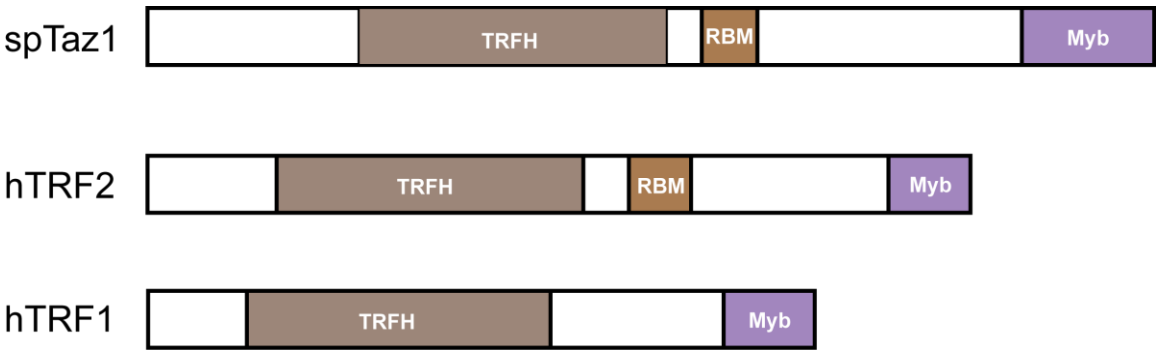


Figure 1.3: Overview of the structural similarities among spTaz1, hTRF1 and hTRF2. sp, *S. pombe*; h, human; TRFH, TRF homology; RBM, Rap1 binding motif; Myb, Myb-related HTH motif.

I.2.2: Rap1

Rap1 is the most conserved telomeric protein across species. Most telomere protein components of *S. cerevisiae* are divergent from humans and *S. pombe*, yet Rap1 is conserved [41]. The protein's name, repressor/activator protein 1, owes to its initial characterization as a transcription factor that binds a number of silencer and activator elements in the genome [42]. Rap1 was found to bind poly(TG_{1-3}) repeats, which are the sequences of budding yeast telomeres, and function in regulating telomere length [41]. In fission yeast, Rap1 does not bind telomeric DNA directly. It is recruited to telomeres mainly via interaction with Taz1 [43, 44], analogous to the situation in humans in which RAP1 localizes to telomeres through interaction with TRF2 [36].

Rap1 in all three species contains a BRCT (BRCA1 C-terminus) domain near its N-terminus (Figure 1.4). The BRCT domain is found in a large number of proteins involved in the DNA damage response (DDR), including BRCA1 and p53BP1, and is frequently used as a protein-protein interaction motif [45]. However, the interacting partners and function of the BRCT domain in Rap1 have not been elucidated. Additionally, both fission yeast and budding yeast Rap1 proteins have two Myb domains. In budding yeast, these domains mediate the interaction between Rap1 and DNA. Conversely, in fission yeast, the two Myb domains (Myb and Myb-L) lack DNA binding activity. Only one Myb domain has been identified in human RAP1, which also lacks direct DNA binding activity. The functions of these domains have yet to be characterized despite their conservation among species.

A conserved motif was identified by aligning hRAP1, scRap1 and klRap1 (*K. lactis*) and named as the RCT (Rap1 C-terminus) domain [36]. This domain was thought to be missing in fission yeast Rap1 when a sequence similarity search failed to identify the region, but biochemical and structural approaches demonstrated a structurally similar motif at the C-terminal region [40]. In addition, it is functionally related to hRAP1 RCT domain, since it mediates interaction with Taz1 and hTRF2, respectively [40, 44]. Taz1/Rap1 is considered the double-stranded binding subcomplex in fission yeast.

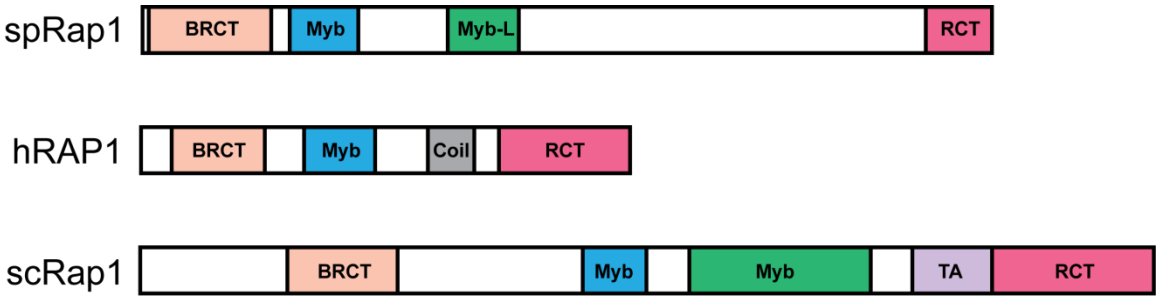


Figure 1.4: Overview of the structure similarities among Rap1 proteins in *S. pombe* (sp), human (h) and *S. cerevisiae* (sc). BRCT, BRCA1 C-terminus; Myb, Myb-related HTH motif; Myb-L, Myb-like; Coil, putative coiled-coil motif; TA, transactivation domain; RCT, Rap1 C-terminus.

I.2.3: Pot1

In budding yeast and ciliate *Oxytricha nova*, the single-stranded telomeric DNA is bound by Cdc13 and telomere end binding protein heterodimer (TEBP-α and β), respectively. [46-50]. Through a homology search, fission yeast Pot1 (protection of telomeres) was identified, which in turn contributed to the identification of human Pot1 through a BLAST search [51]. Pot1 binds the single-stranded overhang of telomeres.

Pot1 contains two oligonucleotide/oligosaccharide binding (OB) fold motif (Figure 1.5A). The first OB fold (OB1, 1-185 a.a.) shows sequence similarity to the OB fold of the *Oxytricha nova* TEBP-α subunit (19% identity and 40% similarity) and has been shown to bind single-stranded telomeric DNA cooperatively with exceptionally high sequence specificity [51-53]. The second OB fold (OB2, 186-389 a.a) is proposed based on secondary structure prediction [54, 55]. Together, Pot1 1-389 a.a binds ssDNA with a higher affinity and longer half-life than Pot1 OB1 alone and more similar to full length

Pot1 [54, 55]. The two domains may be structurally independent to accommodate telomere sequence heterogeneity.

I.2.4: Tpz1

Human TPP1 binds POT1 [56, 57]. Ishikawa lab performed Pot1 immunoprecipitation (IP) followed by mass spectrometry and identified several proteins that interact with Pot1 [58]. They identified a protein that contains an OB fold by secondary structure prediction, which is closely related to those in human TPP1 and ciliate TEBP-β (Figure 1.5B). Subsequent functional characterization confirmed that this gene is the fission yeast TPP1 homolog and was named *tpz1* (TPP1 homolog in *Schizosacchromyces pombe*). Tpz1 directly interacts with Pot1 analogous to the TPP1-POT1 interaction in humans. The N-terminal region of Tpz1 (2-223 a.a.) is sufficient for interacting with Pot1 [58] and its C-terminal region (379-508 a.a.) interacts with Poz1 and Ccq1, a protein that recruits a snf2/histone deacetylase (HDAC)-containing repressor complex (SHREC) and localizes to telomeres by interacting with Tpz1 [59, 60]. Tpz1/Pot1 is considered the single-stranded binding subcomplex.

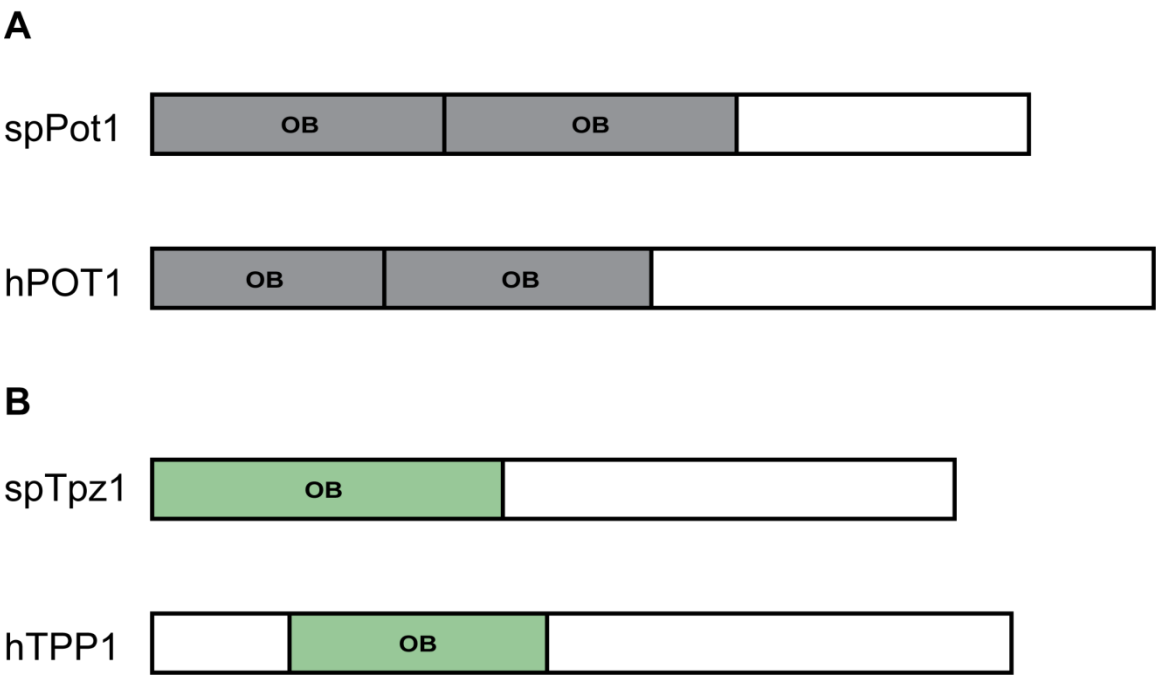


Figure 1.5: Overview of the structure similarities between ssDNA binding subcomplexes. **(A)** Fission yeast and human Pot1 **(B)** Fission yeast Tpz1 and human TPP1. OB, oligonucleotide/ oligosaccharide binding fold motif.

I.2.5: Poz1

In humans, TIN2 interacts with TRF1, TRF2 and TPP1, bridging the double-stranded factors and single-stranded factors [57, 61, 62]. Together these six proteins (TRF1, TRF2, RAP1, TIN2, TPP1 and POT1) are collectively called the shelterin complex. So far, a sequence homolog of TIN2 has not been identified in fission yeast. However, Poz1 (Pot1-associated in *Schizosaccharomyces pombe*), a protein identified through the same Pot1 immunoprecipitation, is considered a functional homolog of TIN2 [58]. It interacts with Rap1 and Tpz1, therefore potentially bridging the proteins bound to the single- and double-stranded regions of the chromosome end. However, little

information about the structure of Poz1 is available. No information can be gleaned from structural predictions or BLAST search.

These five telomere binding proteins (Taz1, Rap1, Poz1, Tpz1 and Pot1) are remarkably similar to human shelterin complex not only structurally, but also functionally.

I.3: Telomeric proteins and telomere length regulation

I.3.1: Models of telomere length regulation

The common model for telomere length equilibrium is a *cis*-inhibitory feedback mechanism by which long telomeres negatively regulate elongation. A study in budding yeast from the Lingner lab has demonstrated that not all telomeres are extended in a cell cycle and shorter telomeres are preferentially elongated by telomerase [63]. They proposed the model that telomeres switch between two states depending on their length: extendible and non-extendible. Short telomeres are preferentially in the extendible state.

Several mutually nonexclusive models of how the extendibility is dictated have been suggested. The most commonly accepted model is the “protein counting mechanism” in which the number of molecules bound to the double-stranded telomeric repeats provide negative feedback for length regulation [64]. This model is based on studies in which targeting budding yeast Rap1 C-terminal domain to a telomere causes it to shorten and the extent of shortening is proportional to the number of targeted Rap1-C molecules [64]. Conversely, mutations that reduce the number of Rap1 binding sites

within a telomere cause elongation [65, 66]. Similar negative feedback phenomenon provided by the double-stranded repeats binding proteins has been suggested for human and fission yeast. In human cells, overexpression of TRF1 or TRF2 results in progressive telomere shortening while expression of a dominant-negative TRF1 mutant leads to telomere elongation [67, 68]. Deletion of Taz1 in fission yeast also results in massive telomere elongation [31], suggesting a possible universal mechanism.

Human telomeres could form higher order structures called T-loops where the single-stranded overhang DNA folds back and invades the duplex telomeric repeats (Figure 1.6) [69]. The T-loop structure could be a potential mechanism to sequester the telomere terminus from telomerase. The formation of T-loop is mediated by TRF2 but independent of TRF1 [70, 71], thus it cannot explain the negative role TRF1 plays in length regulation. Experiments are needed to elucidate whether the T-loop structure *per se* contributes to telomere length regulation. In addition, whether T-loop structures form in fission yeast is not yet known.

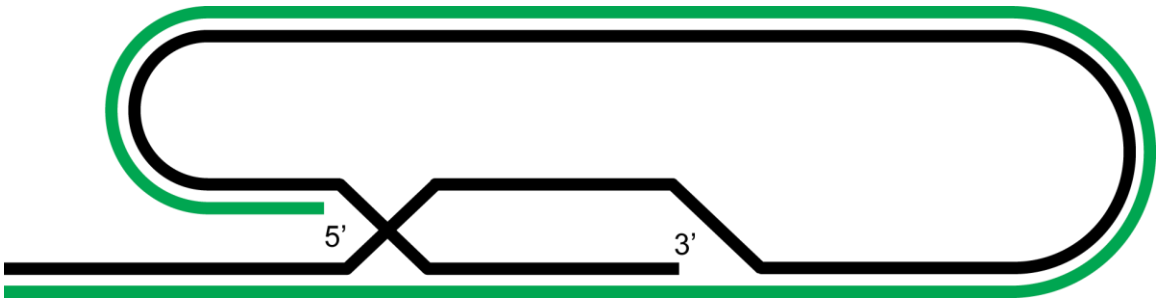


Figure 1.6: Schematic of the T-loop structure. The single-stranded 3' overhang invades internal duplex telomeric repeat array.

In humans, the identification of the interactions between telomeric proteins led to a model that the interaction between TRF1 complex, which binds the double-stranded part, and POT1, the single-stranded overhang binding protein, allows POT1 to transduce information about telomere length to the telomere terminus to regulate telomerase [72]. Similarly, work in fission yeast *S. pombe* has revealed similar protein-protein interactions at telomeres and indicated a possible conserved mechanism of length regulation mediated by telomere architecture [31, 43, 44, 51, 58].

I.3.2: Telomeric proteins in telomere length regulation

In fission yeast, all of the five telomeric proteins contribute to length control based on characterization of the deletion mutants. Deletion of *taz1* leads to dramatic telomere elongation [31]. This elongation is dependent on telomerase [73], indicating Taz1 is negatively regulating telomerase. Similarly, deletion of *rap1* or *poz1* causes massive telomere elongation that slightly exceeds *taz1Δ* cells [43, 44, 58]. In contrast, deletion of either *pot1* or *tpz1* causes immediate telomere loss and genomic instability, resulting in survivors that have circularized chromosomes [51, 58].

Despite these studies revealing the involvement of telomeric proteins in regulating telomerase, how cells transduce the signal from telomere length sensing to telomerase regulation at molecular levels still remains unclear.

I.4: Telomeric proteins in other telomere functions

I.4.1: Telomere end protection

Telomere end protection is a central function of the telomeric complex. Telomeres need to be distinguished from DSBs and prevent a DNA damage response (DDR) at chromosome ends. Paradoxically, many DNA damage response and checkpoint proteins are recruited to telomeres and are required for the normal function of telomeres [74-81]. For example, ATM and ATR, the two checkpoint activating kinases, are required for telomere maintenance [74-76]. Ku70/80, proteins that are required for NHEJ, are also required for telomere length homeostasis and integrity [79-81]. However, the downstream players in the ATM/ATR pathway in fission yeast are not required for normal telomere length [82, 83], suggesting some well-regulated mechanism to engage the DDR activities necessary for telomere maintenance but prevent the full activation of the DDR pathway, which otherwise would be harmful.

Preventing checkpoint activation at chromosome ends

The detection of DSBs will activate checkpoint pathways. There are two major checkpoint inducers: the ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) kinases [84]. ATM senses DSBs and ATR senses single-stranded DNA.

In mammalian cells, ATM and ATR are independently inhibited by distinct telomere proteins. ATM is inhibited by TRF2 [85]. Upon TRF2 deletion or overexpression of a dominant negative allele of TRF2, ATM is activated and

phosphorylates γH2AX, triggering the recruitment of 53BP1. These events lead to activation of p53 and cell-cycle arrest or cell death [86]. The 3' single-stranded telomere overhang could potentially activate ATR. The single-stranded overhang binding protein POT1 is responsible for inhibiting ATR. Correspondingly, loss of Pot1 results in rampant 5' resection and activation of $Rad3^{ATR}$-mediated checkpoint response in fission yeast [87].

Preventing NHEJ at chromosome ends

In fission yeast, NHEJ is restricted to the G1 phase of the cell cycle, while HR dominates in the S/G2 phase [88]. However, fission yeast regular cell cycle has an indiscernible G1 phase. So NHEJ is only a concern when cells are arrested in G1, for example at nitrogen starvation or upon meiotic induction. Under these conditions, deletion of *taz1* or *rap1* leads to chromosome end-fusions, which are dependent on Ku and Lig4, canonical NHEJ machinery components [89], suggesting the critical roles of both Taz1 and Rap1 in preventing NHEJ at telomeres.

In budding yeast, Rap1 is accountable for inhibiting NHEJ. Removal of Rap1 via a degron allele results in NHEJ mediated telomere fusions [90], suggesting a conserved function of Rap1.

In mammalian cells, the Taz1 counterpart TRF2 is the main telomeric protein that inhibits NHEJ. Conditional knockout of TRF2 in mouse cells or expressing a dominant negative allele in human cells gives rise to massive chromosome end-to-end fusions [91-93]. However, characterizing the contribution of RAP1 results in discrepant results. On

one hand, both human TRF2 and RAP1 are required to prevent joining of telomeric DNA substrates *in vitro* [94]. In addition, when a RAP1 fusion protein is tethered to telomeres independently of TRF2, telomere fusions caused by depletion of TRF2 are inhibited [95], further supporting the participation of RAP1 in protection of telomeres from NHEJ. However, on the other hand, deletion of RAP1 in mice and human cells shows no telomere fusion phenotype [96, 97]. Instead, it induces telomeric sister chromatid exchanges (T-SCEs) mediated by recombination when Ku is absent in mouse cells [97]. These results suggest a redundancy in protecting telomeres from end-fusions in mammalian cells. Further characterization of the molecular mechanisms causing these phenotypes would clarify whether the protective role of Rap1 is conserved.

Preventing HR at chromosome ends

In contrast to NHEJ, HR is not prohibited at telomeres *per se*, as it is not necessarily detrimental. Recombination caused exchange between telomere sequences could potentially be invisible or only cause innocuous length changes [98]. However, if the HR occurs within a single telomere, it will result in excision of telomere repeats and may produce a telomere that is too short to be fully functional. In the absence of telomerase, recombination mediated alternative lengthening of telomeres could be crucial for maintaining genomic integrity, making HR a double-edged sword for telomeres.

In fission yeast, HR at telomeres is inhibited by Ku and Taz1. Absence of Ku70 or Taz1 leads to rearrangements of telomere-associated sequences due to hyper-

recombination [79, 99]. Mammalian telomeres rely on three shelterin components TRF2, POT1 and RAP1 as well as Ku proteins to prevent HR [97, 100, 101].

I.4.2: Telomere position effect

Another feature of an intact telomere is the repression of gene transcription near telomeres, called telomere position effect (TPE) [102, 103]. The repression is caused by the heterochromatin state, which involves a variety of factors (Figure 1.6). The heterochromatin structure may be critical for the sub-nuclear localization of telomeres.

In fission yeast, telomere binding proteins Taz1, Rap1 and Poz1 are accountable for the maintenance of TPE [31, 44, 104]. In budding yeast, Rap1, Sir-complex proteins and Ku proteins are required for TPE [105-108].

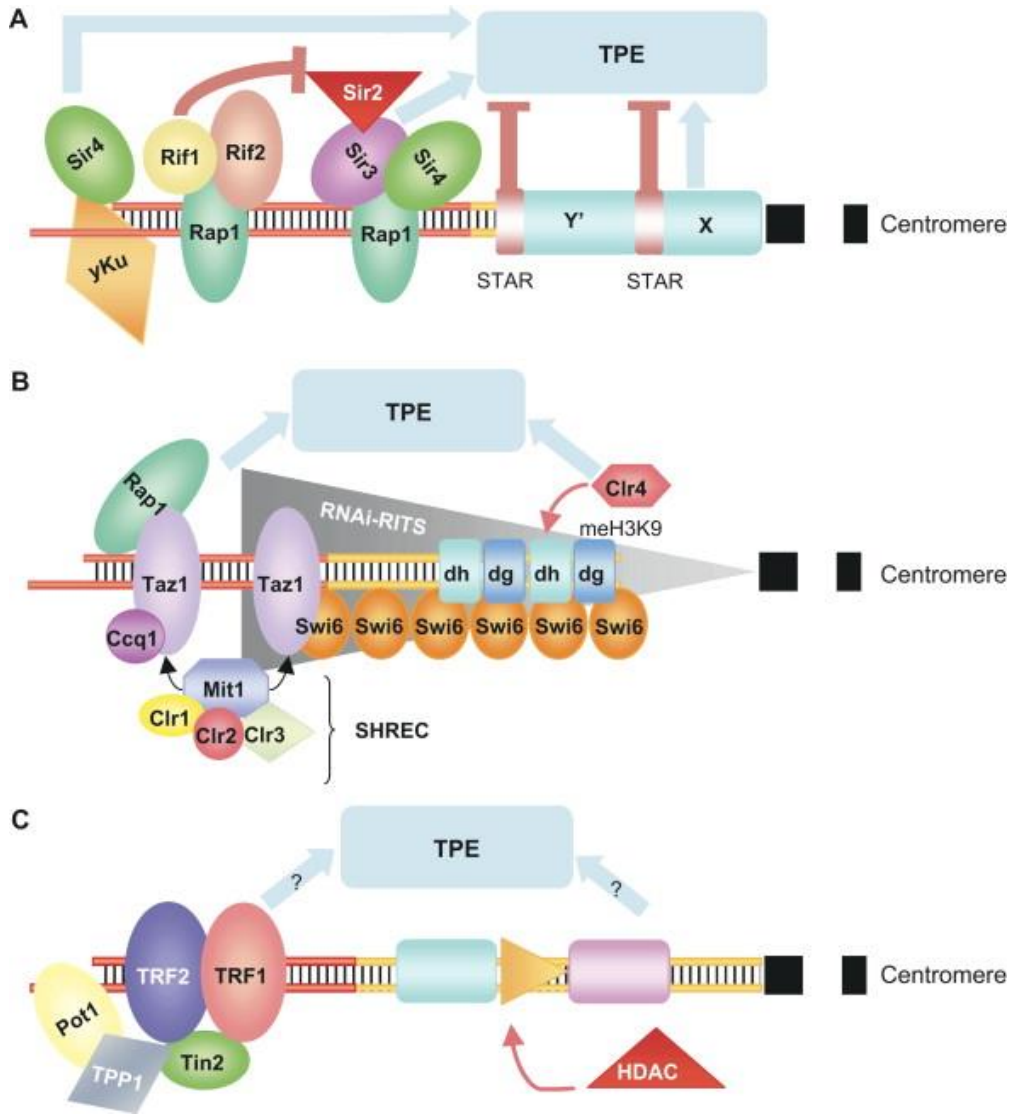


Figure 1.7: Implication of the telomeres and subtelomeres in the regulation of telomere position effect. **(A)** In *S. cerevisiae*, distinct DNA binding factors cooperate for the spreading of the silencing along the telomeric and the subtelomeric region. At natural telomeres, the X element reinforces the silencing while Y' element acts as a boundary. **(B)** In *S. pombe*, the telomeric protein Taz1 and methylation of histone H3K9 residues by the Clr4 histone methyltransferase recruit Swi6 to the telomeric associated sequences and spread silencing toward the centromere to cover the subtelomeric region over 45-75 kb in cooperation with the RNAi-RITS machinery. The SHREC complex containing the Clr3 histone deacetylase and the Mit1 chromatin remodeling factor associates with Ccq1 and Swi6 and cooperates with the Taz1 and RITS pathway to facilitate chromatin condensation and telomeric silencing. **(C)** In human cells, the telomere position effect may involve the cooperation of telomere binding proteins such as TRF1 and classical

chromatin remodeling factors. As described in different species, the identity of the subtelomeric regions might influence TPE and explain different pathologies associated with the rearrangement of these regions. Adapted from [103].

I.4.3: Telomere bouquet and meiosis

Telomeres are not only critical for genome integrity, but are also important for meiosis. A conserved feature observed in a wide range of eukaryotes is that at the early stages of meiosis, telomeres are gathered at a small region of the nuclear envelope in a polarized arrangement that resembles bundled flower stems, and was therefore called the "bouquet" [109].

In fission yeast, the bouquet stage is long and striking. The fission yeast centrosome equivalent, called the spindle pole body (SPB), colocalizes with centromeres during mitotic interphase [110], but in meiosis prophase I, the centromeres separate from the SPB and telomeres localize to the SPB, forming the bouquet structure [111]. The bouquet structure was proposed to facilitate the homology search and the timing coincides with the paring of homologous chromosomes. However, mutants that disrupt bouquet show only a mild recombination defect. Instead, formation of the bouquet structure appears to be critical for proper SPB duplication and spindle formation [112]. Taz1 and Rap1 have been shown to be required for bouquet formation; deletion of Taz1 or Rap1 leads to loss of SPB association of telomeres and defective meiosis [31, 43]. Two meiosis specific proteins, Bqt1 and Bqt2, bridge the SPB and telomeres through interacting with Rap1 [113].

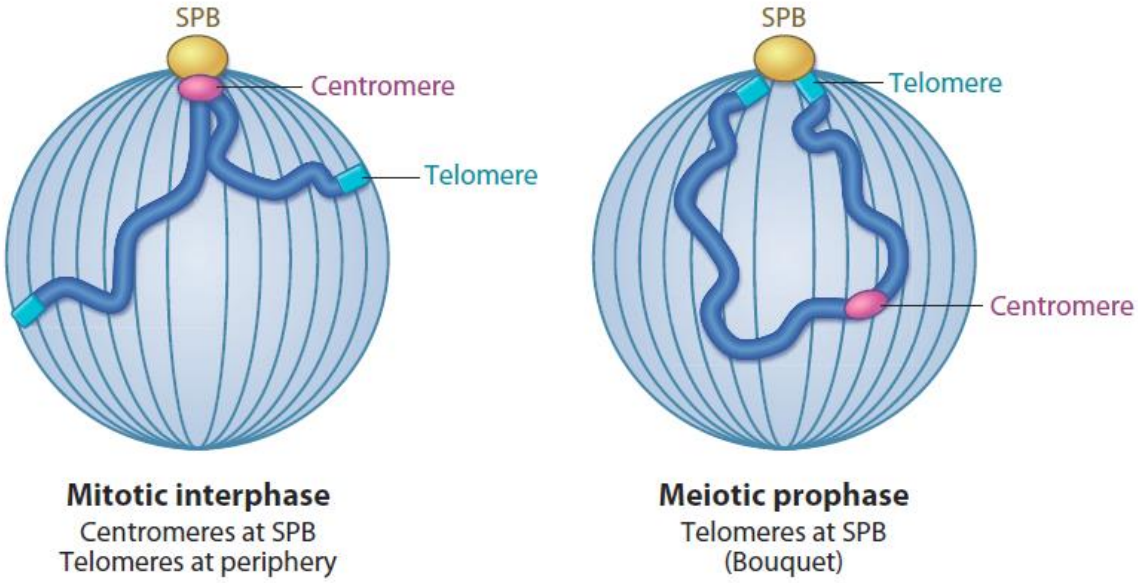


Figure 1.8: Telomere bouquet in fission yeast. During mitotic interphase, centromeres are attached to the spindle pole body (SPB; the fission yeast centrosome equivalent), whereas telomeres are located at the nuclear periphery. Upon induction of meiosis, the telomeres cluster at the SPB, and the centromeres move away from the SPB, forming the bouquet. Adapted from [98].

I.5: Scope of dissertation

The primary goal of my dissertation project is to characterize how telomere architecture contributes to its maintenance in *S. pombe*, whose telomere structure closely resembles that of humans. This chapter outlines current knowledge regarding this study and its importance, including the significance of telomeres, telomeric binding proteins and their functions. Chapter Two includes a comprehensive list of materials and methods used to execute the research described in Chapters Three and Four.

By utilizing the precise and efficient genetic tools developed in *S. pombe*, I created a variety of mutants to dissect the contribution of each telomeric complex component to the overall equilibrium of telomere length. By identifying the minimum functional elements in each protein, I was able to create two minimal complexes (mini-telosomes) that are capable of maintaining wild-type telomere length. Furthermore, I characterized the ability of mini-telosomes to maintain other telomere functions. The results are reported in Chapter Three of this dissertation.

In addition, I worked on a collaborative project with the lab of Dr. Nicolas Thomä. The main aim of this project was to characterize the Poz1-Tpz1 interaction and its role in telomere length regulation through a combination of structural, biochemical and genetic approaches. Cian Stutz from the Thomä lab solved the crystal structure of $Poz1^{30-249}$ in complex with $Tpz1^{475-508}$ which revealed several features including similarity to human TRF1 and TRF2, binding of a zinc ion and the potential to form a higher order structure. My contribution was to characterize the biological significance of these features obtained from the structure by testing their contributions to protein interactions, telomere architecture and telomere length regulation *in vivo*. My data and some of the data generated by the Thomä lab are described in Chapter Four.

Finally, Chapter Five discusses numerous future directions that build upon the studies reported in Chapters Three and Four.

Chapter II: Materials and methods

II.1: Strains and Constructs

Strains used in this study are listed in Table 2.1. Standard media and growth conditions were used [114]. Genomic integration mutant strains were generated by one-step gene replacement [115]. Specifically, the *tpz1-v5-taz1* fusion construct was made by PCR amplifying *v5-taz1* following natMX6 cassette flanked by the last ~750 bp of *tpz1* ORF and ~750 bp of *tpz1* 3'UTR as upstream and downstream homologous regions, respectively. The entire linear fragment was introduced into cells by lithium acetate transformation and integrated at the endogenous *tpz1* locus. Other fusion constructs were made using the same strategy. The *tpz1-v5-taz1* overexpression strain was made by inserting *tpz1-v5-taz1* ORF into the pCST159 plasmid which has a mutated *aur1* gene that confers resistance to aureobasidin A. The plasmid was then linearized and introduced to PP1029 cells where it integrated at the *aur1* locus. All genomic integrations were verified by PCR and sequencing. Telomerase knockout strains were generated by crossing the original strain with *trt1::ura4*$^{+}$ or *trt1::his3*$^{+}$ strain followed by marker selection and PCR verification. Similarly, strains used in Figure 3.15 were generated by crossing the original strain with PP265 (*972 h*$^{-}$) to eliminate auxotrophic markers. Strains containing plasmids were generated by electroporation transformation.

Table 2.1 Strains used in this study

Name	Genotype	Source
FP1138	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 tpz1-V5-natMX6* [*pBG1-tpz1.C482A.H488A-V5*]	This study
FP1139	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 tpz1-C482A.H488A-V5-natMX6* [*pBG1-tpz1.C482A.H488A-V5*]	This study
FP1302	*h^{90} ade6-M210 leu1-32 ura4-D18 taz1-3HA-ura4$^+$* [*pREP81-V5-taz1C(a.a.408-663)*]	This study
FP1303	*h^{90} ade6-M210 leu1-32 ura4-D18 taz1-3HA-ura4$^+$* [*pREP81-V5-taz1Myb(a.a.552-663)*]	This study
PP65	*h^{90} ade6-M210 leu1-32 ura4-D18 his3-D1 his3$^+$-TELO*	Lab stock
PP138	*h$^-$ ade6-M216 leu1-32 ura4-D18 his3-D1*	Lab stock
PP153	*h$^-$ ade6-M210 leu1-32 ura4-D18 his3-D1 taz1::ura4$^+$*	Lab stock
PP265	*h$^-$*	Lab stock
PP858	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1*	Cian Stutz (Thomä lab)
PP859	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 poz1::kanMX6*	Cian Stutz (Thomä lab)
PP860	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 poz1-V5-natMX6*	Cian Stutz (Thomä lab)
PP861	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 poz1-V34E.C37R-V5-natMX6*	Cian Stutz (Thomä lab)
PP863	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 poz1Δ1-29-V5-natMX6*	Cian Stutz (Thomä lab)
PP865	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 poz1Δ1-29.V34E.C37R-V5-natMX6*	Cian Stutz (Thomä lab)
PP867	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 tpz1-V5-natMX6*	Cian Stutz (Thomä lab)
PP868	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 tpz1-C482A.H488A-V5-natMX6*	Cian Stutz (Thomä lab)
PP869	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 tpz1-C482A.H488A-natMX6*	Cian Stutz (Thomä lab)

PP870	*h^{+} ade6-M216 leu1-32 ura4-D18 his3-D1 poz1::kanMX tpz1::tpz1-hApollo$^{495-530}$-V5-hphMX6 rap1::rap1-hTRF2$^{40-245}$-V5-natMX6*	Cian Stutz (Thomä lab)
PP898	*h^{-} leu1-32 ura4-D18 poz1-3FLAG-LEU2*	Ishikawa Lab
PP908	*h^{+} ade6-M216 leu1-32 ura4-D18 his3-D1 tpz1-V5-natMX6 poz1-3FLAG-LEU2*	This study
PP909	*h^{+} ade6-M216 leu1-32 ura4-D18 his3-D1 tpz1-C482A.H488A-V5-natMX6 poz1-3FLAG-LEU2*	This study
PP910	*h^{-} ade6-M216 leu1-32 ura4-D18 tpz1-C482A.H488A-natMX6 poz1-3FLAG-LEU2*	This study
PP931	*h^{+} ade6-M216 leu1-32 ura4-D18 his3-D1 rap1-TAP-kanMX6 poz1-V5-natMX6*	This study
PP932	*h^{-} ade6-M216 leu1-32 ura4-D18 his3-D1 rap1-TAP-kanMX6 poz1-V34E.C37R-V5-natMX6*	This study
PP933	*h^{-} ade6-M216 leu1-32 ura4-D18 his3-D1 rap1-TAP-kanMX6 poz1Δ1-29-V5-natMX6*	This study
PP934	*h^{+} ade6-M216 leu1-32 ura4-D18 his3-D1 rap1-TAP-kanMX6 poz1Δ1-29.V34E.C37R-V5-natMX6*	This study
PP936	*h^{+}/h^{-} ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 his3-D1/his3^{+} poz1-3FLAG-LEU2/poz1-V5-natMX6*	This study
PP937	*h^{+}/h^{-} ade6- M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 his3-D1/his3^{+} poz1-3FLAG-LEU2/poz1-V34E.C37R-V5-natMX6*	This study
PP938	*h^{+}/h^{-} ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 his3-D1/his3^{+} poz1-3FLAG-LEU2/poz1Δ1-29-V5-natMX6*	This study
PP941	*h^{-} ade6-M216 leu1-32 ura4-D18 poz1::kanMX4 rap1::ura4^{+} taz1::taz1-V5-poz1-natMX6*	This study
PP943	*h^{-} ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4^{+} taz1::taz1-V5-rap1.PI-natMX6*	This study
PP945	*h^{-} ade6-M 216 leu1-32 ura4-D18 his3-D1 rap1::ura4^{+} poz1::poz1-V5-Rap1.RCT-natMX6*	This study
PP949	*h^{+} ade6-M210 leu1-32 ura4-D18 poz1::kanMX4 rap1::rap1-V5-poz1-natMX6*	This study

PP993	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::kanMX4 tpz1::tpz1-V5-taz1-natMX6*	This study
PP994	*h$^-$ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::kanMX4 tpz1::tpz1-V5-rap1-natMX6*	This study
PP995	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 poz1::kanMX4 tpz1::tpz1-V5-poz1-natMX6*	This study
PP996	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::kanMX4 tpz1::tpz1-V5-rap1.RCT-natMX6*	This study
PP1029	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::kanMX4 taz1::hphMX6 tpz1::tpz1-V5-taz1-natMX6*	This study
PP1062	*h$^-$ ade6-M216 leu1-32 ura4-D18 his3-D1 tpz1::ura4$^+$ pot1::pot1-V5-tpz1-natMX6*	This study
PP1079	*h$^-$ leu1-32 ura4-D18 tpz1-3Flag-ura4$^+$*	YGRC
PP1087	*h$^-$ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ taz1::kanMX6 poz1::poz1-V5-taz1-natMX6*	This study
PP1090	*h$^-$ ade6-M210 leu1-32 ura4-D18 his3-D1 taz1::ura4$^+$ rap1::rap1-V5-taz1-natMX6*	This study
PP1098	*h$^-$ ade6-M210 leu1-32 ura4-D18 his3-D1 poz1::kanMX rap1::ura4$^+$ taz1::hphMX6*	This study
PP1155	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::poz1-V5-taz1-natMX6*	This study
PP1174	*h$^-$ ade6-M210 leu1-32 ura4-D18 his3-D1 tpz1-C482A.H488A-V5-natMX6 trt1::his3$^+$*	This study
PP1177	*h$^-$ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::kanMX4 tpz1::tpz1-V5-taz1-natMX6 trt1::his3$^+$*	This study
PP1178	*h$^-$ ade6-M210 leu1-32 ura4-D18 his3-D1 poz1::kanMX4 tpz1::tpz1-V5-poz1-natMX6 trt1::his3$^+$*	This study
PP1180	*h$^+$ ade6-M210 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::kanMX4 tpz1::tpz1-V5-rap1-natMX6 trt1::ura4$^+$*	This study
PP1181	*h$^-$ ade6-M210 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::kanMX4 tpz1::tpz1-V5-rap1.RCT-natMX6 trt1::his3$^+$*	This study
PP1184	*h$^-$ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::kanMX4 taz1::hphMX6 tpz1::tpz1-V5-taz1-natMX6 trt1::his3$^+$*	This study

PP1185	*h$^+$ ade6-M210 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ taz1::taz1-V5-rap1.PI-natMX6 trt1::ura4$^+$*	This study
PP1186	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::poz1-V5-Rap1.RCT-natMX6 trt1::ura4$^+$*	This study
PP1189	*h$^-$ ade6-M216 leu1-32 ura4-D18 poz1::kanMX4 rap1::rap1-V5-poz1-natMX6 trt1::his3$^+$*	This study
PP1190	*h$^+$ ade6-M210 leu1-32 ura4-D18 his3-D1 tpz1::ura4$^+$ pot1::pot1-V5-tpz1-natMX6 trt1::ura4$^+$*	This study
PP1191	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 taz1::ura4$^+$ rap1::rap1-V5-taz1-natMX6 trt1::his3$^+$*	This study
PP1225	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::kanMX4 taz1::hphMX6 tpz1::tpz1-V5-taz1-natMX6 aur1$^+$::(pCST159-tpz1-V5-taz1)*	This study
PP1231	*h$^-$ ade6-M210 leu1-32 ura4-D18 his3-D1 taz1-V5-natMX6*	This study
PP1244	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 poz1-V5–natMX6 tpz1-3Flag-ura4$^+$*	This study
PP1245	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 poz1-V34E/C37R-V5-natMX6 tpz1-3Flag-ura4$^+$*	This study
PP1246	*h$^+$ ade6-M216 leu1-32 ura4-D18 his3-D1 poz1Δ1-29-V5-natMX6 tpz1-3Flag-ura4$^+$*	This study
PP1312	*h^{90} ade6-M210 leu1-32 ura4-D18 his3-D1 rap1::ura4 poz1::kanMX4 tpz1::tpz1-V5-taz1-natMX4 his3$^+$-TELO*	This study
PP1313	*h$^+$ ade6-M210 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::kanMX4 taz1::hphMX6 tpz1::tpz1-V5-taz1-natMX6 his3$^+$-TELO*	This study
PP1314	*h$^+$ ade6-M210 leu1-32 ura4-D18 his3-D1 rap1::ura4$^+$ poz1::kanMX4 taz1::hphMX6 his3$^+$-TELO*	This study
PP1399	*h$^-$ ura4-D18 rap1::ura4$^+$ poz1::kanMX4 tpz1::tpz1-V5-taz1-natMX6*	This study
PP1400	*h$^-$ poz1::kanMX4 tpz1::tpz1-V5-taz1-natMX6*	This study
PP1401	*h$^-$ rap1::ura4$^+$ tpz1::tpz1-V5-taz1-natMX6*	This study
PP1402	*h$^+$ tpz1::tpz1-V5-taz1-natMX6*	This study
PP1403	*h$^-$ rap1::ura4$^+$ poz1::kanMX4*	This study
PP1404	*h$^-$ poz1::kanMX4*	This study

PP1459	*h⁻ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4⁺ poz1::kanMX4 taz1::hphMX6 tpz1::tpz1-V5-taz1-natMX6 aur1⁺::(pCST159-tpz1-V5-taz1) trt1::his3⁺*	This study
RP2	*h⁺ ade6-M216 leu1-32 ura4-D18 his2-245 rap1cDNA*	This study
RP3	*h⁺ ade6-M216 leu1-32 ura4-D18 his2-245 rap1ΔBRCT (Δ a.a.5-105)*	This study
RP4	*h⁺ ade6-M216 leu1-32 ura4-D18 his2-245 rap1ΔMyb (Δ a.a. 120-176)*	This study
RP5	*h⁺ ade6-M216 leu1-32 ura4-D18 his2-245 rap1ΔMyb-L (Δ a.a. 249-307)*	This study
RP6	*h⁺ ade6-M216 leu1-32 ura4-D18 his2-245 rap1ΔPI (Δ a.a. 440-490)*	This study
RP7	*h⁺ ade6-M216 leu1-32 ura4-D18 his2-245 rap1ΔRCT (Δ a.a. 641-693)*	This study
RP20	*h⁻ ade6-M216 leu1-32 ura4-D18 his3-D1 taz1::hphMX6 rap1::kanMX6* [*pREP81-rap1-V5*]	This study
RP21	*h⁻ ade6-M216 leu1-32 ura4-D18 his3-D1 taz1::hphMX6 rap1::kanMX6* [*pREP81-rap1-V5-taz1C*]	This study
RP22	*h⁻ ade6-M216 leu1-32 ura4-D18 his3-D1 taz1::hphMX6 rap1::kanMX6* [*pREP81-rap1-V5-taz1Myb*]	This study
RP23	*h⁺ ade6-M216 leu1-32 ura4-D18 his2-245 rap1::ura4⁺* [*pREP81-V5-taz1C*]	This study
RP24	*h⁺ ade6-M216 leu1-32 ura4-D18 his2-245 rap1::ura4⁺* [*pREP81-V5-taz1Myb*]	This study
RP25	*h⁻ ade6-M216 leu1-32 ura4-D18 his3-D1 taz1::hphMX6 rap1::kanMX6* [*pREP81-V5-taz1C*]	This study
RP26	*h⁻ ade6-M216 leu1-32 ura4-D18 his3-D1 taz1::hphMX6 rap1::kanMX6* [*pREP81-V5-taz1Myb*]	This study
RP36	*h⁺ ade6-M210 leu1-32 ura4-D18 his3-D1 rap1::ura4⁺ poz1::kanMX4*	This study
RP44	*h⁻ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4⁺* [*pREP81-rap1C1(a.a.440-693)-V5*]	This study
RP45	*h⁻ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4⁺* [*pREP81-rap1C2(a.a.249-693)-V5*]	This study

RP46	*h⁻ ade6-M216 leu1-32 ura4-D18 his3-D1 rap1::ura4⁺* [*pREP81-rap1C3(a.a.120-693)-V5*]	This study
RP77	*h⁺ ade6-M210 leu1-32 ura4-D18 his3-D1 rap1::ura4⁺ poz1::kanMX4 taz1::hphMX6 tpz1::tpz1-V5-taz1-natMX6 aur1⁺::(pCST159-tpz1-V5-taz1) his3+-TELO*	This study
RP100	*h⁻ ade6-M210 leu1-32 ura4-D18 his3-D1 taz1::ura4⁺* [*pREP81-V5-taz1C*]	This study
RP101	*h- ade6-M210 leu1-32 ura4-D18 his3-D1 taz1::ura4⁺* [*pREP81-V5-taz1Myb*]	This study

II.2: Denatured protein extract preparation

$1x10^8$ cells were harvested and lysed by vortexing for 8 min with 0.5mm glass beads in 10% trichloroacetic acid at 4°C. Beads were washed with 10% trichloroacetic acid and precipitated proteins were collected by centrifugation at 16,000 x g for 2 min. Proteins were then washed once with acetone and resuspended in 120 µl 1x protein sample buffer (1x NuPAGE LDS buffer, 50 mM dithiothreitol, 2% (w/v) sodium dodecyl sulfate). Samples were heated at 75°C for 5 min and centrifuged at 16,000 x g for 1 min. Soluble fractions were used for western blot analysis.

II.3: Native protein extract preparation

Cultures (1.5 L) were grown to a density of 0.5-1.0 x 10^7 cells/ml and harvested by centrifugation. Cells were washed three times with ice cold TMG (300) buffer (10 mM Tris-HCl, pH 8.0, 1 mM magnesium chloride, 10% (v/v) glycerol, 300 mM sodium acetate). The pellet was resuspended in two packed cell volumes of TMG (300) plus supplements (1 µg/ml pepstatin A, 5 µg/ml leupeptin, 5 µg/ml chymostatin, 1 mM benzamidine, 0.5 mM PMSF, 1 mM EDTA and 0.5 mM dithiothreitol) and then frozen as

beads in liquid nitrogen. Cells were lysed in a 6850 freezer mill (SPEX SamplePrep) using eight 2 min cycles at a rate of 10 per second with a 2 min cooling interval between cycles. Lysed cell powder was then thawed and centrifuged at 5,645 x g for 10 min. The supernatant beneath the lipid layer was transferred to new tubes and followed by two additional centrifugations at 16,000 x g and supernatant transfer. The final supernatant was collected and protein concentration was determined by Bradford assay.

II.4: Co-immunoprecipitation

1.2 ml 5 mg/ml native extracts diluted with TMG (300) buffer plus supplements and 0.1% (v/v) Tween 20 (final concentration) were used for immunoprecipitation (IP) with one of the following antibodies: anti-V5 conjugated affinity gel (Sigma-Aldrich, A7345); Ezview Red anti-FLAG affinity gel (Sigma-Aldrich, F2426); Ezview Red anti-HA affinity gel (Sigma, E6779). 120 µl of the samples were taken out and mixed with 120 µl 2x protein sample buffer as input. The remaining extracts were incubated with the agarose for 4 hours at 4°C with gentle rotation and then washed three times with TMG (200) (the same formulation as TMG (300) except for 200 mM sodium acetate) plus supplements and 0.1% (v/v)Tween 20 and once with TMG (50) (as TMG (300) but 50 mM sodium acetate) plus supplements. The agarose was then resuspended in 100 µl 1x protein sample buffer, heated at 75°C for 10 min and centrifuged at 16,000 x g for 1 min. The supernatant was used for western blot analysis.

II.5: Western blot

Protein samples (extracts or IP samples) were loaded onto NuPAGE 4-12% Bis-Tris gel (Life Technologies) and electrophoresis was carried out using the manufacturer's recommended conditions. Proteins were then transferred to Protran BA85 nitrocellulose membrane (Whatman) in a mini Trans-Blot cell (Bio-Rad) at 100 V for 1 hour in western transfer buffer (3.03 g/l Tris, 14.4 g/l glycine, 20% (v/v) methanol). Blots were blocked in 1x TTBS (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20) with 5% (w/v) nonfat milk and probed with one of the following antibodies: mouse monoclonal anti-V5 antibody at 1:5,000 dilution (Invitrogen, 46-0705); mouse monoclonal anti-α-tubulin antibody at 1:20,000 dilution (Sigma-Aldrich, T5168); rabbit polyclonal anti-V5 antibody at 1:2000 dilution (Abcam, Ab9116); rabbit polyclonal anti-FLAG antibody at 1:1000 dilution (Cell Signaling, 2368S); rabbit polyclonal anti-HA antibody at 1:5000 dilution (Abcam, Ab9110). Secondary antibodies used were HRP-conjugated goat anti-mouse IgG (H+L) antibody at 1:5,000 dilution (Thermol Scientific, 31430) and HRP-conjugated goat anti-rabbit IgG (H+L) antibody at 1:5,000 dilution (Thermol Scientific, 31460). Blots were visualized with ECL 2 substrate (Pierce) on a Typhoon 8600 scanner (GE Healthcare Life Sciences) with 457 nm blue laser or on Amersham Hyperfilm ECL (GE Healthcare Life Sciences). Some blots were stripped with stripping buffer (15 g/l glycine, 0.1% (w/v) sodium dodecyl sulfate, 1% (v/v) Tween 20, pH 2.2) for reprobing.

II.6: Genomic DNA preparation

Cells (~$1x10^9$) were harvested by centrifugation and washed once with ddH_2O and Z buffer (50 mM sodium citrate, 50 mM sodium phosphate dibasic, 40 mM EDTA, pH 7.8). Cells were then lysed in 2 ml Z buffer plus 0.5 mg/ml Zymolase T100 (US Biological) and 2 mM dithiotreitol at 37°C for 1 hour followed by addition of sodium dodecyl sulfate to a final concentration of 2% (w/v) and incubation at 65°C for 10 min. The volume of the samples was then raised to 10 ml with 5x TE (50 ml Tris-HCl, pH 8.0, 5 mM EDTA) and proteinase K (Sigma-Aldrich) was added to 50 μg/ml. After 1 hour incubation at 50°C, samples were precipitated with 3 ml 5 M potassium acetate on ice for 30 min and centrifuged at 3200 x g. The supernatant was then transferred and mixed with one volume of 100% isopropanol. After 20 min incubation on ice, DNA was collected by spinning down the samples at 10,500 x g for 10 min and resuspended in 5x TE with 50 μg/ml RNase A. After 1 hour incubation at 37°C, DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, equilibrated with 5x TE) and once with chloroform/isoamyl alcohol (24:1, equilibrated with 5x TE) and then precipitated with 2.5 volumes of 100% ethanol at -20°C for 1 hour. After one wash with 70% ethanol, DNA was solubilized in 1x TE buffer.

II.7: Telomere length analysis

Genomic DNA was digested with EcoRI for 12 hours and then loaded onto a 1% agarose gel and electrophoresed in 0.5x TBE (44.5 mM Tris-borate, 1 mM EDTA, pH 8.3) at 120~160V for 4~6 hours. Gels were stained with 1 μg/ml ethidium bromide and

visualized with a Typhoon 8600 scanner to confirm equal loading. Gels were then depurinated with 0.25 M hydrochloric acid buffer for 10 min, denatured in 0.5 M sodium hydroxide, 1.5 M sodium chloride buffer for 30 min and neutralized with 0.5 M Tris-HCl, pH 7.5, 1.5 M sodium chloride for 30 min. DNA was transferred to Amersham Hybond-N^+ membrane (GE healthcare Life Sciences) by capillary blotting. Membranes were then crosslinked with 120 mJoules of UV. A probe specific for telomeric sequences was generated by PCR from pTELO plasmid (lab stock) using T3 (5'-ATTAACCCTCACTAAAGGGA-3') and T7 (5'-TAATACGACTCACTATAGGG-3') primers. A probe specific for *rad16* gene was used as loading control and was generated by PCR from wild-type genomic DNA using primers XWP9 (5'-ATGGTATTTTTTCGCCATTTACTCG-3') and XWP10 (5'-TAGGCGGATCGTGAAGTTAA-3'). Both probes were labeled by random hexamer labeling with [α-^{32}P]-dCTP and High Prime (Roche). Hybridizations were carried out with 5 million cpm of probe in Church-Gilbert buffer [116] at 65°C. Blots were exposed to phosphor screens and visualized with a Typhoon 8600 scanner.

II.8: Pulsed field gel electrophoresis

Cells were harvested and washed twice with SP1 buffer (1.2 M D-sorbitol, 50 mM sodium citrate, 50 mM sodium phosphate dibasic, 40 mM EDTA, pH 5.6). 4 x 10^8 cells were lysed with Zymolyase T-100 (final concentration 0.375 mg/ml) at 37°C for 2 hours. Cells were centrifuged at 3000 rpm for 2 min and gently resuspended in 140 µl TSE buffer (10 mM Tris-HCl, pH 7.5, 0.9 M D-sorbitol, 45 mM EDTA) and then mixed with

220 µl 1% low melting point agarose (Bio-Rad, 161-3112) in TSE buffer. The suspension was then transferred into four plug molds (Bio-Rad, 170-3706). Solidified plugs were washed in PW1 buffer (50 mM Tris-HCl, pH 7.5, 0.25 M EDTA, 1% (w/v) sodium dodecyl sulfate) at 50°C for 2 hours followed by two rounds of 24 hour treatment with 1 mg/ml proteinase K in PW2 buffer buffer (10 mM Tris-HCl, pH 9.0, 0.5 M EDTA, 1% (w/v) N-lauroyl sarcosine) at 50°C. After three 15 min washes with T10xE (10 mM Tris-HCl, pH7.5, 10 mM EDTA), plugs were stored at 4°C until use.

For NotI digestions, plugs were washed twice for 15 min in 1x TE at 50°C and then incubated with 1x NEBuffer 3.1 (NEB) for 2-5 hours. Plugs were then incubated at 37°C with 500 µl fresh 1x NEBuffer 3.1 containing 100 U NotI (NEB) for 3 hours. 100 U NotI was then added and plugs were incubated for another 3 hours. After rinsing with T10xE and equilibrating in 0.5 x TBE for 30 min, plugs were loaded onto 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad, 162-0137). Electrophoresis was carried out in recirculating 0.5x TBE buffer at 14°C for 24 hours at 6 V/cm with 60 to 120 sec switch ramp at an included angle of 120°. Southern transfer and hybridization was carried out using the same protocol described in telomere length analysis (II.7) with the modification that instead of hydrochloric acid treatment, gels were first irradiated with 120 mJoules of UV to nick the DNA before sodium hydroxide treatment. Probes specific for the end fragments L, I, M, C were generated by PCR from wild-type genomic DNA and labeled by random hexamer labeling with [α-^{32}P]-dCTP and High Prime (Roche). Primers used to amplify these fragments are:

Fragment L (Ch1L):	LT (5’-TTTGTTGACTGGTACAATCAATGCTGGCTG-3’)
	LB (5’-AAGAAGCATATCGATTGGAAAGCAGCTCCA-3’)
Fragment I(Ch1R):	IT (5’-ATGTGCGGAATTTTGGCGTTAATGCTTGCT-3’)
	IB (5’-ACACATGCATAACCACCATTAACGCGATCG-3’)
Fragment M(Ch2L):	MT (5’-GATCGCGTGTCCATCGTCCATTAGCTTCTT-3’)
	MB (5’-GGTAGTGCTAGATGGACTGCGGAACATTGG-3’)
Fragment C(Ch2R):	CT (5’-ATGAGAGAAGTAATTTCTGTTCATGTTGGA-3’)
	CB (5’-CTCAATGTCAAGATTTCGGCGACAGATATC-3’)

Chapter III: Mini-telosomes separate functions of telomeric proteins in end protection and length regulation

III.1: Abstract

Chromosome ends specialize with nucleoprotein structures called telomeres in order to maintain their integrity. Initial characterization of the telomere maintenance machinery in the fission yeast S*chizosaccharomyces pombe* revealed fundamental similarities with the mammalian shelterin complex. In fission yeast, the double-stranded telomeric repeats are bound by Taz1, which recruits Rap1. Pot1, aided by Tpz1, binds the single-stranded DNA and Poz1 forms a bridge between Taz1/Rap1 and Pot1/Tpz1. Deletion of Taz1, Rap1 or Poz1 causes dramatic telomere elongation. In contrast, deletion of Tpz1 and Pot1 results in rapid telomere loss. It is not clear yet how these proteins work together to achieve length homeostasis. The potential to form a bridge between the single- and double-stranded parts by the pairwise protein-protein interactions led us to test the contribution of the telomere architecture to length regulation by dissecting each component in the complex. My data suggest that dynamic interactions between the components are not critical for telomere length regulation. Furthermore, Rap1 and Poz1 serve as interaction modules that bridge the other proteins and are dispensable for the maintenance of wild-type telomere length as long as a static bridge is provided between Taz1 and Tpz1. In this way, I was able to create minimum complexes (mini-telosomes) which maintain wild-type telomere length. The mini-telosome cells display growth rates, a telomere position effect and cold-sensitivities comparable to wild-

type cells. However, these mini-telosomes fail to protect chromosomes against end fusions in G1 arrested cells, thereby providing a separation of telomere length regulation and telomere end protection functions. Further characterization revealed that Rap1, but not Poz1, is required for the end protection. The protection requires the presence of the Rap1 protein itself as opposed to its ability to bridge Taz1 and Poz1.

III.2: Introduction

Telomeres, the nucleoprotein structures at the termini of linear chromosomes, are essential for genome integrity. Telomeric DNA consists of tandem G-rich repeats with a G-rich single-stranded overhang that is extended by a reverse transcriptase called telomerase. This conserved mechanism is used by most eukaryotes to solve the end replication problem caused by the inability of conventional DNA polymerases to fully replicate linear chromosomes.

In contrast to the similarities in telomeric repeat sequences across species, the number of repeats (telomere length) varies greatly. Even within a given species, the length between individual telomere tracts is heterogeneous. However, each organism maintains telomere length within a characteristic range with a defined mean length that varies from as short as 36 bp in *Oxytricha*, to approximately 300 bp in yeast, to 5-15 kb in human cells, to 30-150 kb in some inbred laboratory mouse strains [21, 24-26].

The species-specific equilibrium of telomere length indicates an effort of active regulation. This is largely achieved via a cis-inhibitory feedback mechanism by which long telomeres negatively regulate elongation. Telomere length homeostasis is the result of a balance between telomere shortening and telomere lengthening. Telomere shortening is caused by incomplete replication and nucleolytic degradation and occurs at a length-independent rate. In addition, rapid telomere deletion events caused by intrachromatid recombination sometimes occur in a more stochastic fashion and may favor long telomeres [117]. Replenishment of lost sequences by telomerase is a highly regulated

event and is telomere length dependent [118]. This regulation requires cells to be able to sense the length of different telomeres and regulates when and how many repeats are synthesized by telomerase at a specific chromosome end.

One way to achieve this regulation is to elongate a subset of telomeres during a given cell cycle. The other way is to control the number of nucleotides added per elongation event. The Lingner lab has shown that the number of nucleotides added to a telomere per extension event is independent of its length. However, not all telomeres are extended in a cell cycle and short telomeres are preferentially elongated. They proposed a model in which telomeres switch between two states that is decided by their length: extendible and non-extendible [63].

Several models of how the extendibility is dictated have been suggested. The Shore lab has proposed a protein counting mechanism based on a study in *S. cerevisiae*, whose double-stranded telomere repeats are bound by arrays of Rap1 proteins [64]. The C-terminal region of Rap1 inhibits telomere elongation: longer telomeres render more sites for Rap1 binding which in turn lead to greater inhibition of telomerase action. In contrast, shorter telomeres can bind fewer Rap1 molecules and are thus more accessible to telomerase. This model is based on the result that targeting the Rap1 C-terminal domain to a telomere causes it to shorten and the extent of shortening is proportional to the number of targeted Rap1-C molecules [64]. Conversely, mutations that reduce the number of Rap1 binding sites within a telomere cause elongation [65, 66]. Further experiments have shown that this Rap1 counting mechanism is in fact achieved by Rif1

and Rif2, two additional telomeric proteins that are recruited by the Rap1 C-terminal domain and negatively regulate telomere length [119]. Similar negative regulation by the double-stranded telomeric DNA binding proteins Taz1 and TRF1 were reported in fission yeast and vertebrates respectively, suggesting a possible universal mechanism [31, 67].

A mutually nonexclusive model for telomere length regulation is the fold-back model in which long telomeres form higher order structures that prevent telomere elongation. Telomeres that shortened below a threshold length fail to form these high order structures and become accessible to telomerase action. The Runge lab has proposed that in *S. cerevisiae,* this structure may link the chromosome termini to the telomere-non-telomere junctions [120].

In humans, the identification of the interactions between telomeric proteins led to a model that the interaction between the TRF1 complex, which binds the double-stranded part, and POT1, the single-stranded overhang binding protein, allows POT1 to transduce information about telomere length to the telomere terminus to regulate telomerase [72]. Similarly, work in fission yeast *S. pombe* has revealed similar protein-protein interactions at telomeres and indicated a possible conserved mechanism of length regulation mediated by telomere architecture [31, 43, 44, 51, 58].

Fission yeast has been a valuable model organism for telomere research due to its genetic tractability and remarkable similarities in telomere structure with humans. In fact, the identifications of Trt1, the catalytic component of telomerase, and Pot1, the single-stranded telomeric DNA binding protein in fission yeast, were critical steps in the

identifications of their human orthologs. The most common telomere repeat sequence in fission yeast is TTAC(A)(C)G_{2-8} and the length of telomeres centers around ~250bp.

The double-stranded telomeric repeats are bound by Taz1, a protein that is structurally and functionally related to human TRF1 and TRF2 [31, 34]. Taz1 recruits Rap1 to telomeres [43, 44]. The single-stranded overhang is bound by Pot1 facilitated by Tpz1, a functional ortholog of human TPP1 [51, 58]. Poz1 interacts with both Rap1 and Tpz1, thereby potentially bridging the proteins bound to the single- and double-stranded parts of the chromosome end [58]. Deletion of *taz1*, *rap1* or *poz1* leads to dramatic telomere elongation, indicative of functions as negative regulators of telomere length [31, 43, 44, 58]. The Tpz1/Pot1 complex is required for telomerase recruitment and activity as well as for protection of telomere integrity. Deletion of either *pot1* or *tpz1* causes immediate telomere loss and instability [51, 58]. Disruption of several interactions within the complex leads to telomere elongation [40, 104, 121], suggesting that the interactions are important for the regulation of telomere length. Despite these studies revealing the involvement of telomeric binding proteins in regulating telomerase, it is not clear yet how they work together to achieve length homeostasis. The potential to form a bridge between the single- and double-stranded parts by the pairwise protein-protein interactions led us to test the contribution of the telomere architecture to length regulation.

In addition to the end replication problem, linear chromosomes face another challenge: the ends of chromosomes must not be mistaken for the products of DNA double strand breaks (DSBs) by the DNA repair machinery. If such distinction fails,

telomere fusions generate dicentric chromosomes that cannot be segregated during the next cell division. In this manner, loss of telomere protection promotes genome instability and may drive cancer progression [122]. In fission yeast, deletion of Taz1 and Rap1 causes NHEJ mediated chromosome end fusions in G1 arrested cells, arguing their importance in chromosome end protection [89, 123].

Another feature of an intact telomere is the repression of gene transcription near telomeres, called telomere position effect (TPE) [102]. The repression is caused by the heterochromatin state. Taz1, Rap1 and Poz1 has been shown to participate in the maintenance of TPE [31, 44, 104]. These complex phenotypes caused by deletion of the telomeric proteins further complicate the understanding of the mechanisms of each function.

In this study, I utilized the accuracy and flexibility of molecular genetics to dissect the contribution of each core component of the telomeric complex to the maintenance of telomere length homeostasis. I found that Rap1 and Poz1 serve as interaction modules that bridge the other proteins. They can be replaced by a simple short synthetic linker. In this way, I was able to create minimum complexes (mini-telosomes) which maintain wild-type telomere length. These cells show no growth defect and retain telomere position effect indicative of functional subtelomeres. However, these mini-telosomes fail to protect against chromosome end fusions in G1 arrested cells, thereby providing a separation of telomere length regulation and telomere end protection. Further characterization revealed that Rap1, but not Poz1, is required for the end protection.

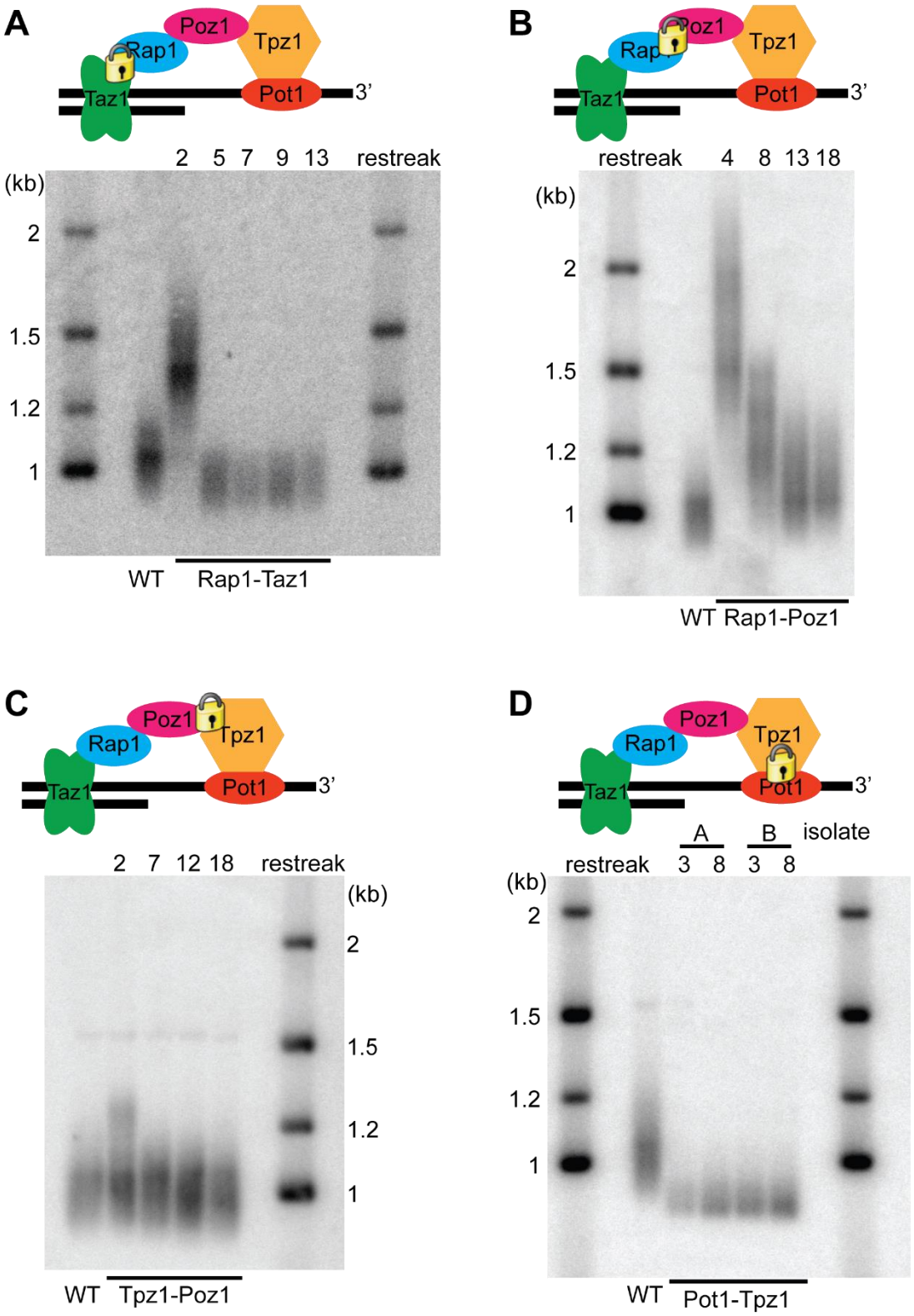
Furthermore, it is a feature of the Rap1 protein itself, not the ability to bridge Taz1 and Poz1, that is critical for end protection.

III.3: Results

III.3.1: Stabilization of individual interaction interfaces in the fission yeast telosome

The mechanistic basis for a switch from a telomerase inaccessible to an accessible conformation is still unknown. An attractive possibility is that a specific protein-protein interaction interface is disrupted in a regulated manner to break the bridge. To test this, I replaced each individual interaction with a covalent linker that permanently joins neighboring proteins (Figure 3.1). gDNA from several restreaks of cells were analyzed to monitor telomere length change over generations. When a Rap1-Taz1 fusion was introduced into *taz1Δ* cells, the long telomere phenotype caused by the *taz1* deletion was rescued and stabilized at near wild-type length (Figure 3.1A). Telomeres in these cells were indeed maintained by telomerase as deletion of *trt1* resulted in telomere loss (Figure 3.2A). Similarly, Rap1-Poz1 or Tpz1-Poz1 cells maintained stable telomeres similar to wild-type (Figure 3.1B and C). Since deletion of *pot1* or *tpz1* causes rapid telomere loss, *tpz1* was deleted after endogenous *pot1* gene was replaced by *pot1-tpz1*. This strain displayed shorter than wild-type yet stable length (Figure 3.1D), which may indicate a role for the Pot1-Tpz1 interface in regulating telomere length. Alternatively, it may simply reflect a functional impairment caused by the protein fusion. In any event, disruption of the Pot1-Tpz1 interface is not critical for stable telomere maintenance as telomeres length remained unchanged between restreak 3 and 8. Deletion of *trt1* in each of the strains confirmed that telomeres were maintained by telomerase (Fig 3.2B-D). The absence of progressive telomere shortening in any of the fusion strains indicates either

redundancy in terms of which protein-protein interaction is disrupted to break the bridge and provide access to telomerase or none of the interactions need be dynamic to provide telomerase access.


A
Poz1
Tpz1
Rap1
Taz1
Pot1
3'
2 5 7 9 13 restreak
(kb)
2
1.5
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Rap1-Taz1
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restreak 3 8 3 8
(kb)
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1
WT
Pot1-Tpz1

Figure 3.1: Replacement of protein interactions with covalent linkers. In each case the construct encoding a fusion between two telomeric proteins was integrated at the genomic locus of the N-terminal partner driven by its native promoter and the genes encoding the individual proteins were deleted. The linker sequence corresponds to the V5 epitope tag. Following the indicated number of sequential restreaks, telomere length was assessed by Southern blot of EcoRI-digested genomic DNA probed with a telomere-specific probe. Each restreak corresponds to approximately 20 generations. **(A)** Rap1-V5-Taz1 (*rap1* promoter), *rap1Δ*, *taz1Δ*. **(B)** Rap1-V5-Poz1 (*rap1* promoter), *rap1Δ*, *poz1Δ*. **(C)** Tpz1-V5-Poz1 (*tpz1* promoter), *tpz1Δ*, *poz1Δ*. **(D)** Pot1-V5-Tpz1 (*pot1* promoter), *pot1Δ*, *tpz1Δ*.

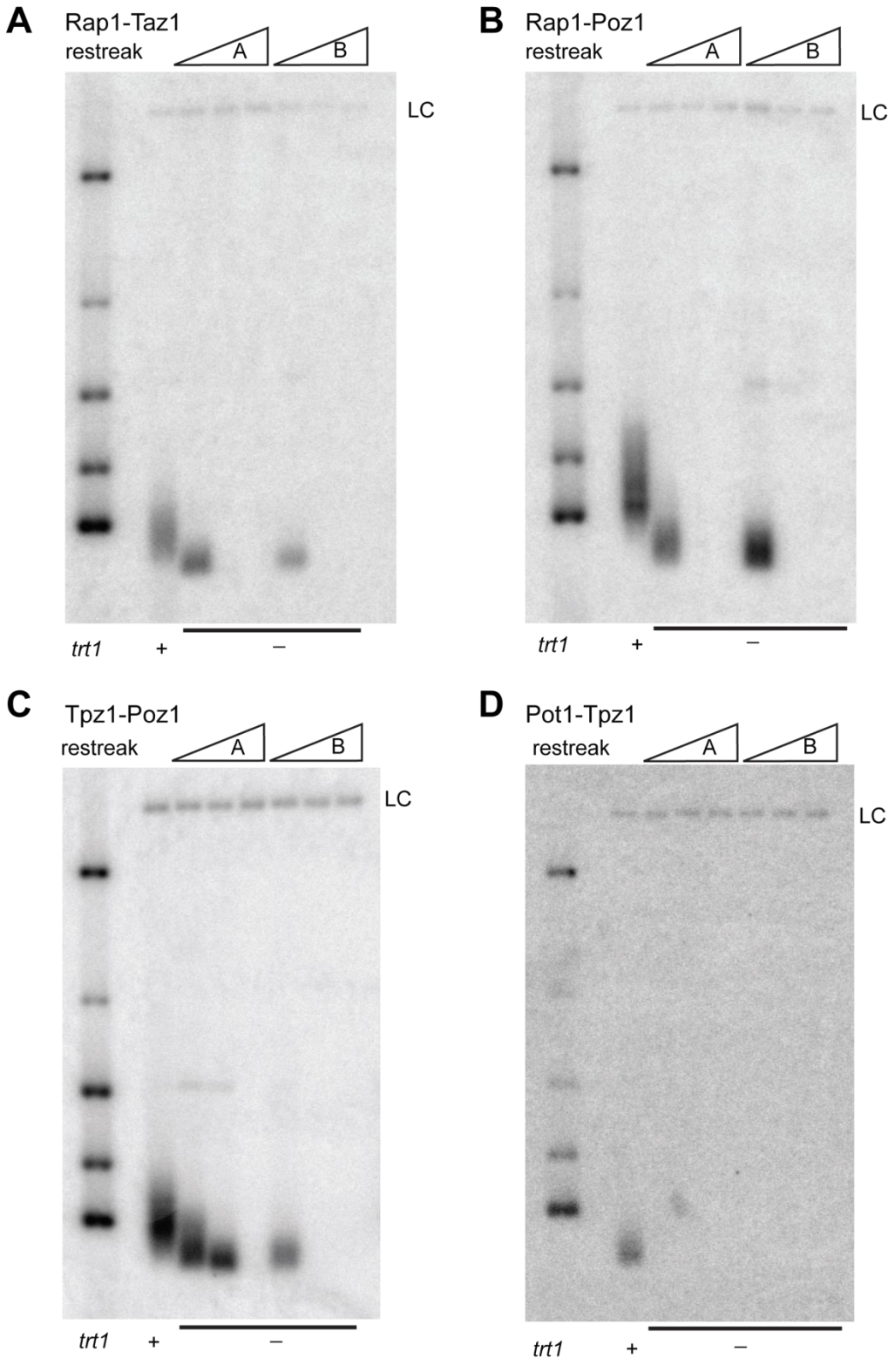

A
Rap1-Taz1
restreak
A
B
LC
trt1
+
–
B
Rap1-Poz1
restreak
A
B
LC
trt1
+
–
C
Tpz1-Poz1
restreak
A
B
LC
trt1
+
–
D
Pot1-Tpz1
restreak
A
B
LC
trt1
+
–

Figure 3.2: Telomere maintenance in the interaction stabilization mutants is dependent on telomerase. Strains indicated in Figure 3.1 were crossed with *trt1Δ* cells. Spores with the fusion proteins and *trt1Δ* were selected and sequentially restreaked. Telomere length was analyzed by Southern blot of EcoRI-digested genomic DNA probed with a telomere-specific probe. **(A)** Rap1-V5-Taz1. **(B)** Rap1-V5-Poz1. **(C)** Tpz1-V5-Poz1. **(D)** Pot1-V5-Tpz1. 1st, 3rd and 5th restreak of two independent isolates were analyzed and indicated by the triangle.

III.3.2: Taz1 DNA binding and dimerization domains are sufficient for telomere length maintenance

To further characterize the role of individual components, I proceeded by mapping the requirements for specific domains of telomeric proteins. Taz1 has a centrally located sequence motif called TRF homology (TRFH) domain, which is conserved in human TRF1 and TRF2 proteins [34]. The 365-396 a.a. region interacts with Rap1 and was hence named the Rap1-binding motif (RBM) [40]. Telomere binding of Taz1 is mediated by the Myb domain located at the C-terminus, which only stably interacts with telomeric repeats as a dimer. Similar to human TRF1 and TRF2, DNA binding by Taz1 thus requires homodimerizations [32, 34, 37, 67]. However, unlike TRF1 and TRF2, which use their TRFH domains for dimerization, Taz1 dimerization is not mediated by the TRFH domain (Figure 3.3A and ref [40]). Co-immunoprecipitation experiments revealed that a C-terminal fragment starting at position 408 is sufficient for dimerization (Figure 3.3B). I then tested whether the DNA binding of Taz1 is sufficient for length regulation. To bypass the requirement of the RBM for Rap1 recruitment, Taz1C was fused to the C-terminus of Rap1 (Figure 3.4). This Rap1-Taz1C fusion protein was sufficient to rescue telomere lengthening caused by the deletion of endogenous *taz1* and

rap1 (Figure 3.3C). Fusing just the Taz1Myb domain to Rap1 partially rescued the lengthening (Figure 3.3C and 3.4), which was probably due to Rap1 mediated dimerization permitting DNA binding [43, 44]. As a control, expressing Taz1C or Taz1Myb alone did not rescue telomere length of *taz1Δ* (Figure 3.5A), *rap1Δ* or *taz1Δrap1Δ* cells (Figure 3.5B). In summary, these results indicate that telomere length regulation requires binding of Taz1 to telomeric repeats as well as the ability to recruit Rap1.

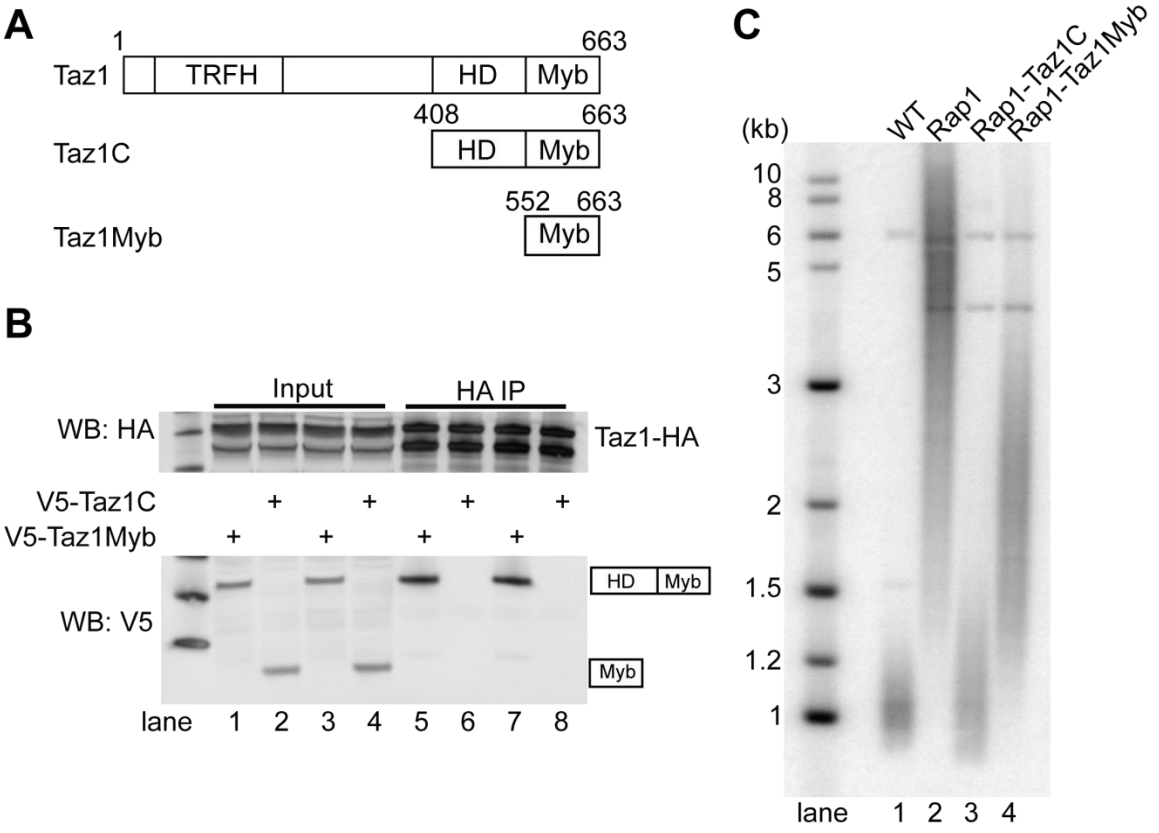


Figure 3.3: DNA binding and dimerization domains of Taz1 are sufficient for near wild-type telomere length maintenance. **(A)** Schematic of Taz1 and Taz1 truncation mutants. Numbers indicate amino acid positions; HD, homodimerization domain; Myb, myb domain. **(B)** Co-immunoprecipitation of V5-tagged Taz1Myb and Taz1C with HA-tagged Taz1. Upper panel: Western blot for input (10%) and immunoprecipitate probed with

anti-HA for full length Taz1. Lower panel: Western blot with anti-V5 to detect Taz1C (lanes 1, 3, 5, 7) and Taz1Myb (lanes 2, 4, 6, 8). Samples shown in lanes 3, 4, 7 and 8 were treated with benzonase prior to immunoprecipitation to assess whether protein interactions are mediated by nucleic acid. **(C)** Telomere length analysis for strains deleted for *rap1* and *taz1* and harboring the indicated constructs under control of the *nmt81* promoter.

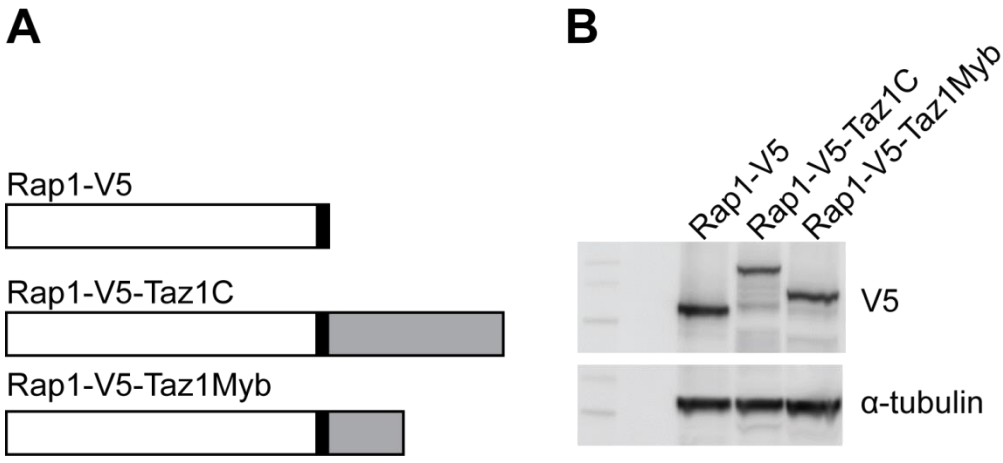


Figure 3.4: Expression levels of the chimeric proteins. **(A)** Schematic of the proteins. Taz1C or Taz1Myb was fused to the C-terminus of Rap1 with a V5 epitope tag as linker. **(B)** Western blot analysis of protein expression levels with anti-V5 antibody. An antibody against α-tubulin was used as loading control.

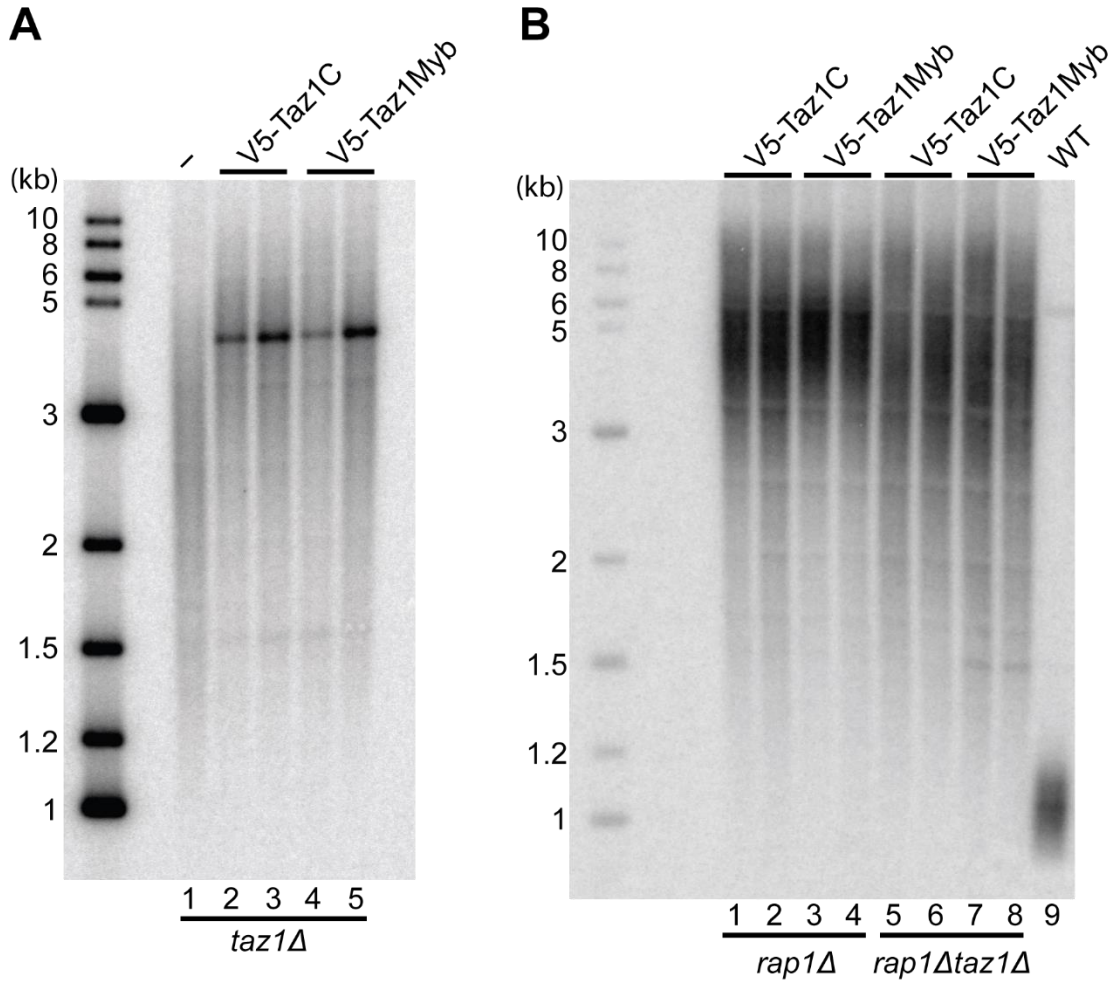


Figure 3.5: Expressing Taz1C or Taz1Myb alone does not rescue telomere length of cells deleted for *taz1* or *rap1*. N-terminal V5 tagged Taz1C and Taz1Myb were expressed from the REP81 plasmid under control of the *nmt81* promoter in **(A)** *taz1Δ* and **(B)** *rap1Δ* or *rap1Δtaz1Δ* cells. Telomere length was assessed by Southern blot. Two isolates of each strain were analyzed.

III.3.3: Replacement of Rap1 with a synthetic linker

Deletion of Rap1 leads to telomere elongation similar to *taz1Δ*. Rap1 interacts with Taz1 via its C-terminal RCT domain [40]. The N-terminus of Rap1 contains a BRCT domain, a Myb and a Myb-like (Myb-L) domain, whose functions have yet to be

characterized despite their conservation among species [43, 44]. Rap1 440-490 a.a. interacts with Poz1 and was therefore named the Poz1 interaction (PI) domain (personal communication with Nico Thomä) (Figure 3.6A), which overlaps substantially with the 457-512 a.a. previously reported by the Kanoh lab [104]. I first characterized the role of each Rap1 domain by making deletion mutants lacking each individual domain (Figure 3.6A). Rap1 mutants lacking the BRCT, Myb or Myb-L domain maintained wild-type telomere length. In contrast, Rap1ΔPI or Rap1ΔRCT cells had elongated telomere length similar to *rap1Δ* (Figure 3.6B). To further define the region of Rap1 that is important for telomere length maintenance, larger truncations were introduced into cells (Figure 3.7A). The entire N-terminus consisting of 63% of the protein (1-439 a.a.) was dispensable for wild-type telomere length maintenance (Figure 3.7A). These results are consistent with a requirement for the Poz1- and Taz1- binding sites, but not the N-terminal 1-456 a.a., in telomere length control [104]. The essential C-terminal region of Rap1 contains Poz1 and Taz1 interaction domains. To further test whether the function of Rap1 in length maintenance is limited to providing interactions with Taz1 and Poz1, each domain was replaced with a 14 a.a. synthetic linker. Fusion of the Poz1 interaction domain (PI) to the C-terminus of Taz1 maintained wild-type telomere length in the absence of endogenous Taz1 and Rap1 (Figure 3.7B, lane 1-4). Similarly, fusing the Taz1 interaction domain (RCT) to Poz1 rescued telomere lengthening caused by deletion of *poz1* and *rap1* (Figure 3.7B, lane 5-9). Both strains lost telomeres following *trt1* deletion (Figure 3.8), suggesting that telomeres were maintained by telomerase.

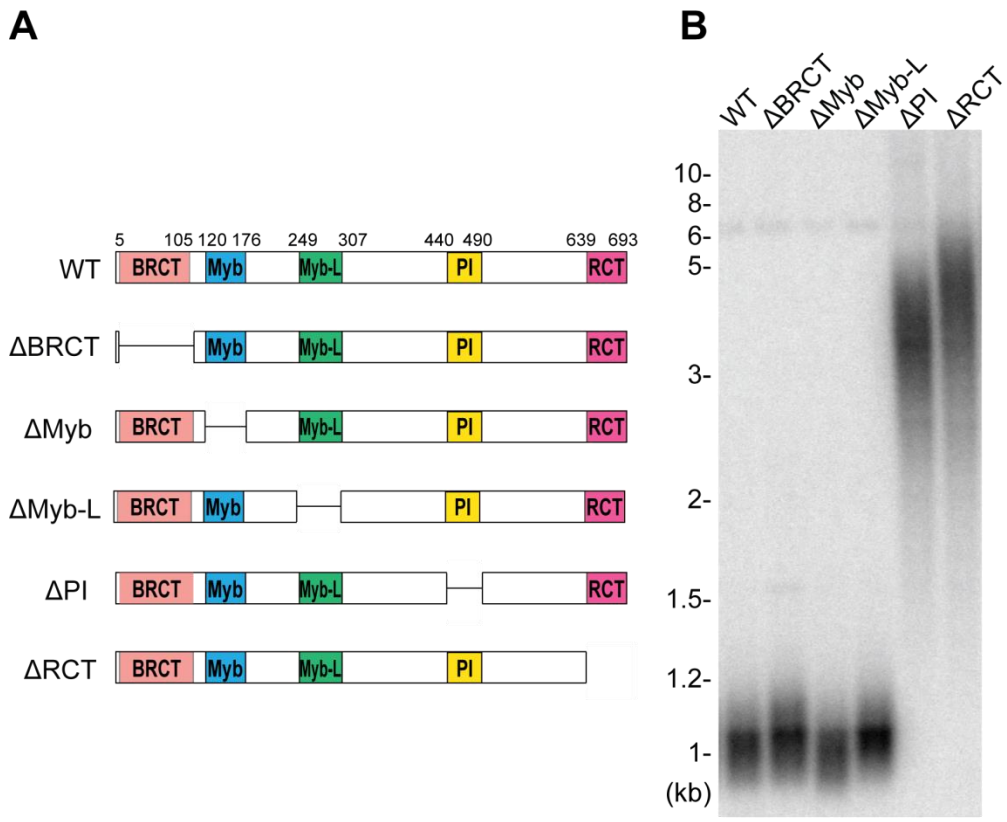


Figure 3.6: Telomere length of a series of Rap1 deletion mutants. **(A)** Schematic of the mutants. **(B)** Telomere length analysis for the Rap1 mutants by Southern blot.

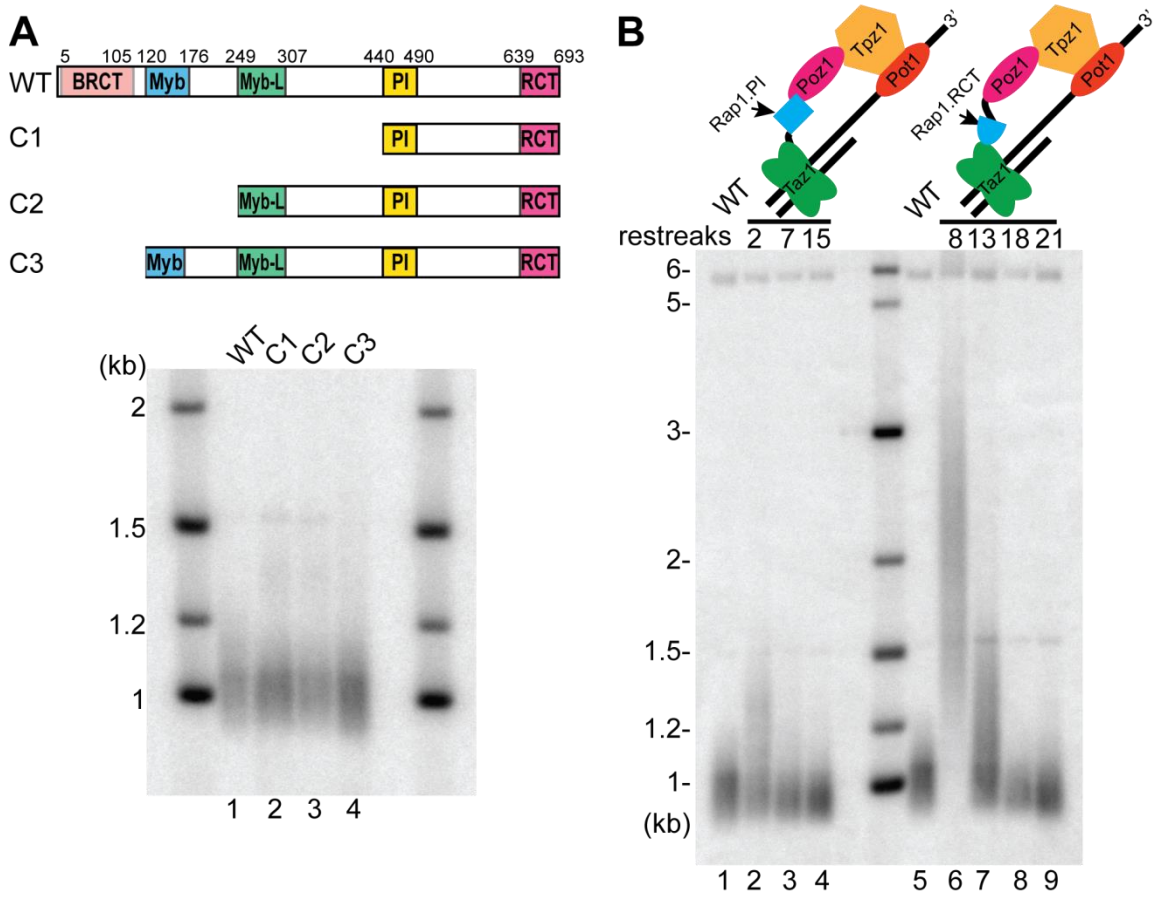


Figure 3.7: Rap1 functions as an interaction module in telomere length maintenance. **(A)** Telomere length analysis for the truncation mutants shown in the schematic. Strains were constructed by introducing Rap1 fragments under the control of the *nmt81* promoter into a *rap1Δ* strain and were subjected to 14 sequential restreaks prior to telomere length analysis. **(B)** Replacement of RCT and PI domains of Rap1 with covalent linkers to Taz1 and Poz1 respectively. The PI domain (440-490 a.a.) of Rap1 was expressed in frame with Taz1 and a V5-epitope tag under the control of the *taz1* promoter in a *taz1Δrap1Δ* strain. The RCT domain (639-693 a.a.) of Rap1 was expressed in frame with Poz1 and a V5-epitope tag under the control of the *poz1* promoter in a *poz1Δrap1Δ* strain. The fusion constructs were integrated into the genome at the respective sites of the *taz1* and *poz1* genes.

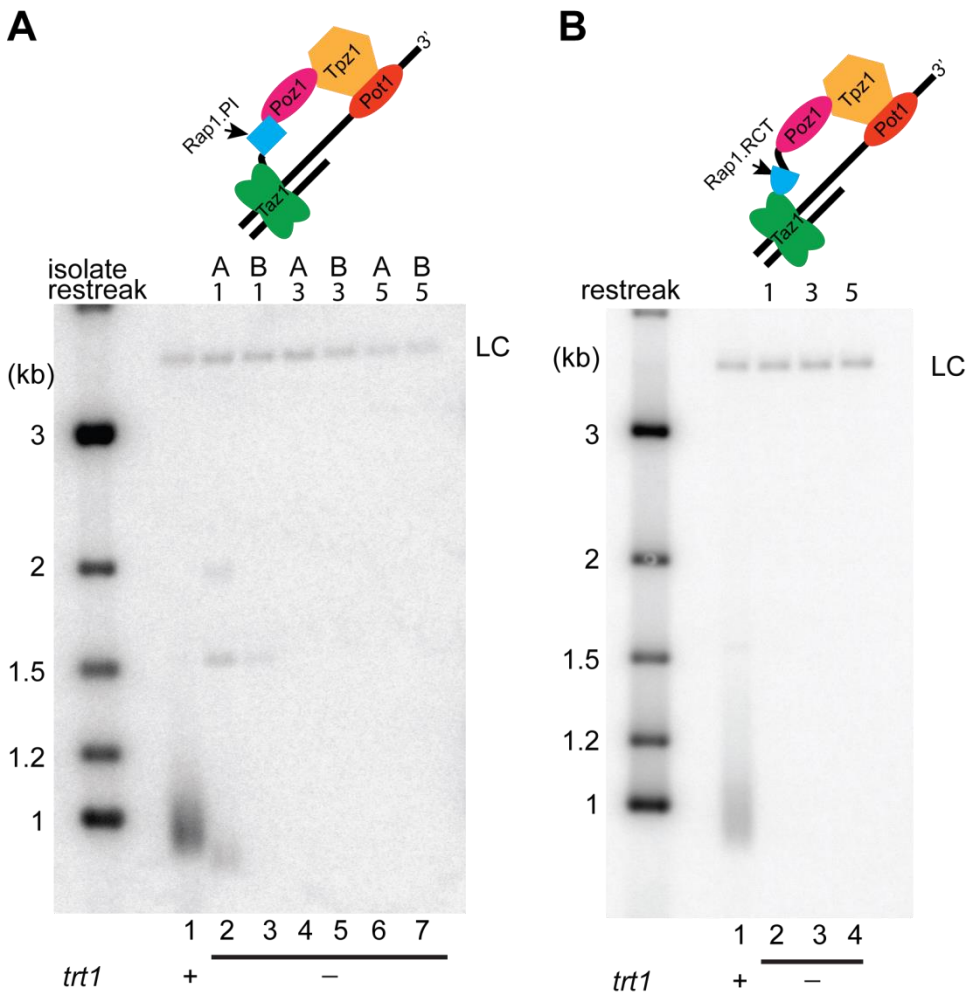
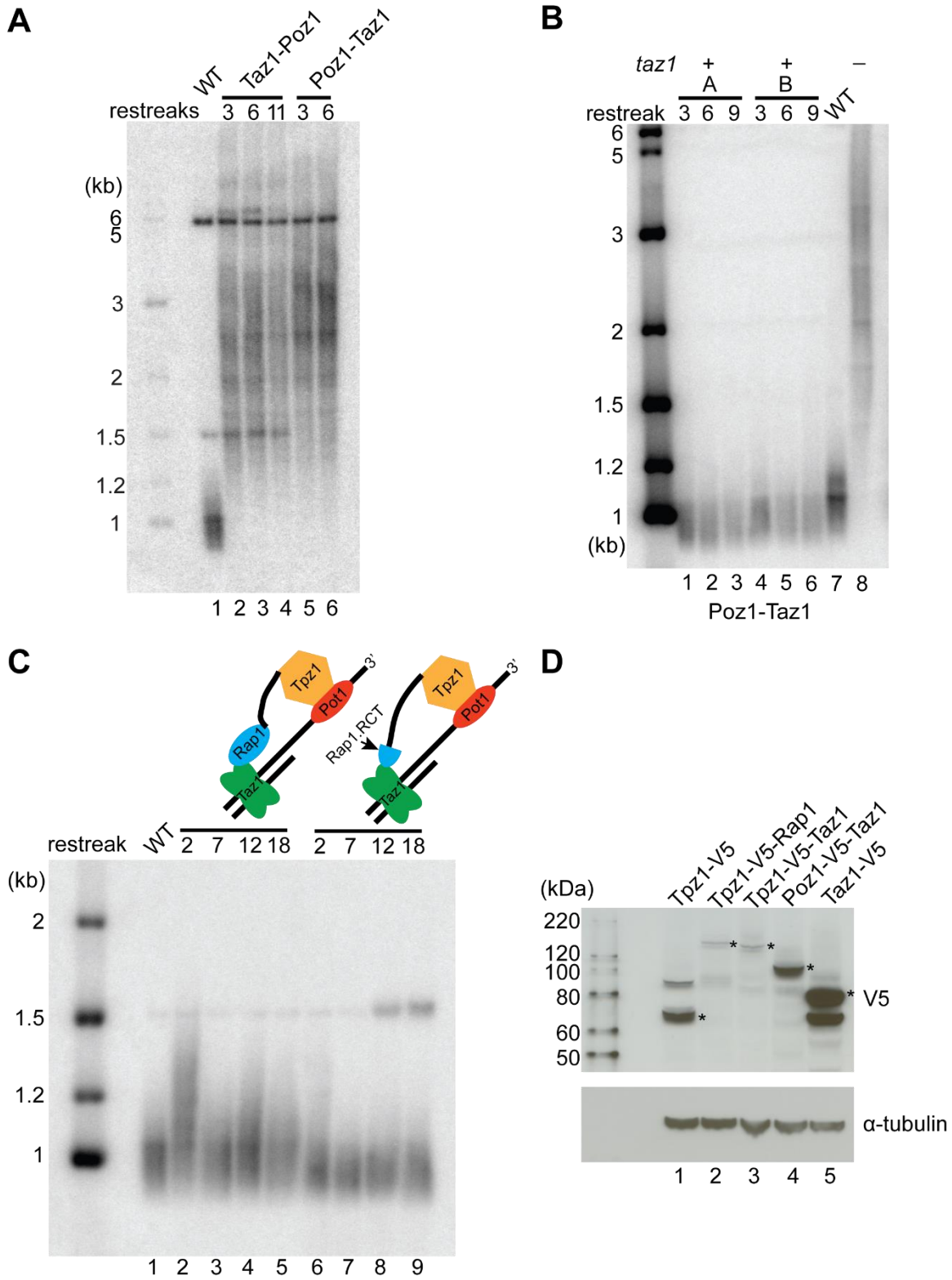


Figure 3.8: Telomere maintenance in Taz1-Rap1.PI and Poz1-Rap1.RCT cells is dependent on telomerase. Deletion of *trt1* was achieved by crossing the strains with a *trt1Δ* strain. **(A)** Taz1-V5-Rap1.PI, *rap1Δ, taz1Δ*. **(B)** Poz1-Rap1.RCT, *rap1Δ, poz1Δ*.

These results strongly suggest that the role of Rap1 in telomere length regulation is to provide a molecular bridge between Taz1 and Poz1. Therefore, we predicted that the entire protein can be replaced with a synthetic linker. However, when I expressed Taz1-Poz1 in *taz1Δrap1Δpoz1Δ* cells, telomeres were as long as in the deletion strains lacking the fusion protein (Figure 3.9A, lane 2-4). In order to make sure this was not the

consequence of the orientation of the fusion, I switched the order of Poz1 and Taz1 in the fusion protein such that Poz1 was at the N-terminus and Taz1 was at the C-terminus. Again, this Poz1-Taz1 fusion protein failed to rescue telomere lengthening (Figure 3.9A, lane 5, 6). We cannot rule out the possibility that the PI and RCT domains have redundant functions in inhibiting uncontrolled telomere elongation. However, two alternative and perhaps more likely explanations are that the synthetic fusion of two proteins affects expression level or protein folding, or that the synthetic linker between Taz1 and Poz1 is too short to accommodate the high order structure required for the telomerase inaccessible state. Interestingly, I found that Poz1-Taz1 maintained wild-type telomere length in *rap1Δpoz1Δ* cells (Figure 3.9B). This repudiates the necessity of any part of Rap1 and instead indicates that the defect relates to Taz1 function or reduced protein level of Taz1when it is part of a Taz1-Poz1 fusion.


A
WT
Taz1-Poz1
Poz1-Taz1
restreaks 3 6 11 3 6
(kb)
6
5
3
2
1.5
1.2
1
1 2 3 4 5 6
B
taz1 + A + B –
restreak 3 6 9 3 6 9 WT
6
5
3
2
1.5
1.2
1
(kb)
1 2 3 4 5 6 7 8
Poz1-Taz1
C
Tpz1
Pot1
Rap1
Taz1
3'
Rap1 RCT
restreak WT 2 7 12 18 2 7 12 18
(kb)
2
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1 2 3 4 5 6 7 8 9
D
(kDa)
Tpz1-V5
Tpz1-V5-Rap1
Tpz1-V5-Taz1
Poz1-V5-Taz1
Taz1-V5
220
120
100
80
60
50
V5
α-tubulin
1 2 3 4 5

Figure 3.9: Telomere length effects of replacing Rap1 and Poz1 with covalent linkers. **(A)** Taz1-Poz1 and Poz1-Taz1 fusions fail to rescue the telomere elongation effect associated with deletion of *rap1*. The fusion constructs were integrated at the genomic locus, and were driven by the promoter of the N-terminal fusion partner. The endogenous copies of *poz1* and *taz1* have been deleted. **(B)** Telomere elongation associated with replacing *taz1*, *rap1* and *poz1* with a *poz1-taz1* fusion is rescued in the presence of endogenous *taz1*. Telomere length analysis for two independent isolates is shown. **(C)** Telomere elongation associated with deletion of poz1 is rescued by fusing Tpz1 via a V5 epitope linker to Rap1 (lanes 2–5) or the RCT of Rap1 (lanes 6–9). The integration strategy was as described in (A). **(D)** Analysis of protein levels for Tpz1, Taz1 and fusion constructs by Western blot. All proteins are V5-epitope tagged at the endogenous locus for Tpz1 and Taz1 and at the locus of the N-terminal partner for fusion constructs. An antibody against α-tubulin was used as loading control. The asterisks indicate bands of the correct size in each lane. The identity of the 90 kDa band in lane 1 is presently unclear, the lower band in lane 5 is the result of translational initiation at an internal ATG in Taz1 (personal communication with Julie Cooper).

III.3.4: Poz1 can be replaced by a covalent linker

Poz1 bridges the factors that bind the double-stranded and single-stranded parts of the telomere. I replaced the entire Poz1 protein with a 14 a.a. peptide linking Tpz1 and Rap1. Tpz1-Rap1 rescued telomere length to wild-type level in the absence of Poz1 (Figure 3.9C, lane 2-5), suggesting that similar to Rap1, Poz1 also functions by providing interaction motifs that bridge the other components in the telomeric complex. Consistent with this, fusion of only the RCT domain of Rap1 to Tpz1 (Tpz1-Rap1.RCT) maintained wild-type telomere length in the absence of both Rap1 and Poz1 (Figure 3.9C, lane 6-9). In both cases, telomeres were maintained via telomerase, as deletion of telomerase caused telomere loss (Figure 3.10). It's worth noting that the protein level of Tpz1-V5-Rap1 was much lower than Tpz1-V5 (Figure 3.9D, compare lane 2 to lane 1), suggesting the endogenous level of Tpz1 is not limiting for its function.

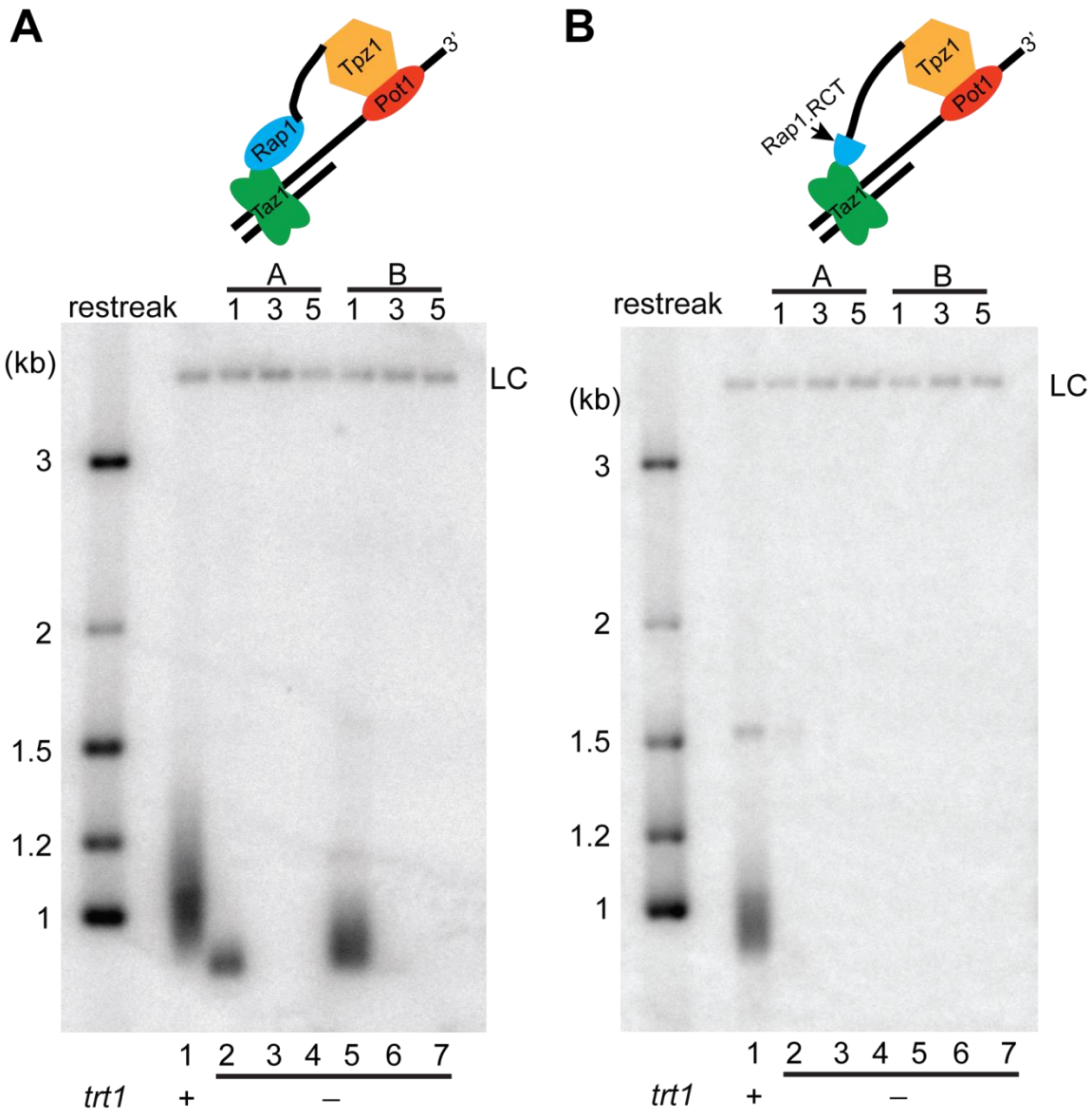


Figure 3.10: Telomerase dependence test for **(A)** Tpz1-V5-Rap1 and **(B)** Tpz1-V5-Rap1.RCT cells.

III.3.5: Tpz1-Taz1 can function in length regulation as a mini-telosome

Since Rap1 and Poz1 can be replaced individually by a synthetic linker, it was tempting to test if a fusion between Tpz1 and Taz1 is sufficient to substitute Rap1 and

Poz1. I integrated *tpz1-taz1* under the control of the *tpz1* promoter into *taz1Δrap1Δpoz1Δ* cells at the *tpz1* endogenous locus. The expression level of the fusion protein was found to be similar to Tpz1-Rap1, but much lower than Tpz1-V5 or Taz1-V5 (Figure 3.9D, compare lane 3 to 1 and 5). Note that Poz1-Taz1 was also expressed at a much lower level compared to Taz1-V5 (Figure 3.9D, compare lane 4 to 5). Similar to what was observed in Poz1-Taz1 cells, telomeres were still elongated in the presence of the Tpz1-Taz1 fusion protein (Figure 3.11A). The long telomeres were maintained by telomerase (Figure 3.12A). However, the presence of endogenous Taz1 rescued the elongation to near wild-type level (Figure 3.11B). These results suggest that Taz1 levels play a key role in length regulation. It further suggests that the number of Tpz1 molecules required for length regulation is much lower than the number present in wild-type cells.

To distinguish whether the availability of total number of Taz1 molecules (either free or part of a fusion protein) is important or the free Taz1 itself is required, I further overexpressed *tpz1-taz1* under the control of the *nmt1* promoter, a strong promoter under induced conditions, but largely repressed by the presence of thiamine in the growth media. Western blot analysis revealed no difference of protein expression levels in the presence or absence of thiamine when cells were cultured in rich media YES, suggesting that YES media contains sufficient thiamine to suppress the *nmt1* promoter (Figure 3.13). Of the two isolates I worked with, one isolate expressed the fusion protein to similar level of Taz1-V5 under *taz1* endogenous promoter (Figure 3.11C, lane 4) and the other isolate expressed twice the amount (Figure 3.11C, lane 3), most likely due to the integration of more than one copy of the plasmid. Expressing Tpz1-Taz1 at similar level to endogenous

Taz1 was sufficient to rescue telomere length (Figure 3.11D, lane 7-11). Cells with the higher levels of Tpz1-Taz1 had a more homogeneous telomere length and the equilibrium was reached faster (Figure 3.11D, lane 2-6). This result demonstrates that the total number of Taz1 molecules is important for length regulation. It also suggests that telomere length regulation is sensitive to Taz1 levels in either the fusion form or the free from. It also further confirms that the entire Rap1 and Poz1 proteins can be replaced with a synthetic linker. They both function as the interaction modules to bridge other proteins. Importantly, the physical distance between Tpz1 and Taz1, thus between the double-stranded region and the single-stranded region, is also not critical, as a 14 a.a linker can substitute for two proteins with a combined molecular weight of 109 kDa. In summary, I have generated two strains, each with greatly simplified telomeric complexes (Low expression Tpz1-Taz1+Taz1 and High Expression Tpz1-Taz1), that maintain wild-type telomere length. The maintenance is dependent on telomerase (Figure 3.12B and C). I refer to them as mini-telosomes.

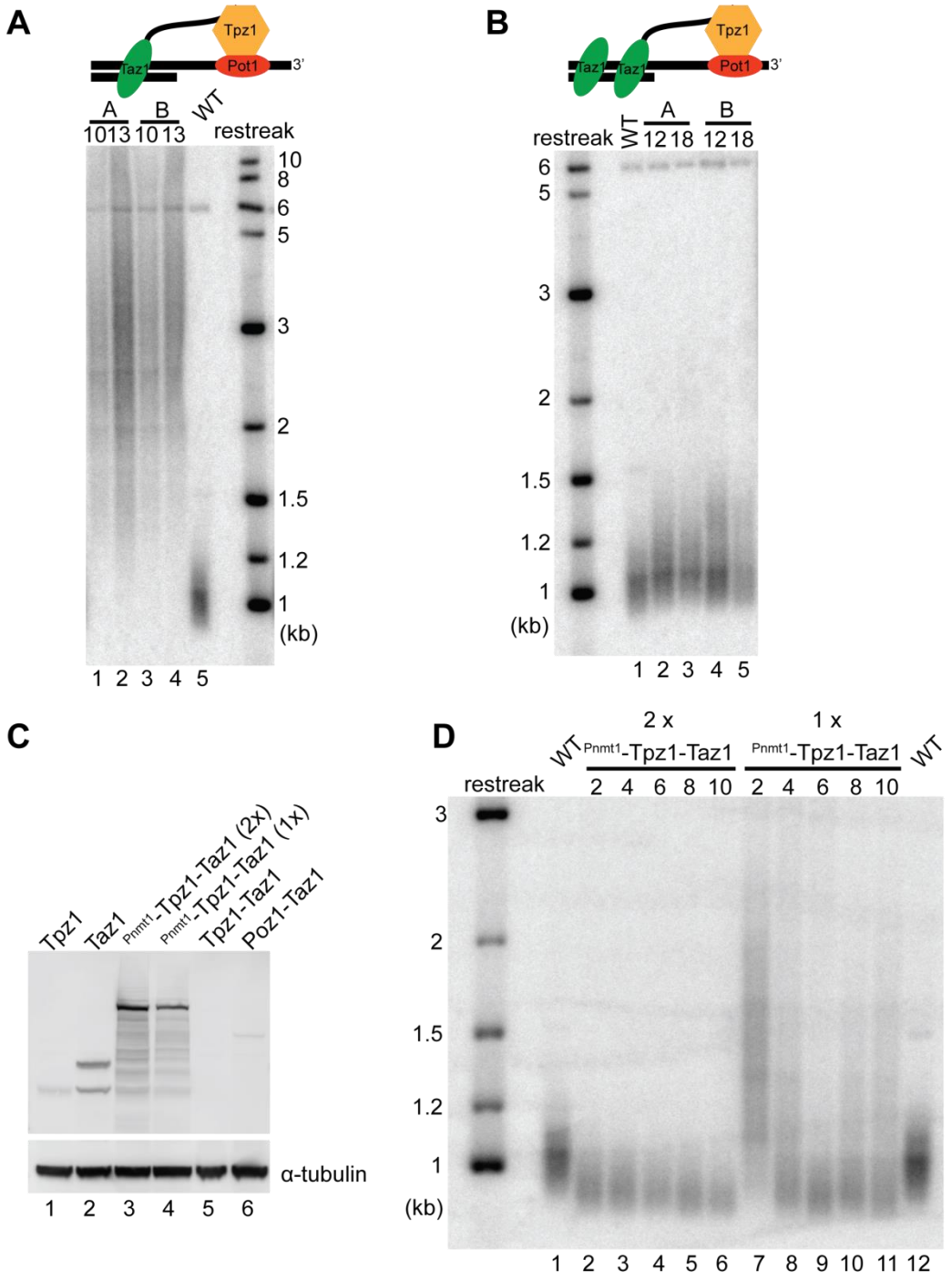


Figure 3.11: Telomere length maintenance is sensitive to the level of Taz1. **(A)** Expression of Tpz1-V5-Taz1 under the control of the *tpz1* promoter is not sufficient to rescue the long telomere length caused by deletion of endogenous *taz1*, *rap1*, *poz1*, and *tpz1*. **(B)** Tpz1-V5-Taz1 maintains normal telomeres in the presence of endogenous *taz1*

and absence of *rap1, poz1* and *tpz1*. **(C)** Determination of protein levels by Western blot analysis. One and multiple copies of *tpz1-v5-taz1* were integrated at the *aur1* locus under the control of *nmt1* promoter. **(D)** Telomere length analysis of strains expressing Tpz1-V5-Taz1 fusions in the absence of endogenous *taz1, rap1, poz1* and *tpz1*.

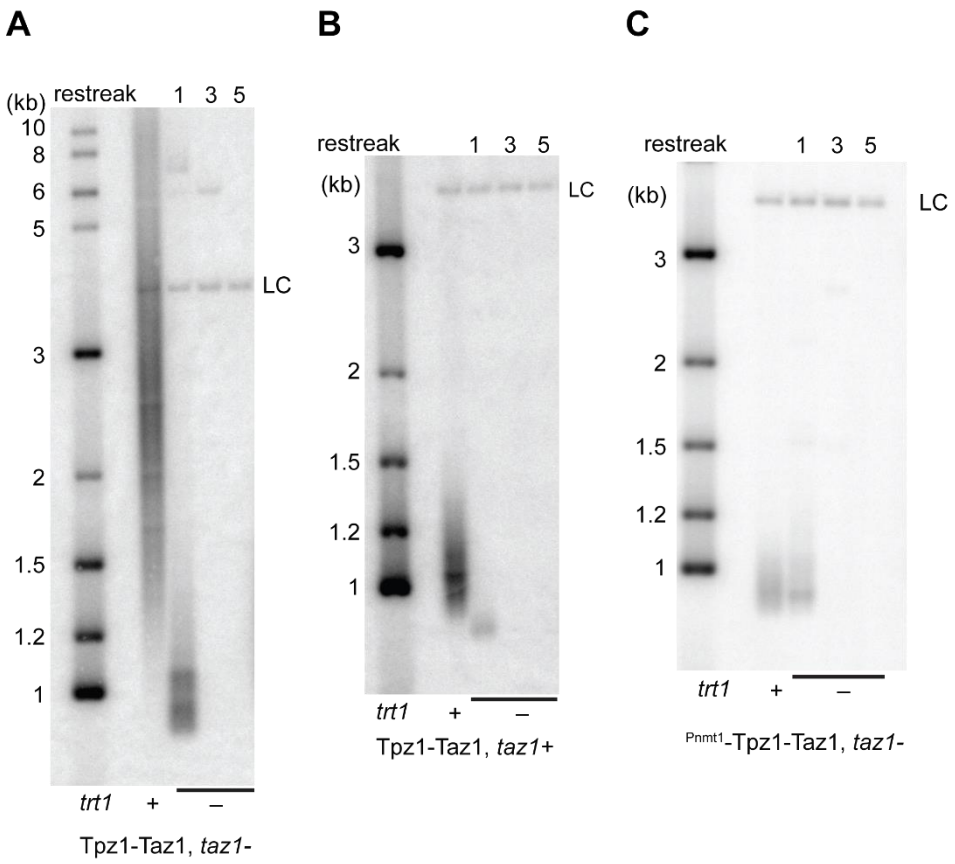


Figure 3.12: Telomerase dependence test for strains expressing Tpz1-Taz1. **(A)** Cells with low expression Tpz1-V5-Taz1 and in the absence of endogenous Taz1. **(B)** Mini-telosome with low expression Tpz1-V5-Taz1 and in the presence of endogenous Taz1. **(C)** Mini-telosome with high expression Tpz1-V5-Taz1**.**

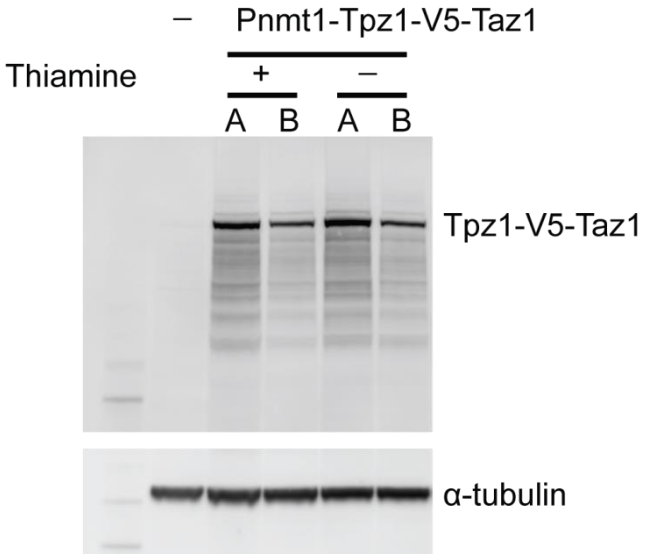


Figure 3.13: Western blot analysis for cells grown in YES media with and without thiamine. Cells harboring *nmt1* promoter driven Tpz1-V5-Taz1 were cultured in rich media YES with or without the addition of 15μM thiamine. A and B represent the higher expression isolate and the lower expression isolate, respectively. Anti-V5 antibody was used for detection of the Tpz1-V5-Taz1 protein and anti-α-tubulin antibody was used as loading control.

III.3.6: Mini-telosomes retain normal growth, TPE and cold-resistance.

Previous characterization of telomeric protein functions with deletion mutants showed coupled phenotypes. For example, end-fusion phenotype or loss of TPE correlates with telomere lengthening [44, 89, 123], making it difficult to dissect the mechanisms of each function. The mini-telosomes provided a unique opportunity to further dissect the molecular basis of length regulation, end protection, and other functions of the telomere. For the high expression Tpz1-Taz1 strain, I used the isolate with higher expression because its telomere length is more homogeneous.

I first compared the growth rate of the mini-telosome cells to wild-type cells. There was no difference in generation time at log phase (Figure 3.14A). I next measured average cell length. While low expression Tpz1-Taz1+Taz1 (referred to as mini-telosome A) cells were indistinguishable from wild-type, high expression Tpz1-Taz1 (referred to as mini-telosome B) cells were 28% longer (Figure 3.14A). Although long cells are generally indicative of G2 checkpoint activation and thus prolonged growth prior to mitosis, there was no difference in doubling times. Even cells with just low expressing Tpz1-Taz1 (and thus long telomeres) showed no significant increase in generation time (~7min longer than wild-type) while revealing ~40% increase in cell length (Figure 3.14A).

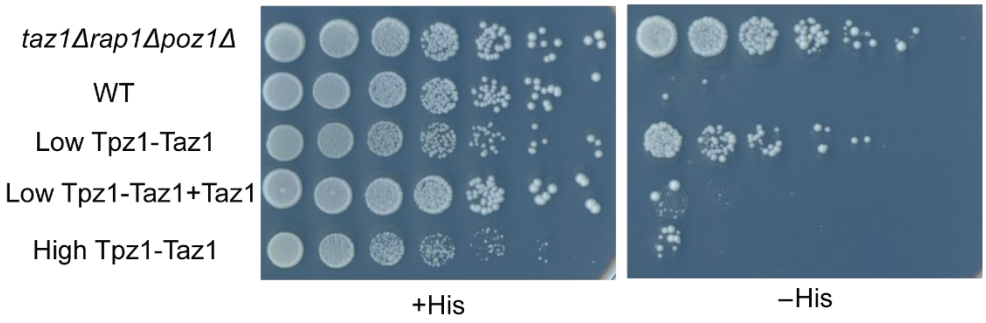
Deletion of Rap1 and Poz1 has been shown to cause loss of TPE in combination with extreme telomere elongation [44, 104]. By providing a covalent linker between Taz1 and Tpz1, the mini-telosomes rescued the telomere length regulation defect caused by the loss of Rap1 and/or Poz1. I next tested if TPE is also rescued by mini-telosomes by examining expression of a *his3*$^{+}$ marker gene integrated near a telomere. Wild-type cells failed to grow in the absence of histidine while *taz1Δrap1Δpoz1Δ* cells were able to grow due to the loss of TPE (Figure 3.14B). Both my mini-telosome cells showed very limited growth in the absence of histidine, similar to wild-type cells (Figure 3.14B). The ability of mini-telosomes to maintain TPE suggests a link between Taz1 and Tpz1 is sufficient to maintain heterochromatin near chromosome ends. The presence of Rap1 and Poz1 proteins is not necessary.

Taz1 has been shown to be critical for cell growth at low temperature by the Cooper lab [123, 124]. Deletion of Rap1 exacerbates the cold sensitivity of *taz1Δ* cells although it has no effect in *taz1*$^{+}$ cells [123]. I compared growth of mini-telosome cells at 18°C and 32°C. In addition to Rap1, Poz1 is also not important for cold-resistance as *rap1Δpoz1Δ* cells grow similarly to wild-type cells (Figure 3.14C). While cells expressing low levels of Tpz1-Taz1 were as sensitive to cold as *taz1Δrap1Δpoz1Δ* cells, both mini-telosomes showed identical growth to wild-type cells (Figure 3.14C). This result confirms that Taz1, but not Rap1 or Poz1, is critical for cell growth at low temperature. Furthermore, the fusion form of Taz1 functions as well as the free Taz1 in promoting cell growth at low temperature.

A

Strain	Generation time (min)	Average cell length (μm)
WT	125.84±0.66	10.69±0.16
Low Tpz1-Taz1+Taz1	123.54±0.48	10.51±0.18
High Tpz1-Taz1	124.72±0.75	13.72±0.30
Low Tpz1-Taz1	132.99±0.65	14.79±0.29

B



C

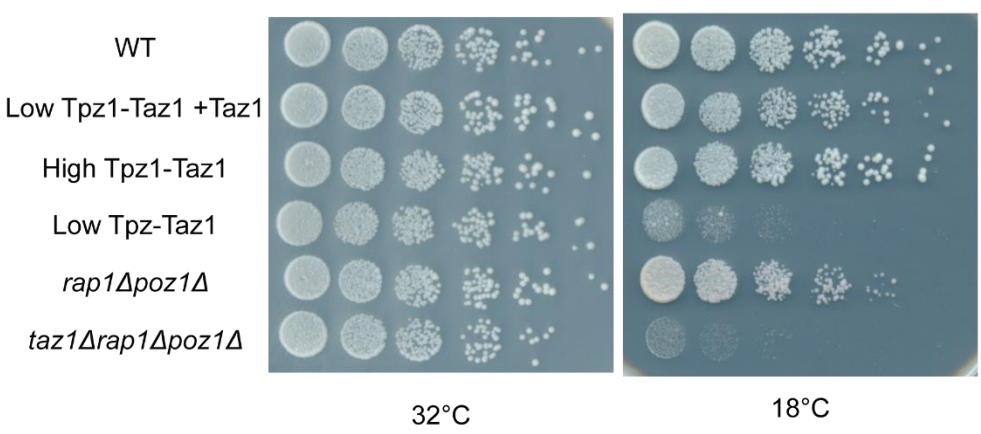


Figure 3.14: Mini-telosomes retain normal growth, TPE and cold-resistance. **(A)** Generation time and average length of mini-telosome cells. **(B)** Telomere position effect of mini-telosome cells. All cells have a *his3*$^{+}$ marker integrated near a telomere whose expression is suppressed in wild-type cells due to TPE. Spotting assays were done with 1:5 serial dilutions starting from 2x10^{6} cells and plated on PMG+ALUH (+His) and PMG+ALU (-His). **(C)** Cell growth in the cold. Serial dilutions were done the same as in (B) and plated on YES and incubated at 32°C and 18°C, respectively.

III.3.7: Mini-telosomes fail to protect cells against NHEJ-mediated chromosome end-fusions.

Another telomere defect that has previously been observed in conjunction with long telomeres are chromosome end-fusions mediated by NHEJ in G1 arrested *taz1Δ* or *rap1Δ* cells [89, 123]. I thus examined G1 arrested mini-telosome cells for the presence of chromosome end-fusion events. Interestingly, neither mini-telosome was sufficient to prevent end-fusions compared to wild-type cells (Figure 3.15A), providing a separation of functions for chromosome end protection and telomere length regulation. To our knowledge, this is the first example of fission yeast cells with wild-type telomere length undergoing chromosome end-fusions.

III.3.8: Rap1, but not Poz1, restores end protection.

The mini-telosomes lack two telomeric proteins, Rap1 and Poz1. To investigate whether the absence of either protein was the cause of compromised end protection, I supplemented mini-telosome A with Rap1, Poz1, and both together. In order to gain a better signal of the bands representing chromosome fusions, I crossed the cells with 972h$^-$ wild-type cells to eliminate auxotrophic markers and gain a better G1 arrest (see Table 2.1). Chromosome end fusions were absent in cells expressing Rap1 or Rap1 and Poz1 together (Figure 3.15B, lane 2 and 4). In contrast, expression of Poz1 alone was insufficient to prevent end-fusions in mini-telosome cells (Figure 3.15B, lane 3). Examination of *poz1Δ* cells arrested in G1 confirmed that this protein is not required for the protection of telomeres against NHEJ (Figure 3.15B, lane 7). These results provide

two fundamental insights: Firstly, Rap1, but not Poz1, is required to prevent chromosome end-fusions in G1. Secondly, Rap1 is not functioning simply by maintaining wild-type telomere length, nor by maintaining a close conformation of the telomeric complex by providing interactions between Taz1 and Poz1. Instead, the protein itself is involved in preventing end-fusions, possibly by interacting with factors that negatively regulate NHEJ.

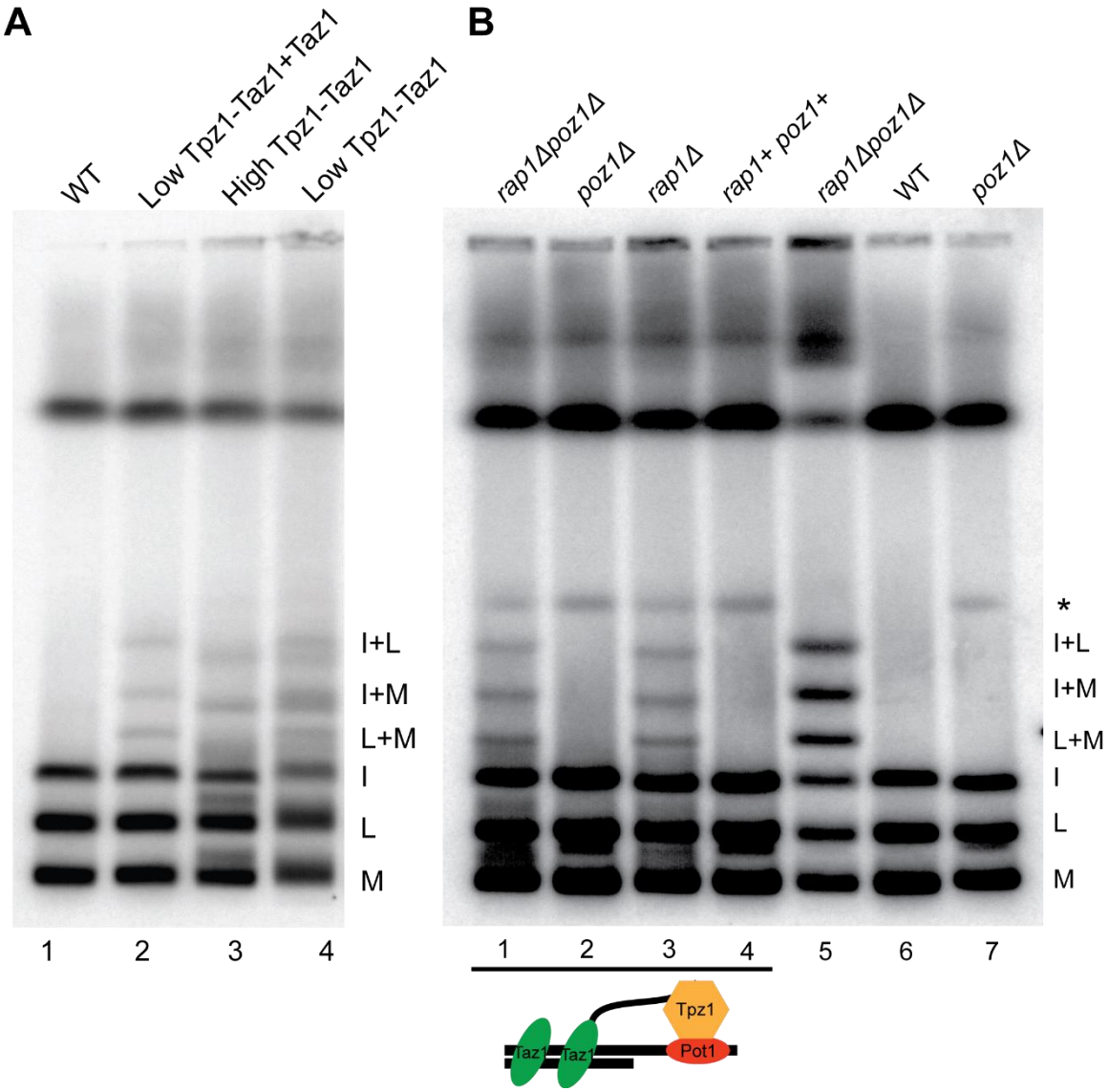


Figure 3.15: Chromosome end-fusion analysis of mini-telosome cells. **(A)** Mini-telosomes are not sufficient to protect cells against NHEJ-mediated chromosome end-fusions. **(B)** Rap1, but not Poz1, restores end protection. Nitrogen starved cells were analyzed by PFGE with internal C, I, L, M probes. The auxotrophic markers (*ade6-M210* or *ade6-M216, leu1-32, ura4-D18, his3-D1*) were eliminated in cells used in (B) to increase G1 arrest efficiency.

III.4: Discussion

Open-closed conformation model: the expected and the surprise

In this study, I have dissected the contribution of different telomeric proteins to the maintenance of telomere length equilibrium in fission yeast and characterized two mini-telosomes lacking Rap1 and Poz1 yet sufficient to maintain wild-type telomere length. Further investigation suggests that they are competent in cell growth, TPE and cold-resistance but defective in preventing chromosome end-fusions compared to the wild-type telomeric complex. Rap1, but not Poz1, is required for the end-protection function. *S. pombe* spends very little time in G1 phase that loss of the protection from NHEJ is not a big issue unless cells are arrested in G1, which allows us to tease apart these functions. An open-closed conformation model for telomere length regulation has been proposed in which the proteinacious bridge across the double- and single-stranded parts of a telomere generates a closed conformation at the chromosome end that is inaccessible to telomerase and disruption of the bridge switches telomere to an open conformation that allows telomerase access [58]. My results agree with this model as both Rap1 and Poz1 function as interaction modules.

Importantly, it suggests that the requirement and regulation of the exact higher order structure is not as complicated and is more flexible than previously thought. Neither the peptide sequence nor the structure of two entire proteins is necessary as long as Taz1 and Tpz1 are linked. Even the physical distance provided by the 80kDa Rap1 and 30kDa Poz1 proteins is not critical and can be reduced to 14 amino acids. In addition, the

dynamic regulation of the interactions seems to not be important either, as in our mini-telosome cells, the only intact interaction is the Tpz1-Pot1 interaction. Although telomere length was shorter than wild-type in the Pot1-Tpz1 cells (Figure 3.1D), the stable telomere length suggests that telomerase can still access and act on telomeres.

Why is length regulation sensitive to Taz1 levels?

Although Rap1 and Poz1 only serve simple bridging functions and the level of Tpz1 for sufficient regulation is much less than the available Tpz1 in cells, the proper length regulation is sensitive to Taz1 levels. It is possible that Taz1 molecules provide the basis for the recruitment of other proteins that are needed for a higher order structure and length regulation. Alternatively but not mutually exclusively, given that Taz1 is the protein that binds double-stranded telomeric repeats, the number of Taz1 molecules bound to telomeric repeats may serve as a gauge for telomere length similar to the "counting" role of Rap1 in budding yeast [64]. Either case, insufficient Taz1 would compromise length regulation.

Separation of telomere functions

Telomeres have several distinct physiological functions including protection of chromosome ends from DNA repair activities, solving the end replication problem, and organizing chromosomes in meiosis via bouquet formation. The characterization of protein-protein interactions among all of the five core telomere binding proteins has led to the view that they function as a unit termed shelterin or telosome in yeast. However, when individual proteins are deleted or truncated, distinct phenotypes are observed. On

one hand, different components are involved in a specific function and on the other hand, each component may participate in several different functions. The recruitment of some proteins depends on the others, adding more complexity to each function. Our study utilized the well-developed genetic tools in fission yeast to tease apart contributions of each protein. As a result, I identified mini-telosomes that are sufficient for telomere length maintenance. This provided us with a unique opportunity to further characterize other functions of telomeres. My results demonstrate that cell growth is normal in the mini-telosome cells. TPE is also maintained, suggesting that both Rap1 and Poz1 proteins are not necessary if interactions between Taz1 and Tpz1 are restored by a linker. Previous studies have shown that deletion of *rap1* or *poz1* leads to loss of TPE [44, 104]. My results suggest that it is not due to the requirement of Rap1 or Poz1 to recruit certain factors. Instead, this may be an indirect effect of telomere length deregulation caused by the loss of either protein. I have also shown that Taz1 is the major player to promote cell viability at low temperature and that the fusion form of Taz1 functions as well as the free form.

Interestingly, I found that mini-telosomes cannot prevent chromosome end-fusions in G1 arrested cells. Instead, Rap1, but not Poz1, is also required. Intriguingly, end protection does not require the interaction between Rap1 and Poz1; in fact, the entire Poz1 protein is dispensable. This surprising result suggests that the molecular bridge between proteins that bind single- and double-stranded telomeric DNA is not sufficient nor required for end protection. My results further demonstrate that the end-fusion phenotype observed in G1 arrested cells lacking Taz1 or Rap1 is not an indirect

consequence of the dramatically elongated telomeres in these mutants, but are directly attributable to functions for Taz1 and Rap1 in end protection independent of their roles in telomere length regulation.

The role of Rap1 in end protection

Rap1 is the most conserved telomeric protein across species. Besides the C-terminal RCT domain, the BRCT, Myb and Myb-like domains are shared features among Rap1 proteins from different organisms, indicating conservation in functions as well. However, these potential functions are not characterized yet. We hypothesize that Rap1 may interact with factors that actively inhibit NHEJ and that this mechanism may be conserved. This is supported by the study demonstrating that in *S. cerevisiae*, Rap1 prevents NHEJ mediated telomere fusions [90] despite the fact that budding yeast Rap1 binds double-stranded telomeric repeat DNA directly. Rap1 provides this protection partly by recruiting Rif2 and Sir4 through its C-terminal domain [125]. However, there are some discrepancies about the involvement of human and mouse RAP1 in inhibiting NHEJ. While our lab has shown that tethering RAP1 to telomeres rescues massive end-fusions caused by TRF2 depletion in human cells [95], the de Lange lab has shown that both human and mouse RAP1 is not required for preventing NHEJ as deletion of RAP1 shows no increase in chromosome end fusions [96, 97]. Instead, they observed T-SCEs due to increased HR at telomeres in Ku70 deficient mouse cells [97]. One explanation to reconcile these results is that the ability to prevent NHEJ is still conserved in RAP1; however, this role becomes dormant as TRF2 takes the major responsibility and may act

upstream of RAP1. In fact, the T-loop structure formed by the help of TRF2 may be one mechanism cells sequester the telomere ends from activating ATM [126, 127]. Even without the T-loop structure, TRF2 itself may still be able to actively prevent ATM and NHEJ [85, 128]. While RAP1 cannot prevent ATM activation, it is able to inhibit the downstream effect which is the DNA fusion mediated by NHEJ [95]. It is not surprising that several layers of protection are used to ensure genome integrity. In fact, the ability of Rap1 to inhibit telomere recombination in mice becomes critical only when Ku70, another HR inhibition factor, is not present [97] supporting the presence of multiple pathways to protect telomere integrity. Further characterization of how Rap1 inhibits NHEJ in fission yeast may provide more information to understand mammalian telomere protection. Considering the BRCT domain is found in a large number of DNA damage response proteins and is frequently used as a protein interaction dock [45], it may be involved in Rap1's role to prevent NHEJ. Some Myb domains also function as protein interaction modules [129, 130]. Characterization of the Rap1 domains involved in protecting fission yeast telomeres and the factors those domains recruit to inhibit NHEJ will help elucidate the mechanism of this function and provide more information to evaluate the conservation of this function.

Chapter IV: The structure of Poz1-Tpz1 reveals conserved interaction modules and potential to form higher order structures

This chapter presents a collaborative project between our group and the lab of Dr. Nicolas Thomä at Friedrich Miescher Institute for Biomedical Research in Switzerland. Cian Stutz from the Thomä lab produced the crystal structure of Poz1-Tpz1 and created some of the mutants used for my *in vivo* experiments (see Table 2.1). Figures 4.1- 4.6, 4.8, 4.12 and 4.13A presented here are adapted from his unpublished thesis [131].

IV.1: Abstract

Telomere repeats recruit specific proteins, collectively termed the shelterin complex to protect their integrity and regulate *de novo* synthesis by telomerase. Fission yeast *Schizosaccharomyces pombe* telomere structures share remarkable similarities to the human structures, including related shelterin components. Fission yeast shelterin consists of the Taz1/Rap1 complex that binds the double-stranded telomere repeats and the Pot1/Tpz1 unit that binds the single-stranded 3' overhang. Homologs of these proteins are found in the human shelterin complex. The only unique protein, Poz1, interacts with Rap1 and Tpz1, potentially bridging the factors that bind double- and single-stranded parts of telomeres. However, little information about Poz1 is available. Here we present the crystal structure of $Poz1^{30-249}$ in complex with $Tpz1^{475-508}$. This structure resembles the TRFH domains of the human TRF1 and TRF2 proteins. In addition, Poz1-Tpz1 form heterotetramers mediated by the helices α1 and α2 of Poz1, further suggesting that a conserved interaction module is used at different places of the

shelterin complexes. Disruption of heterotetramerization compromises telomere length regulation. We also found a zinc ion bound at the interaction interface of Poz1-Tpz1 which stabilizes the Poz1-Tpz1 interaction and limits telomere elongation *in vivo*.

IV.2: Introduction

Telomeres are specialized nucleoprotein structures at the ends of linear chromosomes that maintain genome integrity. Telomeric DNA consists of double-stranded tandem G-rich repeats that terminate in a 3' single-stranded overhang. Both the double-stranded and single-stranded repeats are bound by telomere-specific proteins which are collectively referred to as the shelterin complex.

The shelterin complexes are protective "caps" for telomeres to ensure their integrity. They prevent nucleolytic degradation, distinguish the natural ends from DNA double strand breaks and prevent DNA damage response and undesired repair. Furthermore, they also promote the semi-conservative replication of the telomere region as well as regulate *de novo* synthesis of telomere repeats by telomerase.

Components of the shelterin complexes have been identified and characterized in different species including humans, fission yeast and budding yeast. Fission yeast *S. pombe* share remarkable similarities in shelterin compositions and telomere structures with humans. Homologs of almost all the human shelterin components have been identified in fission yeast. On the other hand, the budding yeast *S. cerevisiae* has more divergent telomere structures (see Chapter I Figure 1.2).

In human cells, the shelterin complex consists of six proteins: TRF1, TRF2, RAP1, TIN2, TPP1 and POT1 [33-36, 51, 56, 57, 61, 62, 132]. TRF1 and TRF2 specifically bind double-stranded telomere repeats as homodimers via their Myb domains [34]. The homodimerization of the proteins is mediated by a conserved centrally located

region named TRFH (TRF-homology) domain [34] whose crystal structures were solved by the Rhodes lab [38]. Despite modest sequence identity, the two TRFH domains have the same entirely α-helical architecture with unique features at the interface that prevent heterodimerization between TRF1 and TRF2. In addition to dimerization, the TRFH domains of TRF1 and TRF2 also interact with TIN2, another shelterin component and Apollo, a shelterin accessory factor, respectively [39]. The structures of these two complexes revealed a shared docking motif. Both TRF1 and TRF2 negatively regulate telomere length [67, 68]. While TRF1 is important for semi-conservative replication of telomeric sequences, TRF2 is vital for protecting telomeres against ATM activation and NHEJ mediated chromosome end fusions [85, 92, 128, 133]. RAP1 interacts with TRF2 and inhibits HR at telomeres [36, 97]. POT1 binds single-stranded telomeric DNA with two OB-fold domains and interacts with TPP1 [56, 57]. They are required for telomerase recruitment and regulation as well as inhibiting ATR activation and HR at telomeres [85, 134, 135]. TIN2 bridges double-stranded and single-stranded factors by interacting with TRF1, TRF2 and POT1 [57, 62]. These interactions are important for the recruitment as well as the function of the complex.

In fission yeast, the shelterin complex is composed of five proteins: Taz1, Rap1, Poz1, Tpz1 and Pot1 [31, 43, 44, 51, 58]. Taz1, the only ortholog of human TRF1 and TRF2, binds double-stranded telomeric repeats through its Myb domain [31]. Rap1, the most conserved component, interacts with Taz1 through a C-terminal RCT domain [31, 44, 136]. The hTPP1/POT1 orthologs Tpz1/Pot1 binds and protects single-stranded telomeric DNA [51, 58]. Poz1, although lacking sequence similarity to human TIN2, is

considered a functional homolog as it bridges the double-stranded and single-stranded factors via interacting with Rap1 and Tpz1 [58]. Previous characterization of these proteins showed similar functions compared to their human homologs. Deletion of Taz1, Rap1 or Poz1 leads to dramatic telomere elongation, suggesting that they are negative regulators [31, 44, 58, 136]. The Tpz1/Pot1 complex is required for telomerase recruitment and activity as well as protection of the chromosome ends. Deletion of them causes immediate telomere loss and genomic instability [51, 58]. The recruitment of telomerase is largely mediated by Ccq1, another protein recruited by Tpz1. Ccq1 interacts with Est1, an accessory factor of telomerase holoenzyme [58, 137].

Structures of some domains of the shelterin complexes have been solved and have promoted our understanding of their molecular mechanisms. For example, the crystal structure of the Pot1 N-terminus binding single-stranded telomeric DNA was solved by the Cech lab [53, 138] and shed light on how Pot1 binds the single-stranded overhang and protects the 3'end of the DNA. Importantly, the conservation of some of these proteins can be observed in the three-dimensional structure rather than the sequence. A conserved C-terminal region known as the RCT (Rap1 C-terminus) domain was identified in both human and *S. cerevisiae* Rap1 proteins. However, this domain was not identified in *S. pombe* Rap1 based on protein sequence. It was not until the structure of the fission yeast Rap1 RCT in complex with Taz1 RBM (Rap1 binding motif) was solved by the Lei lab [40] that the existence of a conserved RCT domain became clear. Remarkable structural similarities are shared between the Taz1-Rap1 and the hTRF2-RAP1 interactions despite low sequence similarities. Furthermore, based on the structure, interaction mutants were

designed to demonstrate the importance of the interaction in telomere length regulation as well as in protection against NHEJ in G1 arrested cells [40].

Poz1 is a relatively small protein with a length of 249 amino acids. Previously, little information about its structure was available as the protein sequence lacked obvious sequence motifs or structural domains. Although interactions between Poz1 and Rap1 or Tpz1 have been identified [58], the domains mediating these interactions are unclear. It has only been shown that the C-terminal 379-508 a.a. region of Tpz1 is sufficient to bind Poz1 via yeast two-hybrid assays [58]. The Poz1-Tpz1 interface is of particular interest as it forms the boundary between negative (Taz1-Rap1-Poz1) and positive (Tpz1-Pot1) regulators of telomere length.

In this study, we present the crystal structure of $Poz1^{30-249}$ in complex with $Tpz1^{475-508}$ at 2.4Å resolution. Intriguingly, this structure shows striking similarities to the TRFH domains of the human TRF1 and TRF2 proteins. A unique feature in this structure is that a zinc ion is bound at the interaction interface of Poz1-Tpz1. The zinc binding stabilizes the Poz1-Tpz1 interaction and limits telomere elongation *in vivo*. In addition, Poz1-Tpz1 forms heterotetrameric arrangement which is also a feature of hTRF1-TIN2 and hTRF2-Apollo, further suggesting that a conserved interaction module is used at different places of the shelterin complexes. The dimerization of the two protomers is mainly mediated by the helices α1 and α2 of Poz1. Mutations disrupting the dimerization of Poz1 lead to telomere elongation but also affect Poz1-Rap1 interaction.

IV.3: Results

IV.3.1: Crystal structure of Poz1 + Tpz1

The attempt to express and purify full-length Poz1 (249 a.a.) in *E.coli* failed due to the insoluble nature of the protein (Figure 4.1A). In order to overcome this obstacle, we optimized the protein boundaries of Poz1 based on secondary structure prediction and designed a series of constructs of Poz1. Among those, Poz1$^{30-249}$, which lacks the first 29 amino acids, showed good expression level and solubility in *E.coli* (Figure 4.1B)

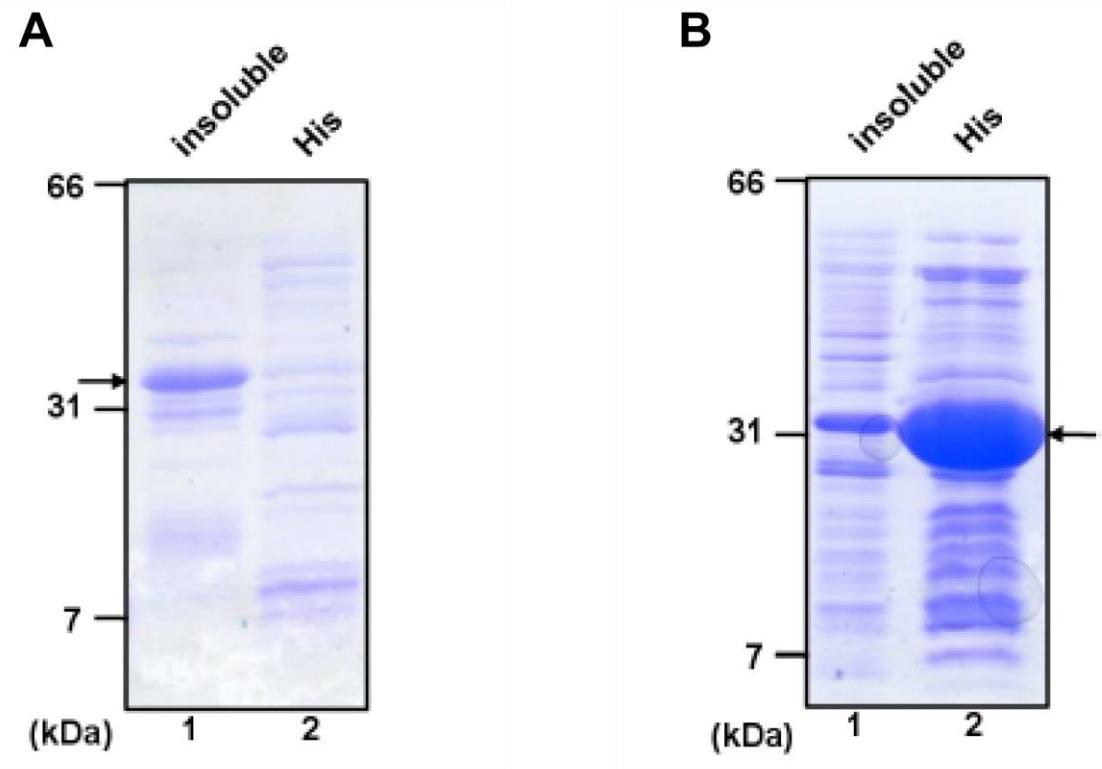


Figure 4.1: Expression and purification of Poz1 in *E.coli*. SDS-PAGE analysis of Ni^{2+}-affinity pulldowns of **(A)** full-length Poz1 and **(B)** Poz1$^{30-249}$. Lanes 1 shows the insoluble fraction and Lanes 2 shows the pulldown fraction.

Similarly, Tpz1 (508 a.a.) is predicted to contain a large unstructured region in the central part of the protein, which is an unwanted characteristic for crystallization as the flexibility of this region would allow Tpz1 to exist in multiple conformations. The full-length protein aggregates during purification further suggested the necessity to narrow down the region. We focused on the C-terminal region of the protein as it has been shown to interact with Poz1 [58]. Limited proteolysis was done to remove unstructured region and eventually $Poz1^{30-249}$ and $Tpz1^{475-508}$ were found to be resistant to 0.1% trypsin and still interact with each other. The boundary of the complex was further validated by co-expressing GST-tagged $Tpz1^{475-508}$ and His-tagged $Poz1^{30-49}$ (Figure 4.2). The complex was purified by pulling down GST first and subsequently pulling down the His tag, which confirms the ability of Poz1 and Tpz1 fragments to interact.

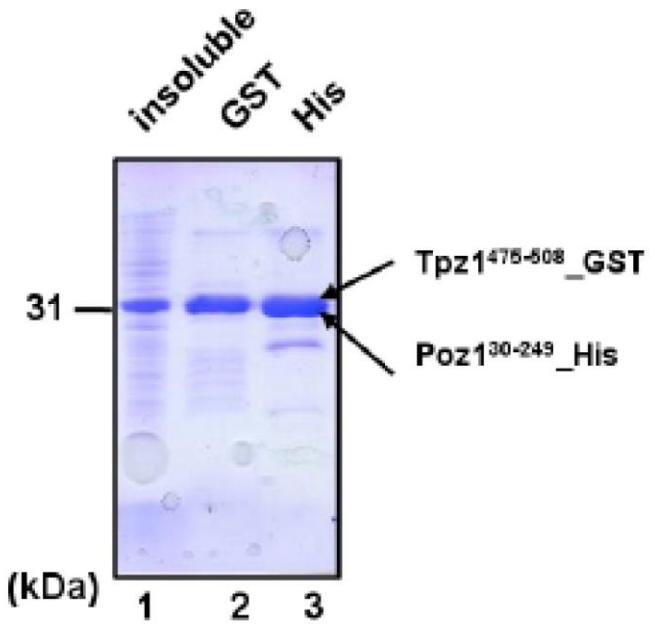


Figure 4.2: Expression and purification of His-tagged Poz1$^{30-249}$ and GST-tagged Tpz1$^{475-508}$. Lane 1 shows the insoluble fraction; lane 2 shows the affinity pulldown with GST resin and lane 3 the subsequent pulldown with Ni^{2+} resin. The sizes of two proteins (30kD and 26kDa) are similar so the bands are barely distinguishable.

In order to obtain enough protein for crystallization, Poz1$^{30-249}$ + Tpz1$^{475-508}$ was expressed in 12 L *E.coli* and purified by three sequential chromatography steps: Ni^{2+}-affinity chromatography, ion exchange chromatography and size-exclusion chromatography (Figure 4.3A). The protein purity was assessed by SDS-PAGE (Figure 4.3B).

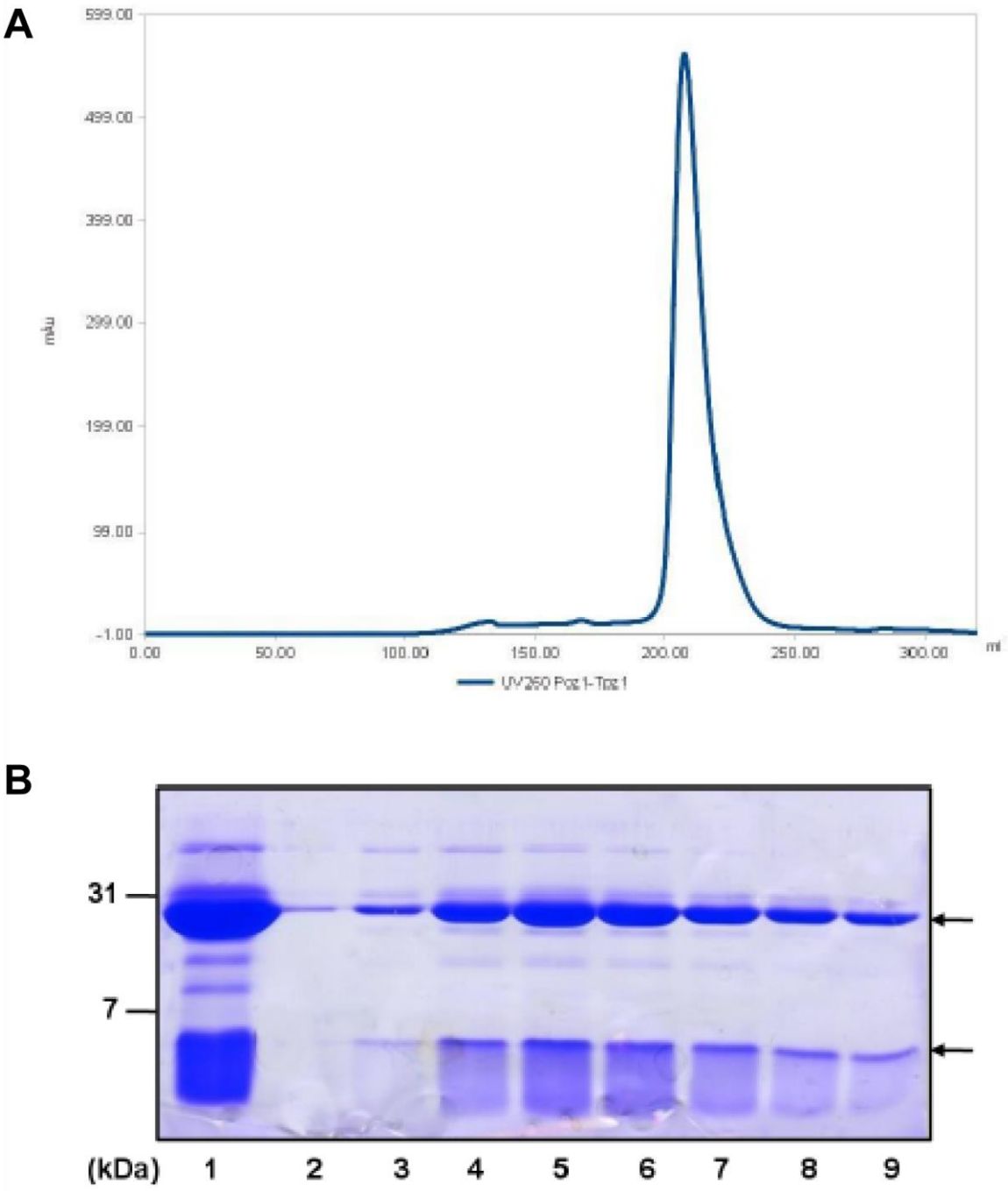


Figure 4.3: Protein purification of Poz1$^{30-249}$-Tpz1$^{457-508}$ complex. **(A)** Chromatogram and **(B)** SDS-PAGE of the gel filtration step. Poz1$^{30-249}$ is indicated by the upper arrow and Tpz1$^{475-508}$ by the lower arrow.

Crystals of Poz1-Tpz1 were then grown and optimized for diffraction test using synchrotron X-ray beam line. The crystal structure was solved using multiwavelength anomalous dispersion (MAD) on selenomethionyl derivatives. After model building and refinement, the crystal structure of $Poz1^{30-249}$ in complex with $Tpz1^{475-508}$ was elucidated at 2.4Å resolution (Figure 4.4 A). In this structure, the complex shows a compact globular, exclusively α-helical structure. $Poz1^{30-249}$ is composed of 8 α-helices. $Tpz1^{475-508}$ is locked in between α1 and α2 of Poz1. The unstructured linker connecting α1 and α2 is held in position by interacting with Tpz1. The flexibility of this region would allow α1 to adopt different conformations in the absence of Tpz1 binding, which may explain the failure of the attempt to crystallize $Poz1^{30-249}$ by itself. In the Poz1 structure, the loops (a.a 70-85 and a.a 118-121) connecting α2-α3 and α3-α4 respectively and the C-terminal a.a. 236-249 were not visible in the electron density and thus not shown in the structure. This was likely due to the flexible nature of these regions. Similarly, three Tpz1 N-terminal residues (475-477) and two C-terminal residues (507-508) were also absent in the electron density. A large number of interactions including hydrogen bonds and non-bonded contacts (hydrophobic and ionic interactions, base-stacking) are formed at the Poz1-Tpz1 interface (Figure 4.4B), suggesting a tight interaction of the complex.

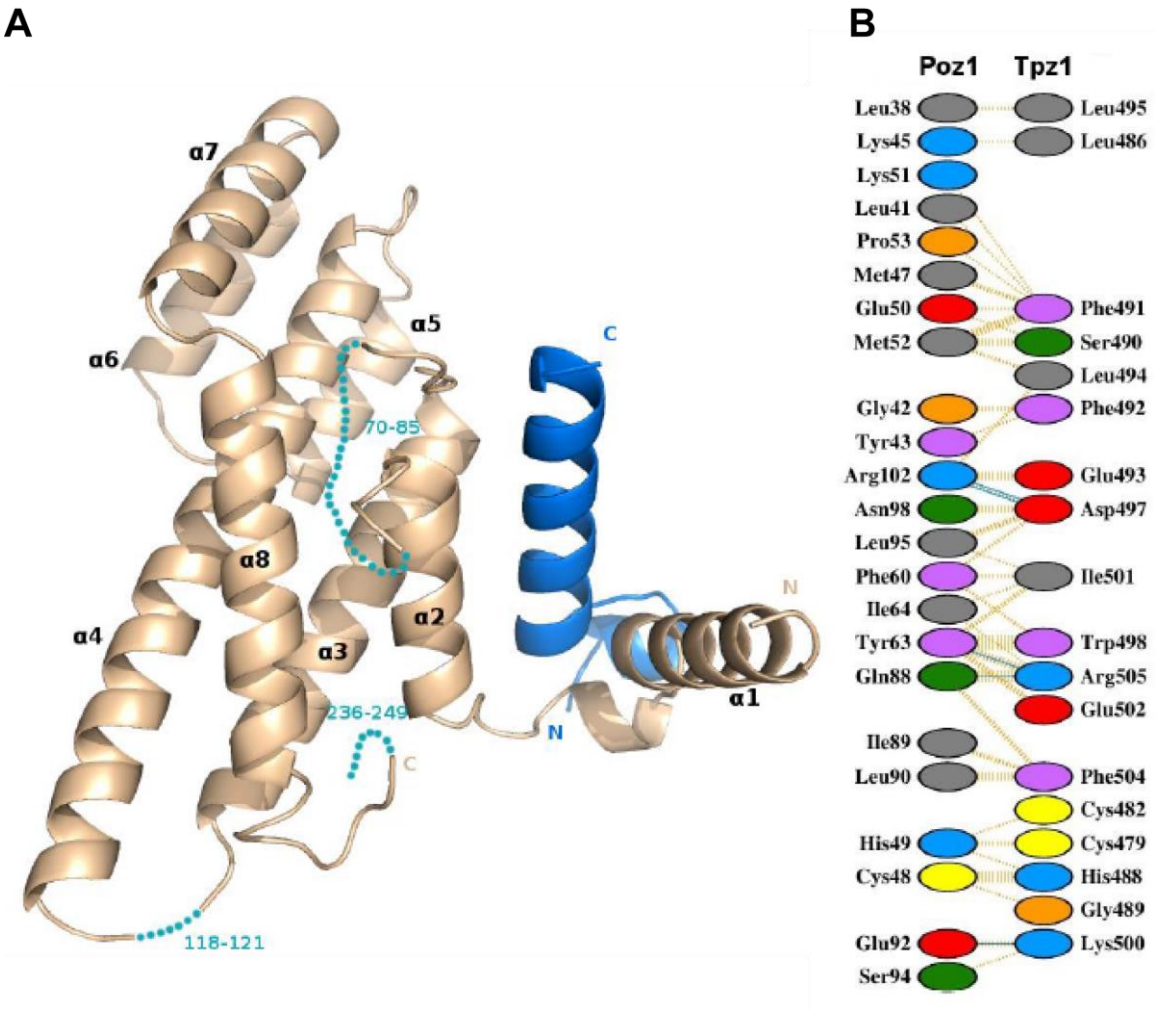


Figure 4.4: Crystal structure of Poz1$^{30\text{-}249}$ in complex with Tpz1$^{475\text{-}508}$. **(A)** The overall structure of the complex shown as ribbon representations. Poz1 is colored in wheat, Tpz1 in blue. Secondary structure elements are labeled in Poz1. The regions that were not built due to missing electron density are sketched in turquoise. **(B)** Interactions formed between Poz1 and Tpz1. Hydrogen-bonds are shown by blue lines while non-bonded contacts are marked by orange dashed lines. Residues are color coded as follows: Positively charged in light blue, negatively charged in red, neutral in green, aliphatic in grey, aromatic in purple, Proline and Glycine in orange and cysteine in yellow. Figure was generated by PDBsum [139] (http://www.ebi.ac.uk/pdbsum/).

IV.3.2: Structural similarity to human TRF1 and TRF2

Based on functional studies, Poz1 has been presumed to be a functional homolog of human TIN2. However, there was no obvious sequence similarity between these two proteins. BLAST searches and structure prediction of Poz1 did not yield any homologous proteins or known protein domains/motifs. The structure of the Poz1-Tpz1 complex allowed us to perform a structure similarity search in the DALI server [140]. This non-biased search of structures in the protein data bank produced the TRFH domains of human TRF1 and TRF2 as the top hits. TRFH domains mediate homodimerization of TRF1 and TRF2 and interactions with other proteins. The structures of these two TRFH domains are almost identical [38, 39].

Superimposition of our Poz1-Tpz1 structure onto hTRF1-TIN2 and hTRF2-Apollo showed remarkable similarities (Figure4.5). With the exception of α0 and α9, all the secondary structures of TRF1 and TRF2 are present in Poz1, with only minor positional shifts (Figure 4.6). The region analogous to helix α0 of the TRFH structures is not present in Poz1 as it was necessary to truncate the first 29 amino acids to increase the solubility of the protein. Based on secondary structure prediction, this region might also form an α-helix. The region corresponding to the α9 of TRFHs is also missing in the Poz1 structure, although secondary structure also predicts a helix structure. This striking similarity was surprising as no apparent sequence similarity between Poz1 and TRF1 or TRF2 had previously been reported.

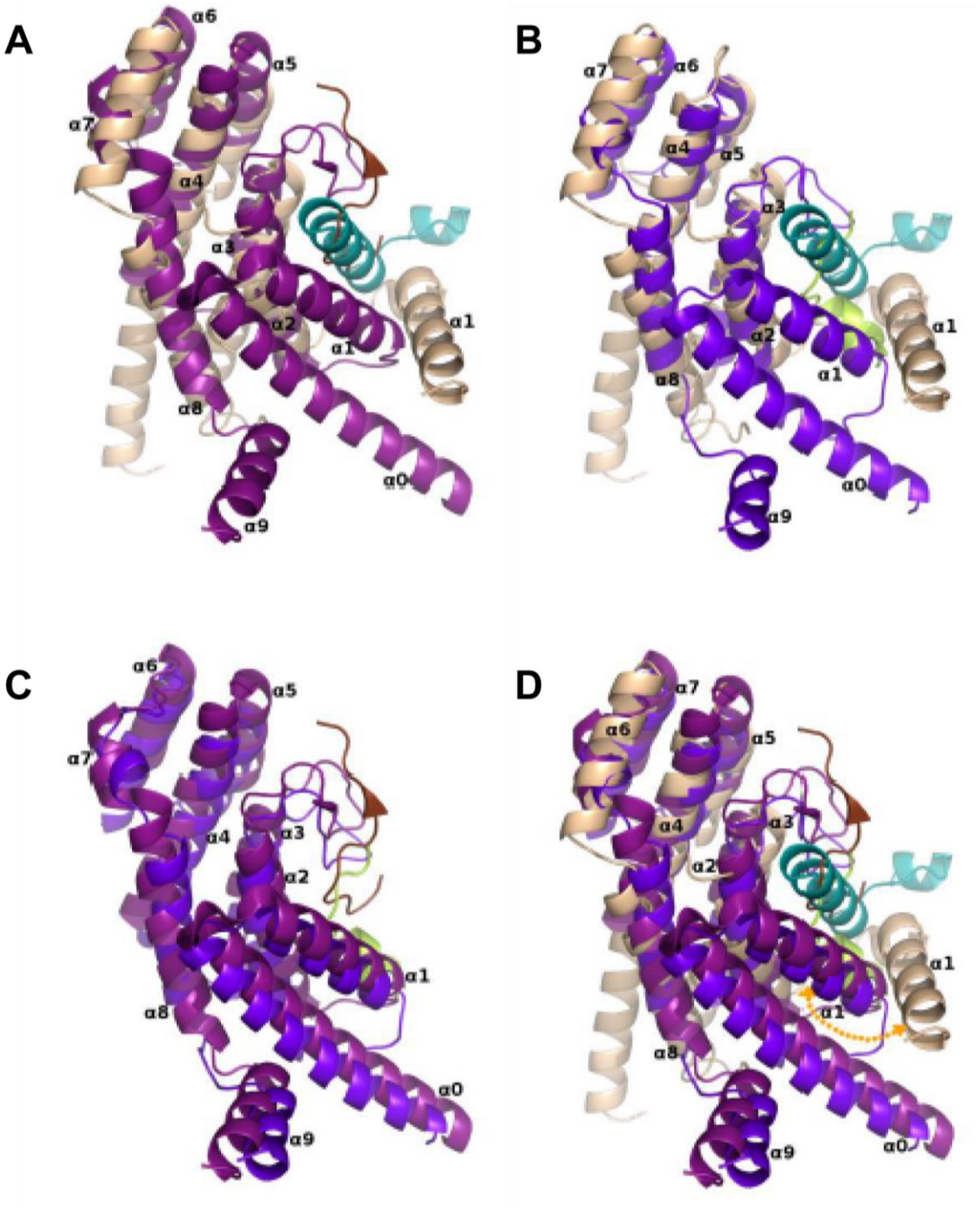


Figure 4.5: Superpositions of spPoz1$^{30\text{-}249}$-Tpz1$^{475\text{-}508}$ onto hTRF1$^{65\text{-}267}$-TIN2$^{256\text{-}276}$ and hTRF2$^{42\text{-}245}$-Apollo$^{496\text{-}532}$. Poz1 is colored in wheat, Tpz1 in blue, TRF1 in magenta, TIN2 in brown, TRF2 in purple and Apollo in green. **(A)** Poz1-Tpz1 aligned with TRF1-TIN2, **(B)** Poz1-Tpz1 and TRF2-Apollo, **(C)** TRF1-TIN2 and TRF2-Apollo and **(D)**

Poz1-Tpz1, TRF1-TIN2 and TRF2-Apollo. An orange arrow indicates the difference in conformation of α1 in Poz1 compared to α1 in TRF1 and TRF2.

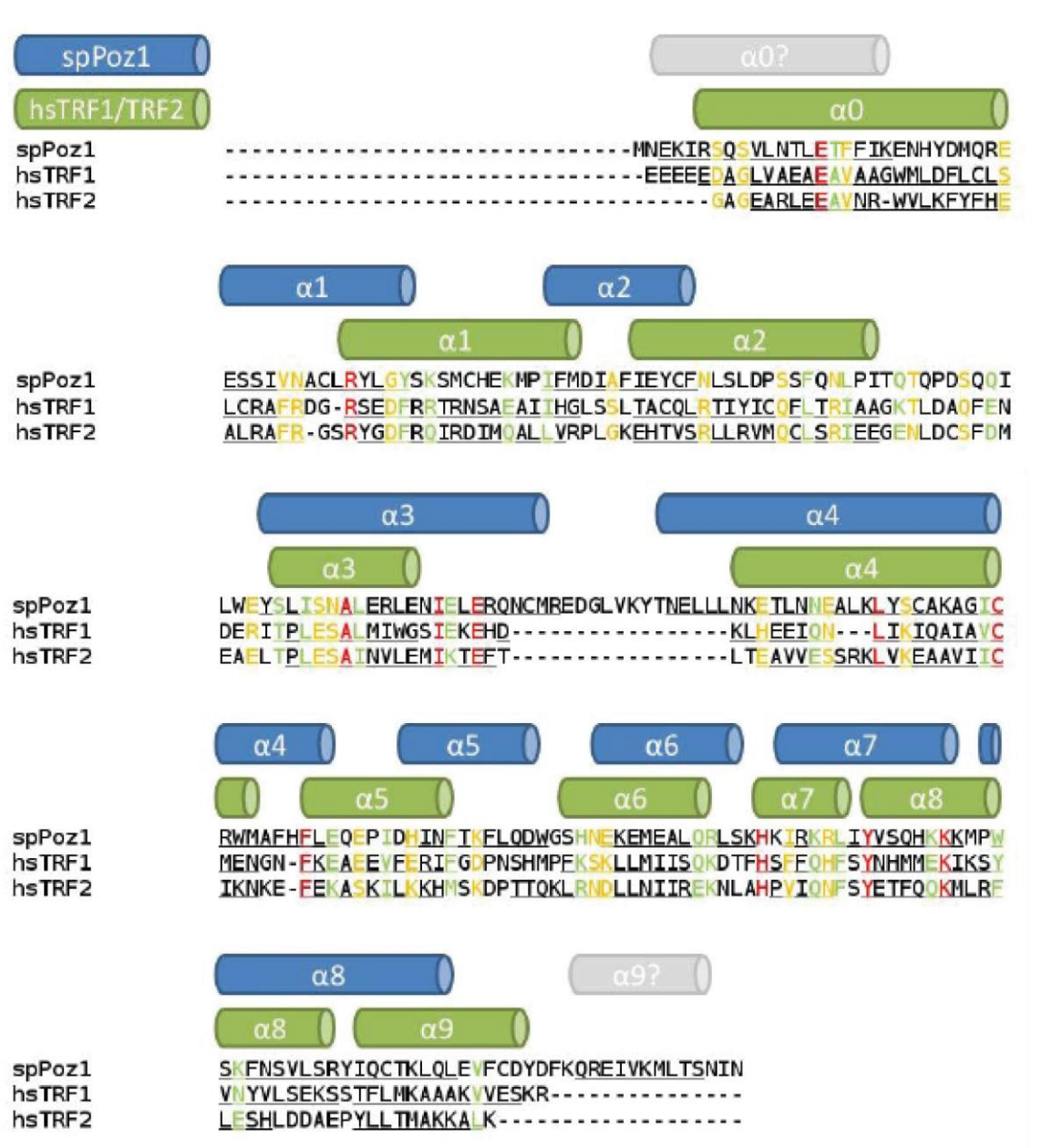


Figure 4.6: Sequence alignment with highlighted secondary structure elements. The sequence of Poz1[1-249] was aligned with the sequence of TRF1[65-267] and TRF2[42-245] in ClustalW [141]. Blue cylinders represent α-helices in Poz1, green cylinders represent α-helices in TRF1 (not shown for TRF2 for simplicity. They are almost identical to those in

TRF1). Grey cylinders represent regions that are not present in the structure of Poz1 but are predicted by PSI-pred [142, 143] as α-helices. Red letters show strictly conserved residues, green letters show residues that are conserved in an amino acid group and yellow letters represent residues that are conserved across groups.

In order to test if the structural similarity indicated functional conservation, we replaced Poz1 with the corresponding part of human TRF2 fused to Rap1 (Rap1-TRF2$^{40-245}$). We also fused the domain of Apollo that interacts with TRF2 to Tpz1 (Tpz1-Apollo$^{495-530}$) (Figure 4.7A). The TRF2-Apollo interaction domains rescued telomere lengthening caused by deletion of *poz1* (Figure 4.7B). After 5 restreaks, telomere length reached equilibrium and showed slightly shorter than wild-type but stable telomere length. This result supports the conservation of the interaction modules in the shelterin complex and Poz1 functions as a molecular bridge to link Rap1 and Tpz1, which in this mutant is reconstituted by Rap1-TRF2 and Tpz1-Apollo.

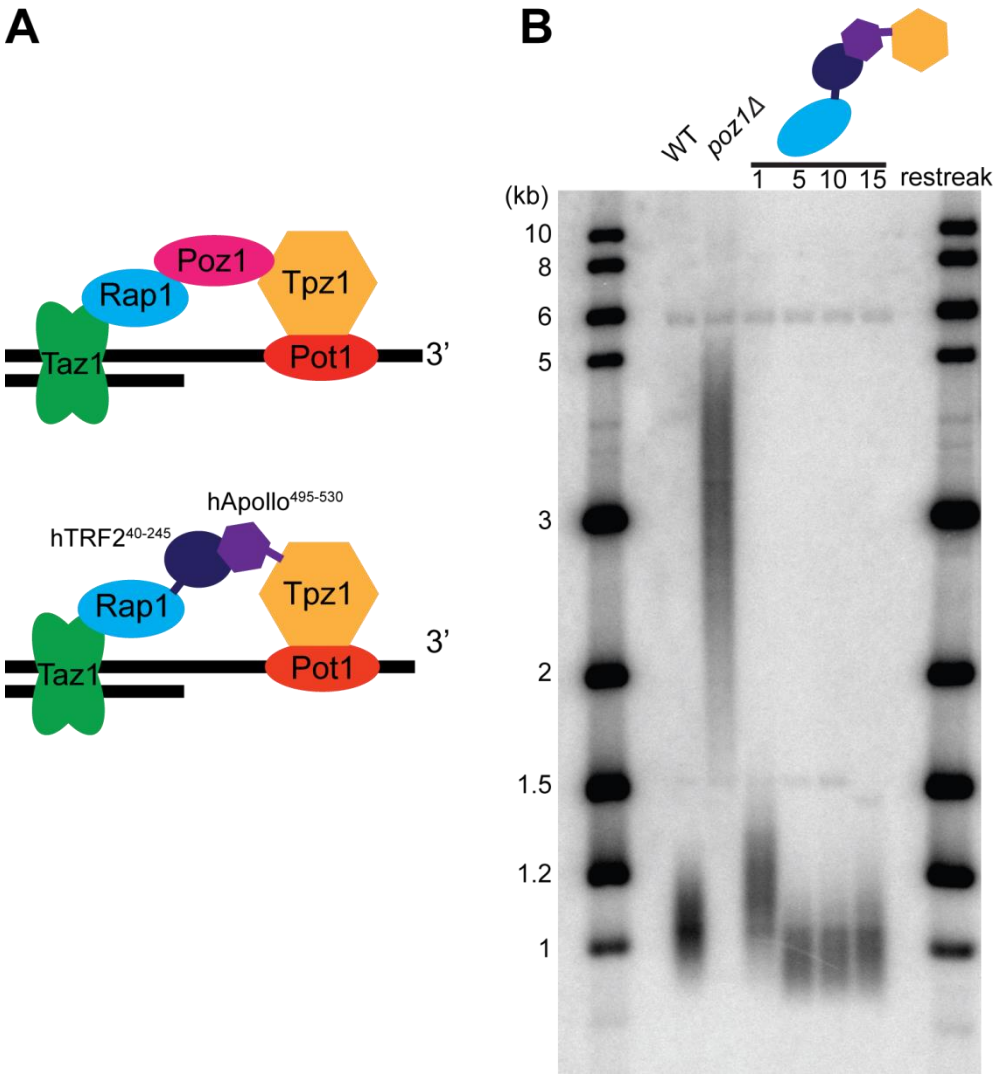


Figure 4.7: Replacement of Poz1 with human TRF2-Apollo interaction domains rescues telomere length. **(A)** Illustration of the mutant strain. Top is the wild-type telomeric complex. Bottom is the mutant: TRF2$^{40-245}$-V5 was fused to the C-terminus of Rap1 with a 6x glycine linker in between and Apollo$^{495-530}$-V5 was fused to the C-terminus of Tpz1 with a 6x glycine linker. *poz1* was deleted. **(B)** Telomere length analysis of the mutant strain. Cells started with extremely long telomeres due to the deletion of *poz1*. Telomeres shortened during restreaks and reached equilibrium after 5 restreaks.

IV.3.3: A zinc ion is coordinated at the interface of Poz1-Tpz1 complex

During the refinement of the Poz1-Tpz1 structure, we encountered a large patch of positive electron density at the interface between Poz1 and Tpz1 (Figure 4.8A). After several steps of model building and refinement, a zinc atom could be correctly placed at this position in the structure coordinated by two histidine residues and two cysteine residues (H49 of Poz1 and C479, C482 and H488 of Tpz1) (Figure 4.8B and C).

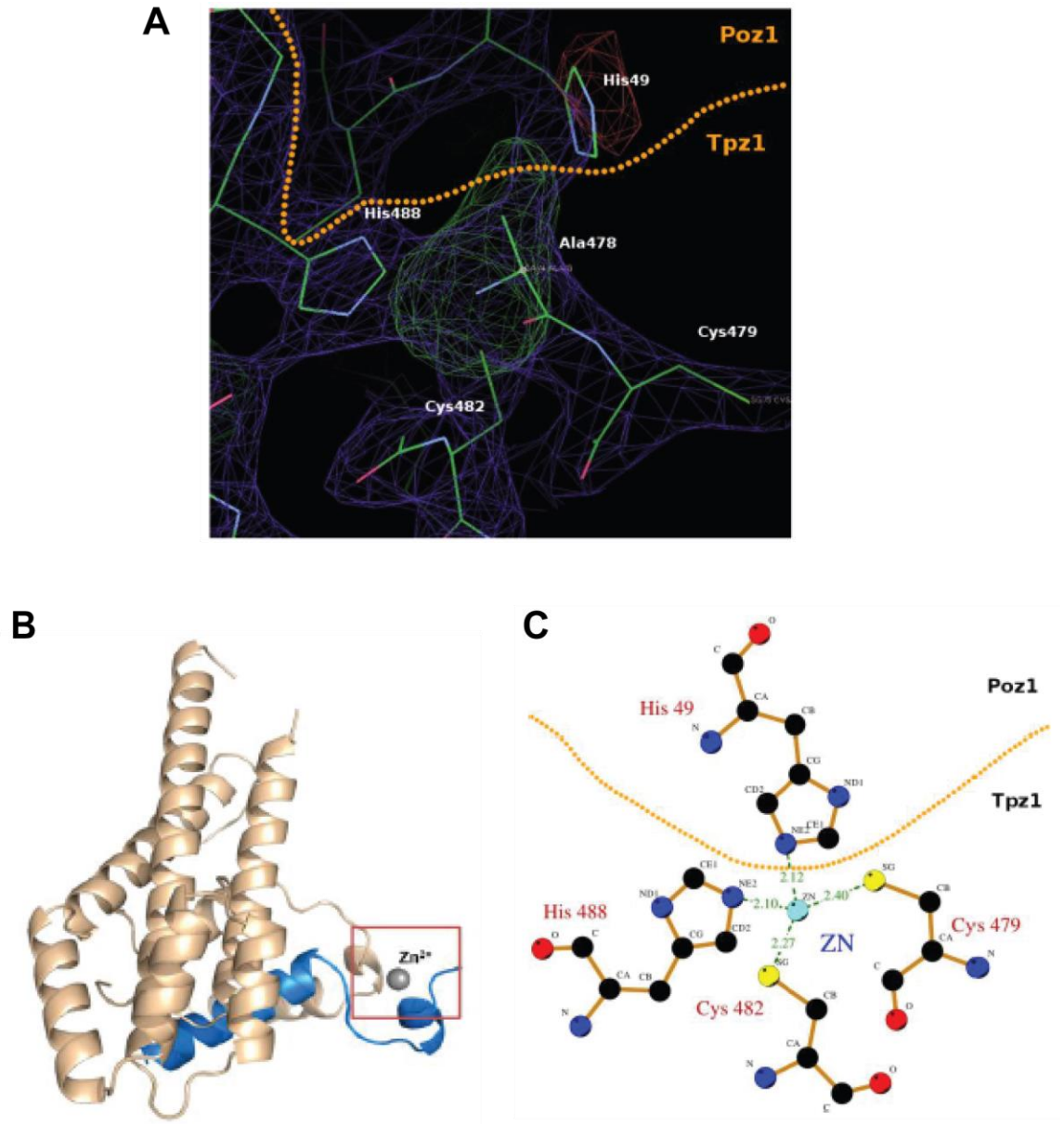


Figure 4.8: Poz1 and Tpz1 coordinate a Zn^{2+} ion at their interphase. **(A)** Positive electron density in the Fo-Fc difference map (green mesh) indicates the presence of a metal ion. Negative electron density is indicated by red mesh and the 2Fo-Fc electron density is shown as a purple mesh. (Figure was generated in COOT [144]. Contour level is at 1.5σ). **(B)** Position of the Zn^{2+} ion in the overall structure. Poz1 is colored in wheat and Tpz1 in blue. **(C)** Schematic representation of the Zn^{2+} coordination (Figure generated by PDBsum [139])

IV.3.4: Zinc coordination is critical for Poz1-Tpz1 interaction and telomere length maintenance

The zinc coordination feature is not observed in the human structure. The function of this is unclear. Since the zinc ion is bound at the interface of Poz1-Tpz1 interaction, it may play a role for complex integrity or function. To investigate this, we first generated a mutant to disrupt the ion coordination. We mutated C482 and H488 of Tpz1, which coordinate the ion, to alanines. Co-immunoprecipitations with extracts from cells expressing FLAG-tagged Poz1 and V5-tagged wild-type or mutant Tpz1 were performed to test the effect on protein interaction (Figure 4.9). The Tpz1 C482A.H488A mutation disrupted Tpz1-Poz1 interaction. However, the interaction with another protein, Ccq1 was not affected (Figure 4.9B).

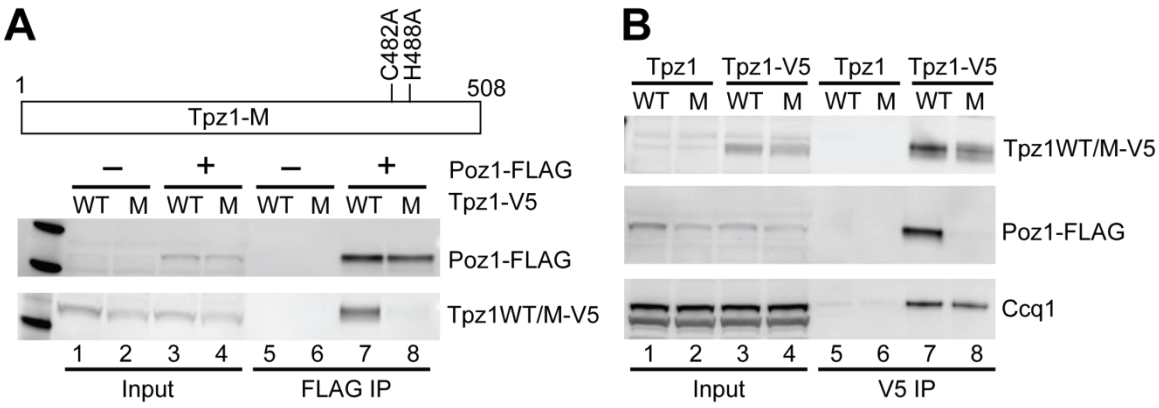
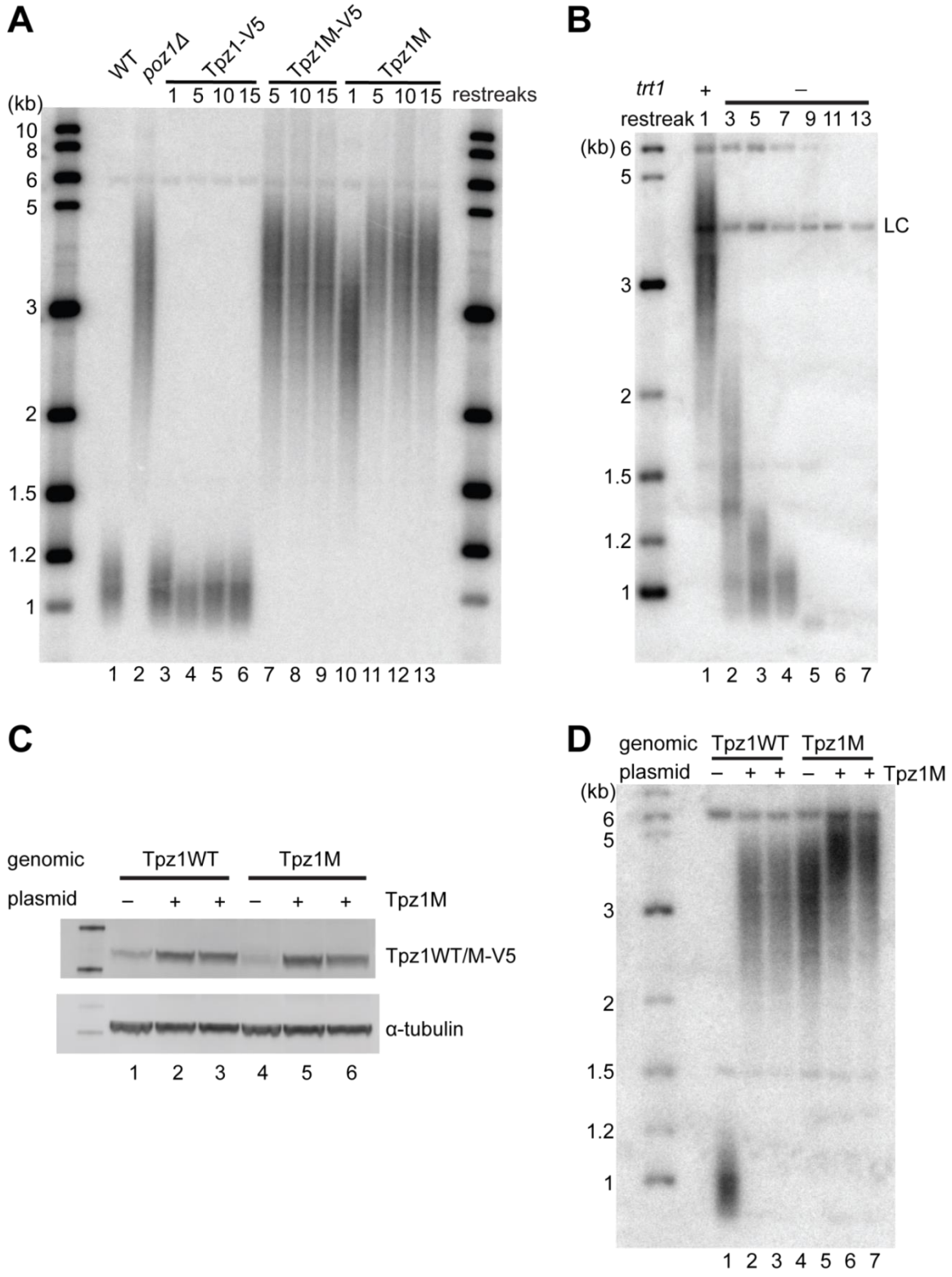


Figure 4.9: Zinc binding stabilizes the Poz1-Tpz1 interaction. **(A)** Changing C482 and H488 to alanines disrupts the interaction with Poz1. Immunoprecipitations (IPs) were carried out with anti-FLAG antibody using extracts from cells expressing FLAG-tagged Poz1 and V5-tagged wild-type (WT) or mutant (M) Tpz1. Proteins were visualized by western blotting with input (10%) or IPs using antibodies against FLAG and V5, respectively. **(B)** Reciprocal co-immunoprecipitation using V5 antibody and detection on western blots with antibodies against V5, FLAG and Ccq1.

We next examined the effect of disrupting zinc binding on telomere length regulation *in vivo*. Cells expressing the C482A.H488A Tpz1 mutant (Tpz1M), V5 tagged or untagged, possess long telomeres comparable with *poz1Δ* cells (Figure 4.10A), suggesting the disruption of proper telomere length regulation. The elongated telomeres were maintained by telomerase as deletion of the catalytic subunit of telomerase (*trt1*) led to shortening and eventually complete loss of telomeres (Figure 4.10B). As the expression level of Tpz1M was slightly lower than Tpz1WT (Figure 4.10C, compare lane 4 to lane 1), we increased the expression of Tpz1M by providing an additional copy of the gene on a plasmid either in the context of wild-type or mutant Tpz1 integrated at the endogenous locus (Figure 4.10C). If the telomere length deregulation was caused by the insufficient level of the Tpz1M, we would expect the rescue of telomere length by the increased expression level. However, we did not observe any telomere shortening in cells expressing higher levels of Tpz1M (Figure 4.10D). Instead, the mutant played a dominant negative role as cells expressing both the mutant (from a plasmid) and wild-type (from the genomic locus) Tpz1 also exhibited telomere elongation. These results suggest the importance of zinc binding for Poz1-Tpz1 interaction and telomere length maintenance.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1 2 3 4 5 6 7

Figure 4.10: Zinc binding is critical for telomere length maintenance. **(A)** Telomere length analysis of strains expressing the C482A.H488A mutant of Tpz1 (Tpz1M) integrated at the endogenous locus with or without a C-terminal V5 epitope tag. **(B)** Maintenance of long telomeres in the Zn^{2+} binding mutant is dependent on telomerase. Southern blot for strains expressing Tpz1M-V5 and with the deletion of *trt1*gene. A probe specific for rad16 gene was used as loading control (LC). **(C)** Western blot for strains expressing Tpz1M from a plasmid in addition to a copy of wild-type or mutant Tpz1 at the genomic locus. All copies of Tpz1 were V5-tagged. anti-V5 antibody was used for detection of wild-type plus mutant Tpz1; an antibody against endogenous α-tubulin was used as loading control. **(D)** Telomere length analysis of strains expressing Tpz1M from a plasmid in addition to a copy of wild-type or mutant Tpz1 at the genomic locus.

The involvement of a zinc ion in telomeric complex integrity led to a question: could telomere length be a sensor of Zn^{2+} levels in cells? To test that, we omitted $ZnSO_4$ from the defined minimal growth media (EMM) and cultured wild-type cells with it. However, telomere length was not affected (Figure 4.11). It is likely that small quantities of zinc were introduced as contaminants in the ingredients or tools we used, which was supported by the fact that cells still grew after 9 days although they were clearly sick. Since zinc is an essential mineral and involved in multiple pathways of cell functions, we were not able to further test whether zinc plays a regulatory role in modulating telomere length.

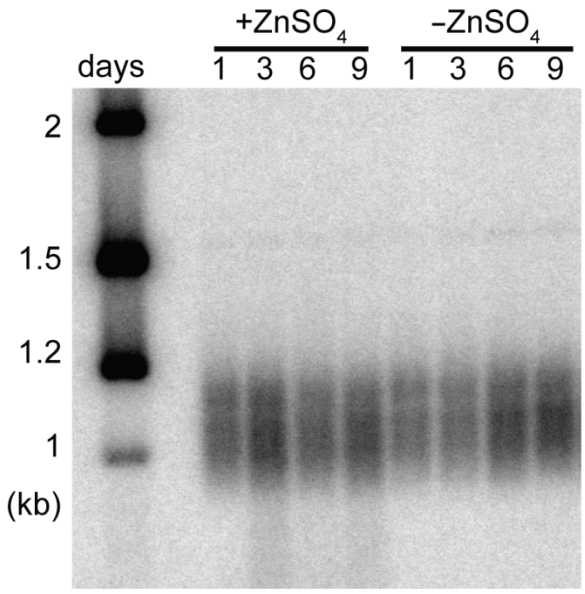


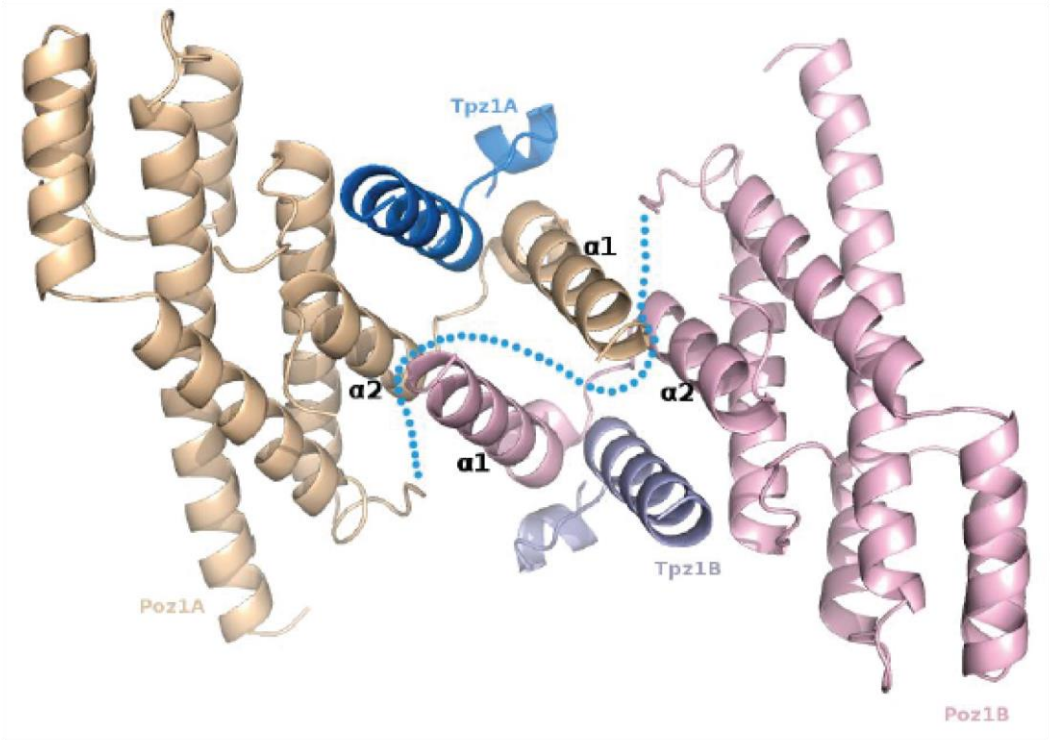
Figure 4.11: Omitting Zn^{2+} from the growth media does not affect telomere length. Wild-type cells were cultured in defined minimal media with or without addition of Zinc Sulfate and diluted at day 1, 3, 6. Telomere length was analyzed for cells from day 1, 3, 6, 9 cultures.

IV.3.5: Poz1-Tpz1 heterotetramer

TRFH domain mediates dimerization of TRF1 and TRF2, and TRF1 with TIN2, TRF2 with Apollo form heterotetrameric arrangments [39]. The similarity of Poz1 to TRF1 and TRF2 led us to examine whether Poz1-Tpz1 also forms heterotetramers. The structure was submitted to PDBePISA, which analyzes macromolecular interfaces and predicts probable quaternary structures [145]. A hypothetical stable heterotetrameric arrangement of two Poz1 and two Tpz1 molecules was predicted by PISA (Figure 4.12A). Close visual inspection of the crystal lattice also revealed two Poz1-Tpz1 monomers that oriented in a head-to-head manner which corresponded to the arrangement predicted by PISA (Figure 4.12B). The dimerization of two protomers is

mainly mediated by reciprocal interactions between helices α1 and α2 of Poz1. Poz1 dimerization was further confirmed by co-immunoprecipitation with extracts from diploid cells expressing both FLAG-tagged and V5-tagged Poz1 (Figure 4.13B).

A



B

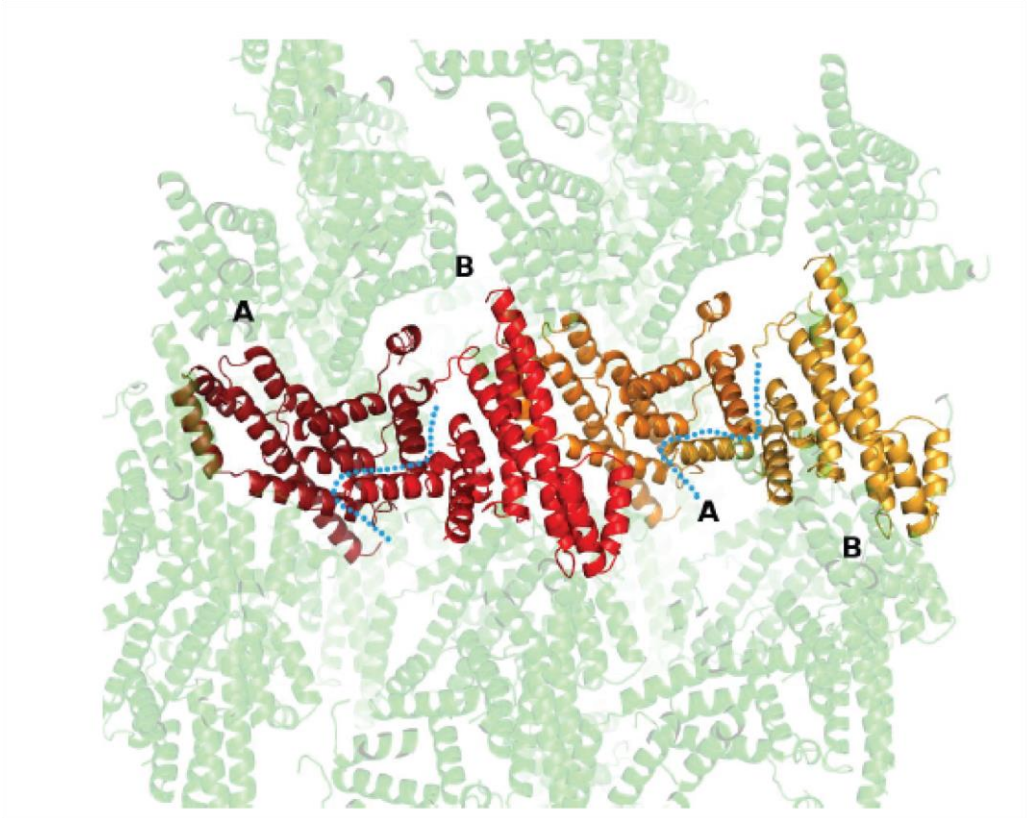


Figure 4.12: Tetramerization of Poz1-Tpz1. **(A)** Poz1-Tpz1 heterotetramer as predicted by PISA. Helices α1 and α2 of Poz1 form reciprocal contacts with the opposite protomer. The interface boundary is indicated by a blue dashed line. **(B)** Poz1-Tpz1 heterotetrameric arrangement present in the crystal lattice.

IV.3.6: Effect of the heterotetramerization mutations on telomere length regulation and telomeric complex integrity

The dimerization of Poz1 further supports its similarity to human TRF1 and TRF2 proteins. However, dimerization of TRF1 and TRF2 is essential for DNA binding, which is not a characteristic of Poz1. In order to understand the biological significance of the dimerization, we mutated V34 and C37 in α1 to glutamic acid and arginine, respectively (Figure 4.13A). The closely positioned mutations were expected to cause a charge mediated repulsion of the helices and thus disrupt Poz1 dimerization. Helix α0 in the TRF proteins also contributes to the dimerization interface [38]. This helix is absent in our Poz1-Tpz1 structure due to the insolubility of the full-length protein. It is possible that the first 29 a.a. is involved in the dimerization of Poz1. So we also generated a truncation mutation, Poz1Δ1-29. Co-immunoprecipitation showed that indeed, both mutations disrupted the dimerization of Poz1 (Figure 4.13B).

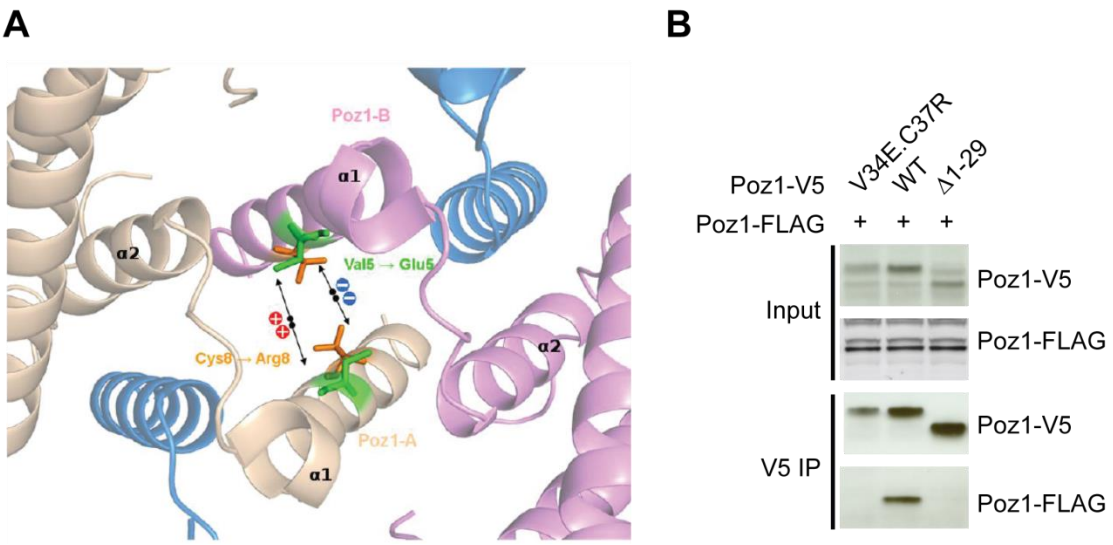


Figure 4.13: Poz1 dimerization mutants. **(A)** Illustration of the dimerization mutant. V34 and C37 within α1 were mutated to negatively charged glutamic acid and positively charged arginine, respectively. The amino acid number labeled in the figure was the relative position in Poz1$^{30-249}$. The mutations are expected to lead to repulsion of the helices and disruption of the dimerization. **(B)** Co-immunoprecipitation for extracts from cells expressing FLAG-tagged wild-type Poz1 and V5-tagged wild-type or mutant Poz1. Besides the point mutant in (A), a 1-29 a.a. truncation mutant was also tested. V5 antibody was used for IP and antibodies against FLAG and V5 were used for western blot analysis. Both mutants disrupt Poz1-Poz1 interaction.

We next examined the effect of dimerization mutations on telomere length regulation (Figure 4.14). While *poz1Δ* cells had elongated telomeres, Poz1-V5 rescued telomere length to wild-type levels, suggesting that the V5 tag does not affect the function of the protein. Telomeres of the Poz1 charge repulsion mutant cells were elongated to the same level as *poz1Δ* cells. Poz1Δ1-29 cells also had elongated telomeres similar to, but slightly shorter than *poz1Δ* cells. The double mutant cells behaved the same as *poz1Δ* cells. This result suggests that the dimerization of Poz1 may play an important role in telomere length regulation.

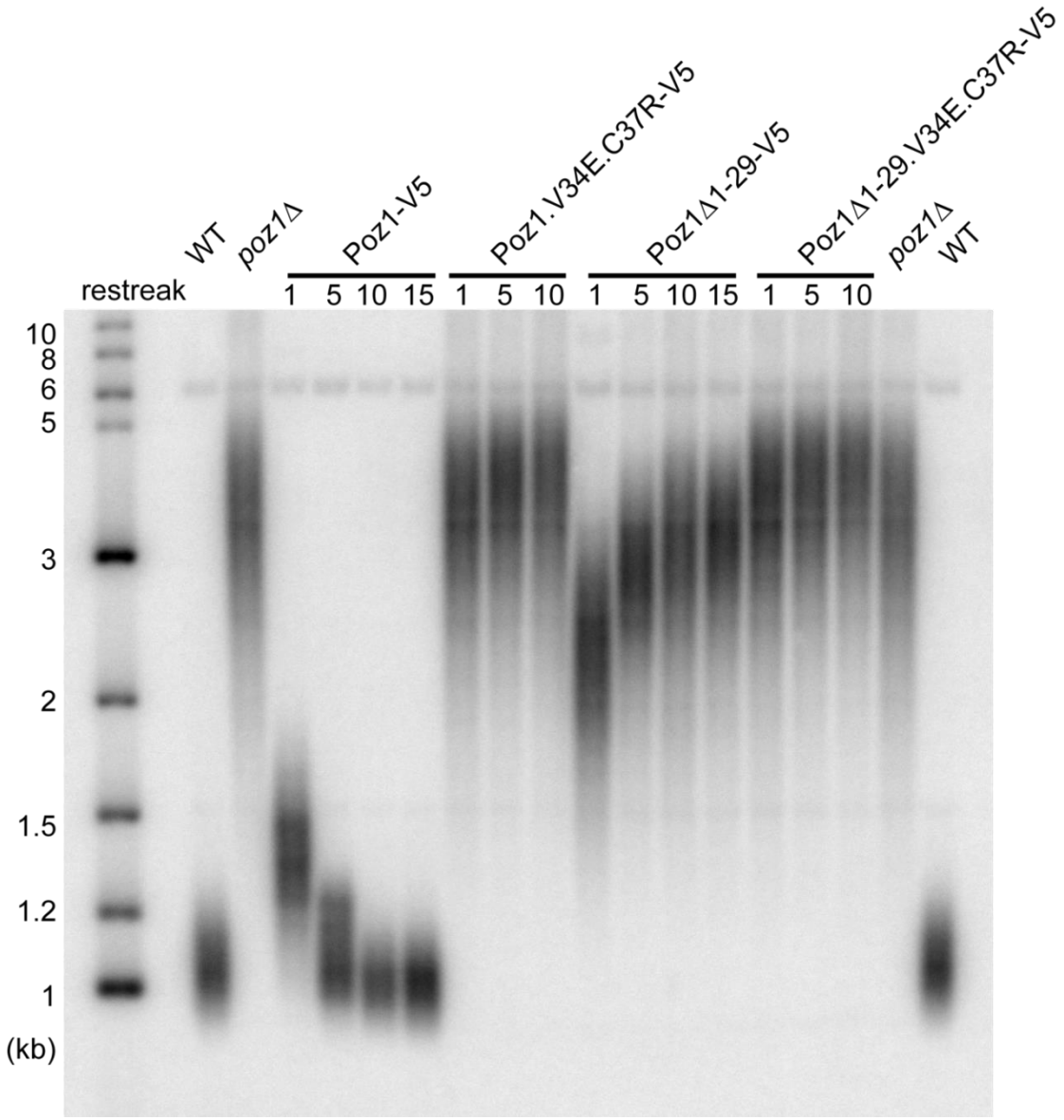


Figure 4.14: Telomere length analysis of Poz1 dimerization mutants. V5-tagged V34E.C37R and Δ1-29 mutants of Poz1 were introduced to *poz1Δ* cells and integrated at the endogenous locus of *poz1*. gDNAs from cells at different restreak points indicated above were analyzed.

As disruption of Poz1-Tpz1 interaction and Poz1-Rap1 interaction both lead to telomere elongation, it is necessary to validate whether the effect of the Poz1 dimerization mutations on telomere length was caused by the loss of Poz1-Tpz1 or Rap1-

Poz1 interaction. To address this, we first performed co-immunoprecipitation experiments using extracts expressing FLAG-tagged Tpz1 and V5-tagged wild-type or mutant Poz1. The V34E.C37R mutation weakened the interaction with Tpz1 considerably, while the Δ1-29 mutant did not (Figure 4.15). Since the same region of Poz1 mediates dimerization and interaction with Tpz1, it is not surprising that disruption of one affects the other. We next analyzed Poz1-Rap1 interaction (Figure 4.16). IgG Sepharose was used to pull down TAP-tagged Rap1. Western analysis showed that while wild-type Poz1 was co-precipitated with Rap1, neither Poz1 dimerization mutant was. Taken together, although V34E.C37R mutation of Poz1 was able to disrupt Poz1 dimerization, it also affected interactions with Rap1 and Tpz1. The other dimerization mutant Poz1Δ1-29 was still able to interact with Tpz1, but lost interaction with Rap1. These results make it more difficult to interpret the telomere lengthening phenotype of the mutants. Further experiments including identifying separation of function mutants will help elucidate the significance of Poz1 dimerization.

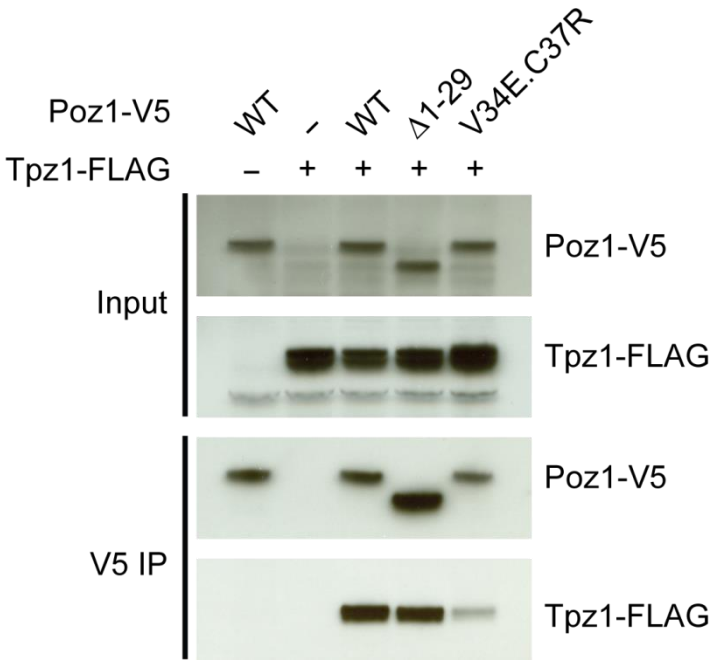


Figure 4.15: Interactions between Poz1 dimerization mutants and Tpz1 analyzed by co-immunoprecipitation. Immunoprecipitations (IPs) were carried out with V5 antibody using extracts from cells expressing FLAG-tagged Tpz1 and V5-tagged wild-type (WT) or mutant (Δ1-29 and V34E.C37R) Poz1. Input and IP samples were analyzed by western blot using antibodies against V5 and FLAG.

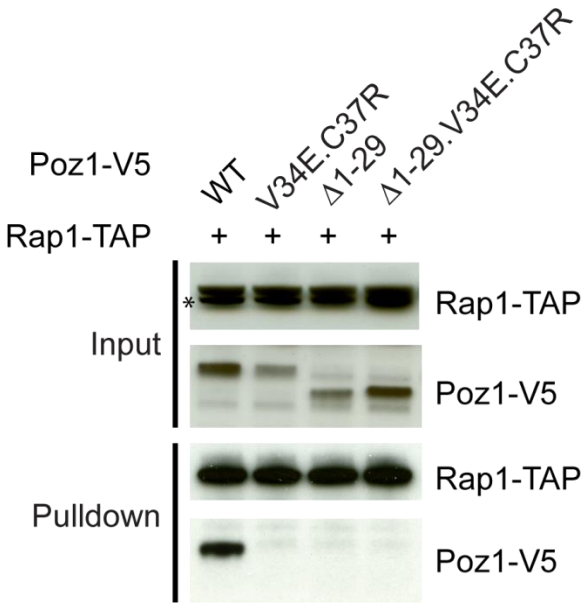


Figure 4.16: Interactions between Poz1 dimerization mutants and Rap1 analyzed by affinity pulldown. Extracts used for pulldown were from cells expressing TAP-tagged Rap1 and V5-tagged wild-type (WT) or mutant Poz1. IgG Sepharose 6 Fast Flow (GE healthcare) was used for pulldown of TAP-tagged Rap1. Input and pulldown samples were analyzed by western blot using an antibody against V5 which recognized both Rap1-TAP and Poz1-V5.The lower band (indicated by the asterisk mark) in the input panel was Rap1-TAP based on comparison with an untagged control.

IV.4: Discussion

Preceding this work, little information was available about the structure and function of Poz1. Being part of the fission yeast shelterin complex, Poz1 has been shown to be important for telomere length regulation and telomere position effect. However, the molecular mechanisms of those functions are unclear. Poz1 is unique in the fission yeast shelterin complex as it is the only protein that is not a homolog of the human shelterin components based on sequence prediction. No known protein or protein domain was returned from BLAST searches or secondary structure prediction.

By solving the crystal structure of Poz1 in complex with Tpz1 ($Poz1^{30-249}$ + $Tpz1^{475-508}$), we uncovered several features of Poz1 that promote the current understanding of telomere architecture and related functions.

The conservation of shelterin complexes from fission yeast to humans

Our structure revealed unexpected similarities to human shelterin proteins. Although human TIN2 has been considered a functional homolog of Poz1 as both proteins bridge the proteins binding the double- and single-stranded parts of telomeres, the Poz1 structure is strikingly similar to the TRFH domains of human TRF1 and TRF2 proteins. All the secondary structure elements found in Poz1 are present in human TRF proteins with only some differences in length despite no obvious sequence similarities. The replacement of Poz1 with the corresponding region of hTRF2-Apollo interaction modules rescued the telomere length regulation defect caused by *poz1Δ*. Our results provide further support of the conservation of the shelterin complexes.

However, this does not exclude the possibility that Poz1-Tpz1 is also structurally similar to hTIN2-TPP1, especially since Tpz1 is the ortholog of hTPP1. On the other hand, it is reasonable to speculate that Taz1, fission yeast homolog of human TRF1/TRF2, may also possess a similar structure since it has a centrally located region corresponding to the TRFH domain based on sequence similarity [36], whose function or interaction partner has not been uncovered yet. We postulate that similar interaction modules are used at different places of the shelterin complexes. The structures of hTIN2-TPP1 and fission yeast Taz1 are awaited for further understanding the conservation and diversity of the shelterin complexes in different species.

Zinc, a constitutive component or a dynamic regulator?

Although striking similarities were identified, Poz1-Tpz1 structure revealed a unique feature that is absent in the TRF1/TRF2 structure, which is the binding of a zinc ion at the interaction interface. Mutant disrupting zinc binding compromises Poz1-Tpz1 interaction and telomere length maintenance. Zinc is an integral component for a large number of proteins and it is critical for numerous vital cellular processes [146]. Poz1-Tpz1 is the first telomere subcomplex identified that binds zinc, which raises the question that whether zinc is a modulator of telomere length. It is not known whether the binding of zinc is dynamic. The attempt to detect telomere length change by omitting zinc from the minimal media used for culturing fission yeast cells was not successful most likely due to inevitable contaminations from tools and reagents. It is also possible that the

association and dissociation of zinc is not controlled by the concentration change but by some active regulation in response to the need of telomerase access.

Dimerization and potential to form higher order structures

The main function of the TRFH domain is to mediate homodimerization of TRF1 and TRF2. The similarity in structures prompted us to examine the possibility of Poz1 dimerization. Prediction by PISA as well as direct visual inspection in the crystal lattice both returned the heterotetrameric arrangement of Poz1-Tpz1 mediated by the helices α1 and α2 of Poz1. *In vitro* size exclusion chromatography and light scattering experiments suggest that Poz1 by itself is monomeric and only dimerizes upon binding to Tpz1 (not shown in this dissertation), although whether the same is true *in vivo* is unclear yet.

We designed two mutants that disrupt Poz1 dimerization *in vivo* and observed elongated telomere length. However, one mutant decreases Tpz1 interaction greatly and both mutants affect Rap1 interaction. Disruption of Poz1-Tpz1 interaction (Figure 4.10) or Rap1-Poz1 interaction (Chapter III, Figure3.6) also leads to telomere elongation, making it inconclusive whether there is a direct relationship between Poz1 dimerization and telomere length regulation. Separation of function mutants of Poz1 is required to dissect the mechanistic cause for the telomere length phenotype.

Intriguingly, dimerization is a repeated theme among telomeric complex components. It is known that Taz1 binds telomeric DNA as homodimers [37], which could recruit two molecules of Rap1. Rap1 itself has been shown to have self-interaction activity by yeast two-hybrid assays [43, 44]. A recent study reported that Pot1 also

dimerizes upon binding to single-stranded telomeric sequences [147]. The addition of our results that two molecules of Poz1 and two molecules of Tpz1 form a heterotetramer suggests that instead of having one copy of each component, the entire fission yeast shelterin complex may be dimeric: two columns of Taz1-Rap1-Poz1-Tpz1- Pot1 interacting to bridge the double- and single-stranded region of telomeric DNA (Figure 4.17B). However, the interactions may not necessarily occur within a complete parallel dimeric complex, instead, they could form networks between different sub-complexes, providing the potential to form higher order structures. Poz1 could bridge arrays of Taz1/Rap1 complexes along the telomeres (Figure 4.17C). This may provide a molecular mechanism to regulate the compactness or accessibility of telomeres.

A second type of network could form between different telomeres. Intriguingly, human telomerase is also dimeric [148-150]. The dimeric telomerase contains two catalytic active sites and can bind two telomeric DNA substrates [150]. Although it is not known yet whether fission yeast telomerase is also dimeric, this feature is conserved in *S. cerevisiae* and ciliates [151, 152]. The dimerization of shelterin components in fission yeast could facilitate the coordinated extension of both telomere ends in the sister chromatids (Figure 4.17D). Alternatively, the network could form between different chromosomes which would promote telomere clustering. Currently, all these models are possible and they are not mutually exclusive.

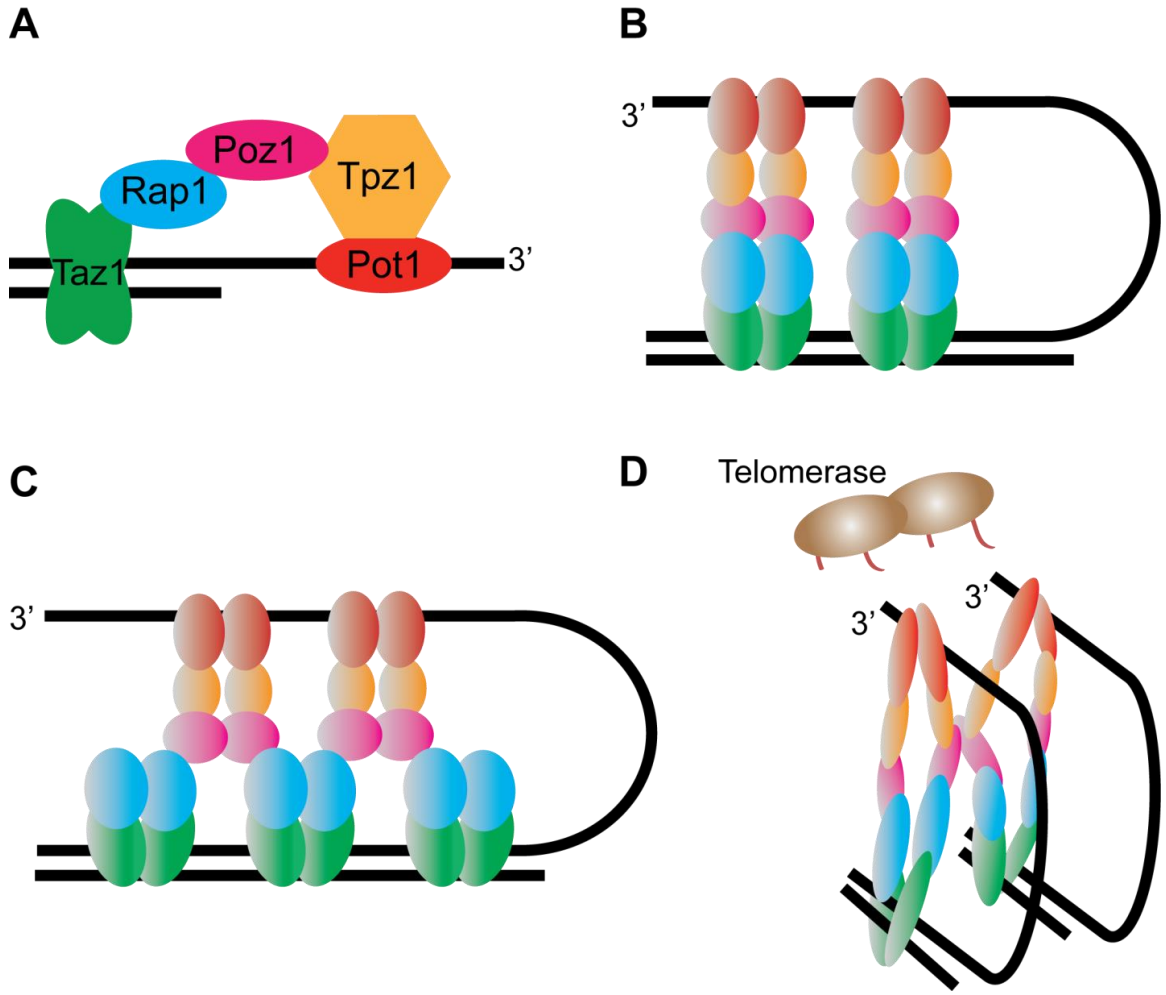


Figure 4.17: Models of potential higher order structures at telomeres. **(A)** A simplified version of the fission yeast shelterin complex demonstrating the interaction relationships. **(B)** Dimeric shelterin complexes. **(C)** Poz1 dimers bridging arrays of Taz1/Rap1 complexes (D) Poz1 dimers promote networks between different telomeres. In the case that networks form between sister chromatids, coordinated telomere synthesis could be carried out by telomerase dimers.

Chapter V: Conclusions and future directions

V.1: To further investigate different telomere functions

Chapter Three describes how I dissected the contribution of different telomeric proteins to the maintenance of telomere length homeostasis in fission yeast. Two mini-telosomes that are sufficient to maintain wild-type telomere length were created in the process. These mini-telosomes provide great opportunities to further dissect telomere functions. I analyzed cell growth rate in rich media, average cell size, the ability to maintain heterochromatic structures at sub-telomeric regions, viability at low temperature, and NHEJ in G1 arrested cells. With the exception of NHEJ inhibition, the other functions are similar to wild-type cells. The strains created by this project can be used for further characterization of other functions to improve our understanding of the molecular details of telomere functions.

One feature that can be evaluated is the ability of mini-telosomes to prevent DNA damage in logarithmically growing cells. As *S. pombe* spends little time in G1 phase in the laboratory, loss of protection during G1 phase does not affect the overall growth of vegetative cultures. However, an increase in average cell size was observed in one of the mini-telosome strain suggesting a cell cycle delay. GFP-tagged DDR proteins can be used to evaluate the activation of DDR in those cells. Chromosome segregation defects at mitosis, which have been observed in *pot1Δ* cells and *taz1Δ* cells when cultured at 20ºC [51, 124], can be evaluated by microscopy.

In addition, the mini-telosomes might be fully functional under optimal conditions but not under sub-optimal conditions. Similar growth between mini-telosome cells and wild-type cells were observed at low temperature (18ºC). However, low Tpz1-Taz1 fusion cells and cells lacking Taz1, grown at 18ºC, demonstrated an obvious growth defect. We can further characterize the sensitivity to DNA damaging reagents. Several DNA damaging reagents are widely-used to study the DNA damage response. Hydroxyurea (HU) depletes nucleotide pools and leads to S-phase arrest [153]. Methyl Methanesulfonate (MMS) is an alkylating agent that damages DNA [154]. Bleomycin (BLM) mimics gamma irradiation and produces double strand breaks [155]. Ultraviolent radiation (UV) causes covalent bonds between pyrimidine bases [156]. Besides these DNA damaging agents, Thiabendazole (TBZ) is a microtubule-depolymerizing agent that induces the spindle checkpoint [157]. By comparing sensitivity to these reagents, we will have a better understanding of proteins that are required for telomere integrity. Another adverse condition is heat. Several telomeric complex components can dimerize, indicating the possibility of a higher order structure. These higher order structures may be less stable at higher temperature in the mini-telosome context. The optimal growth temperature for wild-type *S. pombe* cells is 32ºC and cells grown above 36.5 ºC are sick. Future experiments to compare growth rates of mini-telosome cells and other mutants to wild-type cells at temperatures above and below 36.5ºC can determine the heat sensitivities of these mutants.

Deletion of Rap1 or Taz1 leads to meiotic defects due to compromised telomere bouquet formation [31, 43]. Two meiosis specific proteins Bqt1 and Bqt2 interact with

Rap1 and Sad1, a SPB component, thereby bridging telomeres and SPB [113]. Because Rap1 is missing, the mini-telosomes are unlikely to be sufficient for the tethering of telomeres to SPB during meiotic prophase. But in order to further understand the contribution of other proteins, we can reintroduce Rap1, Poz1, or both back into the mini-telosome system. In addition, the Rap1-Taz1C fusion can be used to define whether the N-terminal region of Taz1 is required.

In summary, by performing these experiments, a more comprehensive dissection of telomere functions can be obtained and will improve our knowledge about how telomeres contribute to diverse physiological processes.

V.2: The mechanism by which Rap1 prevents NHEJ

My mini-telosome provides a separation of functions between telomere length regulation and end protection. It maintains wild-type telomere length but is defective in inhibiting NHEJ at telomeres. On the other hand, cells harboring a deletion of *poz1* also harbor long telomeres, but are protected from NHEJ. My results further demonstrate the importance of Rap1 in protecting chromosome ends from NHEJ activity. Notably, in contrast to the role of Rap1 as a molecular bridge in length regulation, the ability of Rap1 to bridge Taz1 and Poz1 is not critical for end protection. Instead, an inherent feature of Rap1 appears to be responsible for the protein's contribution to end protection. This observation provides a basis for future study of the mechanism by which Rap1 prevents NHEJ activity.

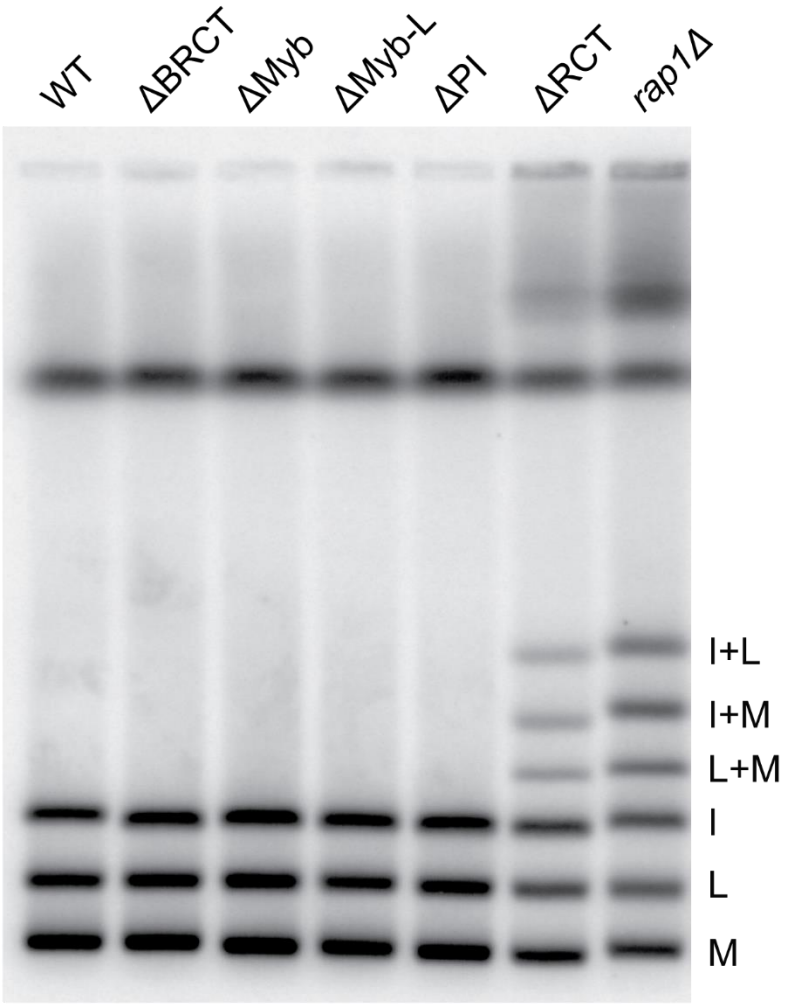


Figure 5.1: Rap1 deletion mutants in preventing chromosome end-fusions. Rap1 constructs lacking each individual domain indicated above were integrated at the endogenous locus of *rap1*. Nitrogen starved cells were analyzed by PFGE with internal C, I, L, M probes.

This study can be further divided into several questions. First, what is the functional element in Rap1 that accounts for the inhibition? Preliminary results were obtained with Rap1 deletion mutants lacking each individual domain including BRCT, Myb, Myb-L, PI and RCT. Rap1 mutants that lack one of the BRCT, Myb, Myb-L or PI domains showed no defect in the inhibition of NHEJ (Figure 5.1). The Rap1ΔPI mutant

further confirms the dispensability of the Rap1-Poz1 interaction. The only mutant that revealed an end-fusion phenotype was the Rap1ΔRCT mutant. This is not surprising since the RCT domain interacts with Taz1 and recruits Rap1 to telomeres. Although the BRCT domain is found in many DDR factors [45] and therefore is an attractive candidate to function in end protection, it was found to be dispensable for NHEJ inhibition. Similarly, in *S. cerevisiae*, Rap1 also inhibits NHEJ irrespective of its BRCT domain [90, 125]. In conclusion, larger truncation mutants are needed to characterize the region in Rap1 responsible for the inhibition of NHEJ.

Second, what are the interaction partners of Rap1? In *S. cerevisiae*, Rap1 recruits Rif2 and Sir4 to establish NHEJ inhibition [125]. Rap1 interacting partners in *S. pombe* can be identified by immunoprecipitation followed by mass spectrometry. Factors that are involved in NHEJ inhibition can be specified by using the functional region of Rap1 as bait for pulldown.

Third, are post-translational modifications (PTMs), such as phosphorylation, ubiquitination and SUMOylation, involved in the NHEJ inhibition pathway? Phosphorylation is required for the DDR pathways [3]. SUMOylation plays a role in regulating DNA repair and many DDR factors are subject to this type of modification [158]. In addition, SUMOylation has been suggested to play a role in telomere maintenance. Deletion of *pmt3*, the gene that encodes SUMO, or *pli1*, a SUMO E3 ligase, causes a striking increase in telomere length [159, 160]. In *S. cerevisiae*, Uls1, a non-essential Swi2/snf2-related translocase and SUMO-targeted ubiquitin Ligase (STUbL), is

required for the maintenance of NHEJ inhibition at telomeres. Loss of Uls1 results in telomere-telomere fusions and is alleviated by rap1 alleles lacking SUMOylation sites [161]. It is worth inspecting, by mass spectrometry followed by mutational study, whether Rap1 is subject to PTMs. Additionally, we can investigate whether there is a genetic interaction between Rap1 and PTM pathways by applying mutants defective in PTM pathways (e.g. ubiquitin, SUMO).

The fourth question that extended from this dissertation is whether inhibition of NHEJ by Rap1 is telomere sequence specific and dependent on other telomeric proteins. Deletion of Taz1 also causes NHEJ mediated chromosome end-fusions in G1 arrested cells [89]. It is not clear yet whether this is because Rap1 needs Taz1 to localize to telomeres or whether Taz1 itself is important. By targeting Rap1 to non-telomeric DNA ends of a linear plasmid, we can evaluate whether the ability of Rap1 to prevent NHEJ is sequence-sensitive. The widely used UAS/Gal4 system or genome editing system TALEN, ZFN or CRISPR [162-165] could be used as the targeting strategy. Furthermore, comparing the NHEJ inhibition efficiency between a *taz1*$^{+}$ and a *taz1Δ* background would uncover the contribution of Taz1.

The fifth question that put forth by the work in *S. pombe* is whether the protective mechanism is conserved in human cells. Both *in vitro* and *in vivo* approaches can be applied to address this question. An *in vitro* NHEJ assay with a linearized plasmid harboring telomere sequences at the ends was utilized by Bae *et al* to show TRF2/RAP1 complex inhibits NHEJ at telomeric DNA ends [94]. We could utilize this system to

further characterize key regions in RAP1 involved in telomere protection and compare to the functional region in fission yeast. In addition, we can compare the efficiency of inhibition between telomeric DNA ends and non-telomeric DNA ends using Rap1 fusion proteins that target the ends. Furthermore, by immunodepleting TRF2 or expressing a Rap1 mutant that fails to bind TRF2, we can test whether RAP1 is sufficient to inhibit NHEJ in general. Complementarily, targeting RAP1 functional domain identified by the *in vitro* assay or RAP1 mutant that lacks the functional domain to natural telomere ends would further confirm the functional domain *in vivo*. Teb1 is a *S. pombe* protein that binds vertebrate telomeric DNA and its DNA binding domain has been successfully used to tether human RAP1 to telomeres independently of TRF2 [95]. Separately, a mammalian artificial chromosome (MAC) could be utilized [166]. By inducing a site specific DSB in the artificial chromosome and tethering RAP1 to the break, we can further investigate the ability of RAP1 to prevent NHEJ independently of telomere sequences and other telomere proteins *in vivo*.

V.3: Is the mini-telosome minimal?

The mini-telosomes created in this study are Rap1 and Poz1 free. The other three proteins in the complex serve indispensable functions. Taz1 is the only double-stranded telomeric DNA binding protein and Pot1 is the only single-stranded DNA binding protein, both of which cannot be simply replaced by a linker. Tpz1 interacts with Ccq1, which in turn recruits telomerase [58, 137, 167, 168]. The DNA binding domain of a *S. pombe* protein Teb1 (Teb1DB) has been used to target hRAP1 to telomeres in human

cells [95]. However, an alternative protein that directly binds *S. pombe* telomeres has not been identified, which may be due to the degenerative nature of the telomeric repeat sequences. However, I was able to show that Taz1 N-terminal region is also dispensable for telomere length regulation if the C-terminal region including the DNA binding and the homodimerization domains is fused to Rap1. One question to pursue is whether we can further trim down the complex through generating truncation and fusion mutants. Is the Ccq1 interaction domain of Tpz1 sufficient to recruit and regulate telomerase if fused to Pot1? Is the DNA binding domain of Pot1 sufficient if fused to Tpz1 or part of Tpz1? It is not very likely that we can simply make a fusion protein that links arrays of necessary domains together (DNA binding domain from Taz1-Ccq1 binding domain from Tpz1-DNA binding domain from Pot1). But such stepwise truncations would identify key domains that are involved in regulating telomerase, which would in turn shed light on the next question: What is the molecular mechanism that accomplishes the communication between telomeric complex and telomerase? The mini-telosome is a good tool to address this. Phosphorylation of Thr93 of Ccq1 by $Tel1^{ATM}$ and $Rad3^{ATR}$ stabilizes Ccq1-Est1 interaction and is therefore critical for the telomere-telomerase interaction [137, 168]. Deletion of Taz1, Rap1 or Poz1 causes hyperphosphorylation of Ccq1, which may be the reason why telomeres are over-elongated in those mutants. How these three proteins affect Ccq1 phosphorylation is not known. With the mini-telosome in the absence of Rap1 and Poz1, this question can be further pursued. It is possible that Rap1 and Poz1 only function to transduce the negative signal from Taz1 to Tpz1-Ccq1. Ccq1

phosphorylation and the telomerase recruitment pattern in mini-telosome cells can be characterized. Interaction profiles between Taz1 and Tel1/Rad3 can also be tested.

V.4: Future prospects for Chapter IV

Through our structural, biochemical and genetic approaches, the molecular details of Poz1 structure and Poz1-Tpz1 interaction has been investigated and led to the identification of a conserved structure. The Poz1 structure shares striking similarities with the TRFH domains of hTRF1 and TRF2. TRFH domain not only mediates interactions with other proteins, but is also a homodimerization domain. This dimerization feature is shared by Poz1 as well. This result was unexpected since Poz1 is considered to be the functional homolog of human TIN2. In addition, the TRF1/TRF2 fission yeast counterpart Taz1 also contains a TRFH domain based on sequence prediction [36]. The evolution of these two similar domains is not well understood. A possible explanation is that this conserved structure is used at different places of the shelterin complexes. Solving the structure of Taz1 TRFH domain would test this possibility. On the other hand, the structure of hTIN2-TPP1 is also needed for comparison to Poz1-Tpz1 structure.

Two mutants that disrupt Poz1 dimerization were created in this study. However, one mutant affects interactions with both Rap1 and Tpz1 and the other mutant affects the interaction with Rap1. More dimerization mutants should be designed based on the structural data to obtain separation of function mutants. The region of Poz1 that interacts with Rap1 has not been identified yet. It will be informative to map that region and possibly solve the structure in order to understand the significance of Poz1

homodimerization. Additionally, tethering the dimerization mutant of Poz1 to Rap1 would bypass the need for the Poz1-Rap1 interaction and would therefore provide insights into the significance of Poz1-Poz1 interaction.

A unique feature in the Poz1-Tpz1 structure is the binding of a zinc ion at the interaction interface. A mutant with disrupted zinc binding compromises Poz1-Tpz1 interaction and telomere length maintenance. However, whether these two effects are independent or the length deregulation is the secondary effect of loss of protein-protein interaction is not known. My speculation leans towards the latter based on my results in Chapter three demonstrating that disruption of other interactions in the complex also leads to telomere elongation and that mini-telosomes lacking poz1, thus lacking zinc binding, maintain wild-type telomere length. Tethering the zinc mutant of Tpz1 to Poz1 would distinguish between the possibilities. Whether zinc binding is dynamic and serves as a modulator of telomere length is not known. Zinc is a necessary mineral for cells, making it difficult to address the question *in vivo*. However, *in vitro* approaches by modulating zinc concentration and using EDTA to chelate zinc or adding competing ions can be applied to investigate this question.

Poz1 dimerization is part of a larger theme of dimerization among telomere complex components. Homodimerization could occur between two copies of Taz1, Rap1, Poz1 and Pot1 [37, 43, 44, 147]. However, the functional significance of these homodimers is not known. Are these interactions the basis for the formation of a higher order structure? Several molecular networks could form between telomeric complexes

(Figure 4.17). Although it is technically challenging to visualize such large complexes, several experiments can be done to test different models. If the networks occur interchromosomally between different telomeres, we would expect to observe telomere clustering, which can be assessed by quantifying telomere foci using GFP-tagged telomeric proteins. If the networks occur within the same telomere, then the compactness of telomeres may be affected. An *in vitro* assay using a synthetic telomeric DNA fragment labeled with different dyes (e.g. Cy3, Cy5) at each end could be established to test this possibility. The distance between two ends which is indicative of the compactness of the DNA could be evaluated by Fluorescence correlation spectroscopy (FCS) and Fluorescence resonance energy transfer (FRET).

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