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Role of *Saccharomyces cerevisiae* Rif1 and Rif2 proteins in protection of telomeres

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A tutti quelli che mi hanna aiutato,

ad arrivare qui dove sono io oggi.

To everyone who has helped me,

to be here where I am today…

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Abstract

Eukaryotic cells distinguish their chromosome ends from accidental DNA double-strand breaks (DSBs) by packaging them into protective structures called telomeres that prevent DNA repair/recombination activities. In this work, we investigated the role of key telomeric proteins in protecting *Saccharomyces cerevisiae* telomeres from degradation. We show that the shelterin-like proteins Rif1, Rif2, and Rap1 inhibit nucleolytic processing at both *de novo* and native telomeres during G1 and G2 cell cycle phases, with Rif2 and Rap1 showing the strongest effects. Also Yku prevents telomere resection in G1, independently of its role in non-homologous end joining. Yku and the shelterin-like proteins have additive effects in inhibiting DNA degradation at G1 *de novo* telomeres. In particular, while Yku plays the major role in preventing initiation, Rif2 and Rap1 act primarily by limiting extensive resection. Finally, Rap1 and Rif2 prevent telomere degradation by inhibiting MRX access to telomeres, which are also protected from the Exo1 nuclease by Yku. Thus, chromosome end degradation is controlled by telomeric proteins that specifically inhibit the action of different nucleases.

Since Rif1 plays a very minor role in protecting wild type telomeres from degradation, we further investigated whether Rif1 participates in telomere protection in combination with other capping activities, like those exerted by the CST complex (Cdc13-Stn1-Ten1). We found that, unlike *RIF2* deletion, the lack of *RIF1* is lethal for *stn1ΔC* cells and causes a dramatic reduction in viability of *cdc13-1* and *cdc13-5* mutants. Both *cdc13-1 rif1Δ* and *cdc13-5 rif1Δ* cells display very high amounts of telomeric single-stranded DNA and DNA damage checkpoint activation, indicating that severe defects in telomere integrity cause their loss of viability. In agreement with this hypothesis, lethality in *cdc13 rif1Δ* cells is partially counteracted by the lack of the Exo1 nuclease, which is involved in telomeric single-stranded DNA generation. Like *CDC13*, *RIF1* also genetically interacts with the Polα-primase complex, which is involved in the fill-in of the telomeric complementary strand. Thus, these data highlight a novel role for Rif1 in assisting the essential telomere protection function of the CST complex.

Riassunto

Le cellule eucariotiche distinguono le proprie terminazioni cromosomiche dalle rotture a doppio filamento di DNA (*Double Strand Breaks*), impacchettandole in strutture chiamate telomeri, i quali limitano eventi di riparazione/ricombinazione del DNA.

In questo lavoro, abbiamo indagato il ruolo di diverse proteine telomeriche nella protezione dalla degradazione dei telomeri del lievito *S. cerevisiae*. Abbiamo dimostrato che le proteine *shelterin-like* Rif1, Rif2 e Rap1 inibiscono il processamento nucleolitico sia ai telomeri *de novo* che ai telomeri nativi durante le fasi G1 e G2 del ciclo cellulare. Anche il complesso Yku inibisce la degradazione nucleolitica in fase G1, indipendentemente dal suo ruolo nel Non-Homologous End Joining (NHEJ). L'inattivazione di entrambi i complessi Yku e *shelterin-like* comporta un effetto additivo sull'inibizione della degradazione del telomero *de novo* in G1. In particolare, mentre il complesso Yku inibisce principalmente l'inizio del processamento, Rif2 e Rap1 agiscono limitando la degradazione estensiva dei telomeri. Infine, abbiamo mostrato che le proteine Rap1 e Rif2 inibiscono la formazione del DNA a singolo filamento, limitando l'accesso del complesso MRX (costituito dalle proteine Mre11-Rad50-Xrs2) alle estremità telomeriche, le quali sono anche protette da parte di Yku dall'attività nucleasica di Exo1. Quindi, possiamo concludere che la degradazione delle estremità cromosomiche è regolata da diverse proteine telomeriche che impediscono specificamente l'azione di diverse nucleasi.

Poichè la proteina Rif1 ha un ruolo minoritario nella protezione dei telomeri dalla degradazione, abbiamo verificato se essa potesse avere un'azione protettiva in combinazione con altre proteine, come Cdc13, Stn1 e Ten1 (che insieme formano il complesso CST), le quali è noto svolgano un'attività di *capping* dei telomeri. Abbiamo osservato che, al contrario della delezione di Rif2, la mancanza di Rif1 è letale in cellule *stn1ΔC* e causa una forte riduzione della vitalità in mutanti *cdc13-1* e *cdc13-5*. Sia cellule *cdc13-1 rif1Δ* che cellule *cdc13-5 rif1Δ* mostrano una grande quantità di DNA telomerico a singolo filamento e attivazione del *checkpoint* da danno al DNA, suggerendo che la perdita della vitalità cellulare sia dovuta a gravi danni all'apparato protettivo dei telomeri. In accordo con questa ipotesi, il difetto di crescita di cellule *cdc13-1 rif1Δ* è parzialmente soppresso dalla mancanza della nucleasi Exo1, implicata nella formazione del DNA telomerico a singolo filamento. Inoltre, come Cdc13, anche Rif1 interagisce geneticamente con il complesso Polα-primasi, coinvolto nel processo di *fill-in* ai telomeri. Quindi, questi dati mettono in luce un nuovo ruolo di Rif1 nel supportare la funzione di protezione dei telomeri esercitata dal complesso CST.

Introduction

Marginotomy – End replication problem

In 1960's Leonard Hayflick observed when confluent cells of different origins were continuously subcultured, the cells age and stops dividing after a certain number of cellular divisions. Based on this observation, he hypothesized that aging or senescence might occur at cellular level [1]. Meanwhile, Alexey Matveyevich Olovnikov refined the Watson and Crick's classical model of DNA replication. Based on Watson and Crick's model, he expected that with every round of replication the resulting "replica" or daughter strand will be shortened and he called this problem "Marginotomy" (or "End replication problem") [2]. He proposed two hypotheses: the first one was based on the structural constrain of the DNA polymerase catalytic domain and the second was based on the removal of the RNA primer. Olovnikov very well realized that Marginotomy might be the reason for Hayflick's observation of limited cellular doubling's. He hypothesized that the chromosome ends should have some kind of "telo-genes" or "buffer genes", which could be sacrificed during every successive replication. After the exhaustion of these "telo-genes", the cell might age or die because it loses essential genes near "telo-genes". He also hypothesized that cell survival during evolution requires "Anti-Marginotomy", which can occur when factors regulating Marginotomy are reintroduced into cells and can delay ageing by lengthening "telo-genes". Overall according to Olovnikov, the terminus of a chromosome was the Achilles Heel and it could be protected by Anti-Marginotomy [2].

Telomeres and telomerase

Unaware of Olovnikov's hypothesis, Elizabeth Blackburn observed that the ends of ciliate *Tetrahymena* chromosomes are made up of repetitive DNA sequences (3' strand with T_2G_4) [3] and few years later the telomere terminal transferase (telomerase enzyme) which can add repetitive DNA was identified. The repetitive nature of the telomeric DNA was indeed the "buffer gene" and telomerase can be considered the Anti-Marginotomy factor hypothesized by Olovnikov [2]. From ciliates to humans, telomeres have conserved features and telomeric sequence are made up of short tandem repeats. Telomeres in different organisms vary by repeat consensus, length, structure and diversity of bound proteins.

In *Saccharomyces cerevisiae*, telomeres are 300 ± 75 bp of simple repeats (TG_{1-3}). In the telomeric DNA, the 3' strand is G-rich and so referred as G-strand, whereas the 5' C-rich complementary strand is called C-strand. The G-strand extends beyond its complementary C-rich strand to form a single-stranded overhang, referred to as the G-tail [4] (for a detailed review on budding yeast telomeres, please see [5]).

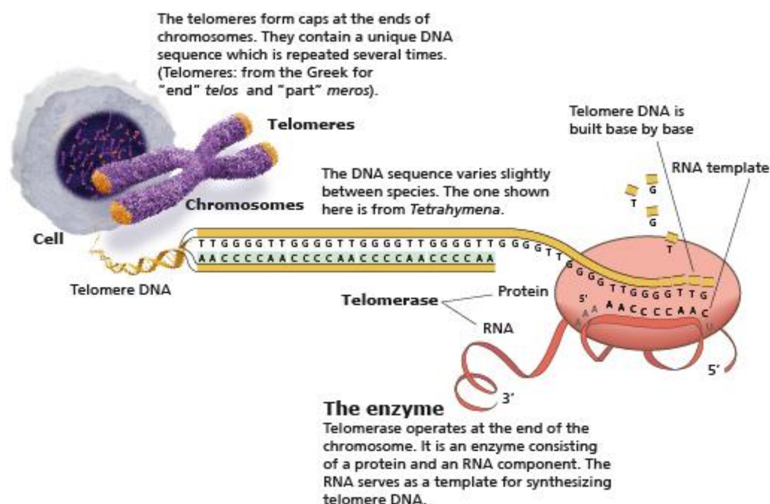

Figure 1: Diagram showing the repetitive sequence of telomeric DNA and telomerase reverse transcriptase using RNA template to elongate G-rich telomeric DNA (Source: NobelPrize.org)

In budding yeast, *EST2* encodes the reverse transcriptase subunit (telomerase). Est2 uses the RNA encoded by the *TLC1* gene as a template for telomere elongation [6]. The stem loop of TLC1 RNA serves as scaffold for the binding of Est2, Est1 (telomerase regulatory subunit), Ku heterodimer complex (See DNA damage proteins section for more details) [7–9]. Est1 can bind to telomeric ssDNA independently of its interaction with *TLC1* RNA [10]. Cells lacking *EST2*, *TLC1* or *EST1* undergo progressive loss of telomeres and senescence [11,12].

Cdc13 binds to telomeric TG_{1-3} tails using its OB (oligonucleotide/ oligosaccharide binding) domain [13] and interacts with Est1 to promote recruitment and activation of Est2. The strong association of Est2 to telomeres

during late S/G2 phase occurs concomitantly with Cdc13 and Est1 binding at telomeres and telomerase action [14].

In fission yeast, *TER1* encodes the RNA template that is used by the telomerase Trt1 for telomere elongation (for a review fission yeast telomere maintenance, see [15]). In humans, the telomerase enzyme is encoded by *TRT* and it adds telomeric repeats using RNA template encoded by hTR (for a review on human telomere maintenance, see [16])

Telomere structure

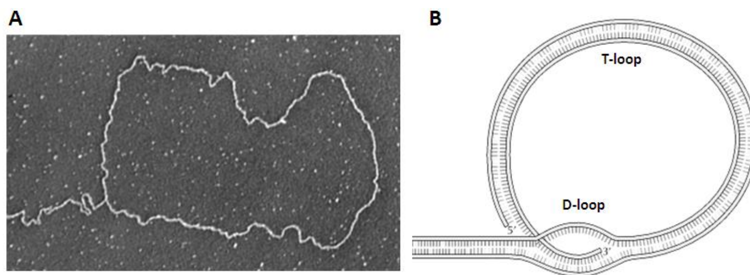

Figure 2: A: Scanning Electron micrograph of mammalian t-loop structure; B: Diagram representing overhang invasion (D-loop) required to form the t-loop. (Source: [17])

In mammalian cells, telomeres exist in a t-loop structure formed by the invasion of the 3' telomeric overhang into the duplex telomeric repeat array [17]. Similar structures are also observed in *Ciliates* and plants [18,19]. Due to natural abundance of guanine in the telomeric DNA, G-Quadruplex (G4) structures has been observed in telomeres from ciliates and humans [20,21]. G4 can form by Hoogsteen hydrogen bonding between four guanine bases.

Scarce data from *in vitro* and *in vivo* analysis suggest that telomeric G4 formation can be regulated by telomere binding proteins [22–24]. In budding yeast direct evidence of such structures are lacking so far, although it was recently observed that rudimentary G4 based and/or fold back loop structures might exist [25,26].

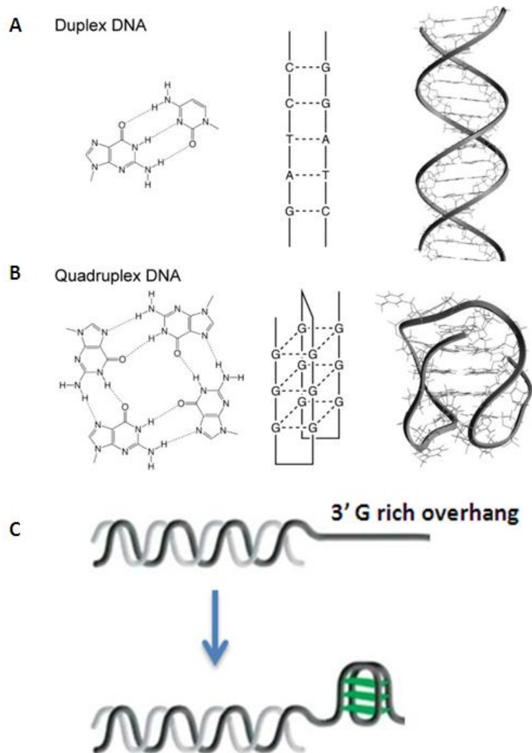

Figure 3: A: Diagram showing Duplex DNA secondary structure. B: Diagram showing G-Quadruplex structure with Hoogsteen hydrogen bonding between four guanine bases. C: Diagram showing potential G-Quadruplex structure at telomeric 3' G rich overhang. (Source:[27]).

Telomeric ssDNA binding proteins

Apart from Est1 and Est3, telomeric ssDNA is bound by CST complex. The CST complex is involved in telomere protection and consists of Cdc13, Stn1 and Ten1 proteins. *CDC13* is an essential gene and encodes a less abundant Cdc13. Cdc13 can bind to telomeric TG_{1-3} tails using its OB domain and protect telomeres from degradation [13]. Stn1 and Ten1 are DNA-binding proteins with specificity for telomeric DNA substrates [28]. Cdc13, Stn1 and Ten1 proteins physically interact with each other by both coimmunoprecipitation and two-hybrid assays [29,30], indicating that these three proteins function at chromosome ends as a heterotrimeric complex.

Based on the finding that at higher temperature *cdc13-1* mutant accumulates telomeric ssDNA and undergoes DNA damage checkpoint activation, CST was proposed to function as a telomere capping complex that protect telomeres from degradation [31]. Cdc13 also physically interacts with the DNA polymerase α and this interaction is important for telomere length regulation [32]. Telomeric 3' G strand synthesis by telomerase is tightly co-regulated with 5'C synthesis by the DNA polymerase α-Primase complex and Polymerase δ [33]. *cdc13* mutants have impaired telomere length regulation [34,35].

Cdc13 is SUMOylated in cell cycle regulated manner. Cdc13 SUMOylation is high during early to mid S phase before telomerase is activated. Cdc13 SUMOylation site overlaps with Stn1 interaction region of Cdc13 and SUMOylation enhances interaction with Stn1. *cdc13* mutants which cannot be SUMOylated have overelongated telomeres probably due to reduced Stn1 mediated control over elongation. Cdc13-SUMO fusion has increased Stn1 interaction and exhibit shorter telomeres. SUMOylation and Cdk1-phosphorylation of Cdc13 act antagonistically on telomere length regulation [36].

STN1 is an essential gene and was identified as a partial suppressor of the *cdc13-1* temperature sensitivity [29]. As Stn1 physically interacts with Cdc13, it is possible Stn1 can compete with Cdc13 for binding to Est1 or Est2, and thereby Stn1 can control Cdc13-mediated telomerase recruitment and elongation. Stn1 interact with Pol12 - the B subunit of the DNA polymerase α Pol1-Primase complex - by two-hybrid and biochemical assays [37]. *stn1* mutants have increased telomeric ssDNA and/or long telomeres [29,38].

It was proposed Pol12 and Stn1 provide a link between telomere elongation by telomerase and *fill-in* synthesis by the lagging strand replication machinery.

TEN1 is an essential gene and was identified as partial suppressor of the temperature sensitivity of *stn1* mutants. Like *cdc13* and *stn1*, *ten1* mutants also have increased telomeric ssDNA and/or longer telomeres [30]. Stn1 and Ten1 can regulate telomere capping in Cdc13-independent and DNA replication-dependent manner [39]. Similar to *cdc13-1*, temperature sensitive *stn1* and *ten1* mutants undergo telomeric degradation, G2/M cell cycle arrest at restrictive temperatures.

The Replication Protein A (RPA) heterotrimeric complex is the major single-stranded DNA-binding complex in eukaryotic cells. RPA can bind with high affinity to single-stranded DNA all over the genome and has multiple roles during DNA replication, repair, and recombination [40]. RPA has multiple oligosaccharide/oligonucleotide binding (OB) folds, distributed among all three subunits, which are used for both DNA and protein recognition [41]. In budding yeast, Cdc13 is structurally similar to Rpa1, whereas Stn1 and Ten1 are structurally similar to Rpa2 and Rpa3 subunits of the RPA complex. Thus, it was proposed Cdc13, Stn1 and Ten1 function as a telomere-specific RPA-like complex [28,42].

In the hypotrichous ciliate *Oxytricha nova*, an α-β protein heterodimer binds specifically to telomeric single-strand DNA and protects telomeres [43,44]. Fission yeast Pot1 (Protection of Telomeres) is the structural homolog of budding yeast Cdc13. OB folds are present in α-β proteins of *Oxytricha nova* and also in fission yeast and human Pot1 [45]. Fission yeast *POT1* null cells undergo nucleolytic degradation of telomeres and lose telomeres within one cell cycle [46]. In mouse, two Pot1 orthologs exist namely Pot1a and Pot1b. Pot1a protects telomeres from uncontrolled resection and DNA damage repair activities which are detrimental to normal telomeres [47]. Similarly human hPot1 can bind and protect telomeric ssDNA and regulate telomere length [45,48].

Recently, CST homologs were also identified in plants and in mammals. Arabidopsis plants lacking At-STN1 display developmental defects and reduced fertility and these phenotypes are accompanied by catastrophic loss of telomeric and subtelomeric DNA, high levels of end-to-end chromosome fusions, increased G-overhang signals, and elevated telomere recombination [49]. *Xenopus laevis* xCST protein complex is involved in priming DNA synthesis on single-stranded DNA template for replication [50]. Depletion of human *CTC1* by RNAi triggers a DNA damage response, chromatin bridges, increased telomeric G overhangs, and sporadic telomere loss [51]. Similarly *STN1* knockdown cells have increased telomeric G overhangs [52,53].

A recent study found that mutations in *CTC1* cause a rare human genetic disorder called “Coats plus”, characterized by neurological and gastrointestinal defects. Patients suffering coats plus have shortened telomeres with spontaneous γH2AX-positive cells in cell lines indicative of DNA damage

response [54]. *CTC1* is also one of the 8 telomere maintenance genes with mutations observed in patients with dyskeratosis congenita, a rare inherited bone marrow failure syndrome [55]. Altogether, the above data indicates that CST complex is essential for telomere protection and maintenance from yeast to plants and humans.

Telomeric duplex DNA binding proteins

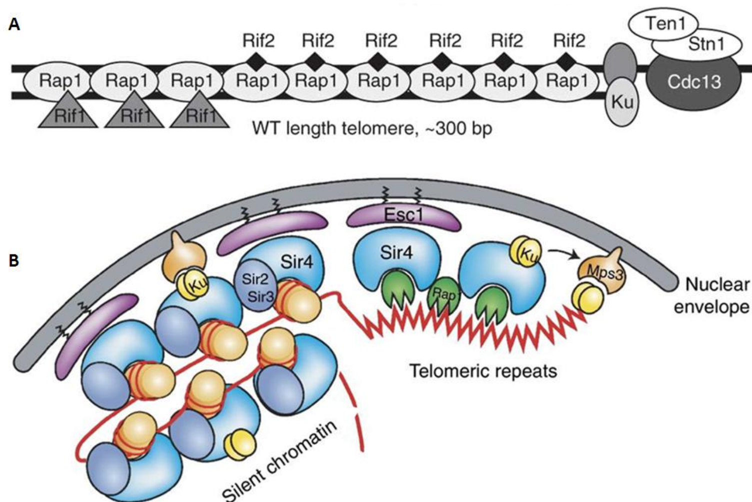

Figure 4: A: Schematic representation of budding yeast telomere. Rap1 binds all the telomeric duplex DNA. Rif2 binding at telomeres is more proximal, whereas Rif1 binding is more distal. Ku heterodimer binds between Rap1 and the CST complex, which binds to the terminal ssDNA region. (Source: [56]) B: Diagram showing telomere anchoring to the nuclear envelope through protein protein interactions. (Source: [57]).

In budding yeast, the telomeric double stranded DNA is bound a Shelterin-like complex, comprising of Rap1 and its interacting factor Rif1 and Rif2. The essential gene *RAP1* was first indentified to regulate gene expression (**R**epressor **A**ctivator **P**rotein 1). *RAP1* encodes Rap1, an abundant nuclear protein that can bind to transcriptionally repressed mating-type genes and to telomeric TG repeats through two Myb type DNA binding domain and regulate telomere length [58].

Rap1 C terminal is essential for the silencing of HML mating loci and telomeres and it is also involved in telomere length control [59]. Through its C terminal part, Rap1 recruits Rif1 and Rif2 (**R**ap1 **i**nteracting **f**actors). It is observed that Rif1 plays a mediator role for Rap1 in silencing and length regulation [60]. Cells lacking *RIF1* or *RIF2* have overelongated telomeres, whereas deletion of both *RIF1* and *RIF2* leads to longer telomeres similar to Rap1 with truncated C terminal. Rif1 and Rif2 are also involved in regulating telomere silencing [61]. Rif proteins can regulate telomere length even in *rap1*ΔC mutant, indicating that they can be recruited to telomeres independently of Rap1 [62].

At telomeres, Rap1 interacts with Sir4 and regulates the recruitment of Sir3, which deacetylates the subtelomeric histones to establish the heterochromatic environment at telomeres, maintain telomere length, and also cluster telomeres in foci near the nuclear periphery [63,64]. Rap1, Rif2 and Sir4 also inhibit non-homologous end joining (NHEJ), which could lead to detrimental telomere-telomere fusions [65,66]. (for a review on budding yeast telomere binding proteins see [67]).

In fission yeast, Taz1, like scRap1, contains a Myb DNA domain and binds telomeric DNA, regulates telomere length and silencing. Taz1 is essential for the stable association between telomeres and the spindle pole bodies during the meiotic prophase. Taz1 recruits spRap1 and spRif1 to telomeres and like Taz1, spRap1 and spRif1 are involved in regulation of telomere length, silencing and meiosis. Fission yeast Pot1 interacts with Ccq1, Tpz1 and Poz1; all of them are involved in telomere length regulation and protection (for review on fission yeast telomeres see [15]).

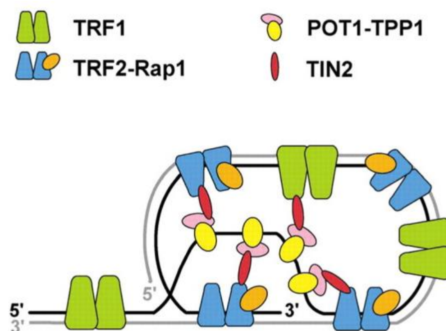

Figure 5: Schematic representation of the mammalian telomere t-loop structure with bound proteins (Source: [68]).

Human telomeres are bound by TRF1 and TRF2, both containing a Myb type DNA-binding domain that specifically binds to the duplex telomeric TTAGGG repeat array. TRF1 recruits TIN2, which interacts with TRF2. TPP1 (homolog of fission yeast Tpz1) localizes to telomeres and recruits POT1 and TIN2. PIP1 also stimulates the interaction between POT1 and TRF1. Human Rap1 is the only protein that has conserved motifs with scRap1. hRap1 is recruited to telomeres though TRF2 and negatively regulates telomere length (for review on mammalian telomeres see [16,69]).

Telomere length regulation

Telomere length regulation is essential for the maintenance and propagation of stable chromosomes. Telomere length is maintained as equilibrium between lengthening and shortening events. Telomere length is cell cycle regulated and requires Clb/Cdk1 kinase activity, which increases at the G1/S transition reaching its maximum level in late G2 to drive entry into mitosis [70].

Usually, telomere lengthening occurs by the action of the telomerase enzyme Est2, which uses TLC1 RNA template on the telomeres [11]. At individual telomeres, telomerase-mediated elongation is restricted to few base pairs per generation and this elongation rate decreases with increasing telomere length, indicating a progressive *cis*-inhibition of telomerase action during telomere elongation [71]. It was observed that telomere elongation is a stochastic process that is limited for few telomeres in every cell cycle. Shorter telomeres have a higher probability of being elongated than longer telomeres [72].

Telomere shortening can occur through three mechanisms: end replication problem (the lagging strand replication machinery is unable to fully copy the parental strand); nucleolytic degradation [73]; increased transcription [74] near telomeres leading to generation of TERRA (long noncoding telomeric repeat containing RNA). TERRA transcripts contain G-rich telomeric and subtelomeric RNA and localize to telomeres [75,76].

TERRA at telomeres

The transcription of telomeric DNA into TERRA is a conserved process observed from yeast to humans [77]. In budding yeast, TERRA levels are lowest in late S/G2 phase and increases as the cells passes through G2 and M phases

and highest at G1 phase. Yeast TERRA can be stabilized by polyadenylation by Pap1 and this is negatively regulated by 5′ to 3′ RNA exonuclease, Rat1. Defective Rat1 degradation of TERRA leads to shorter telomeres, possibly due to defective replication fork progression [75].

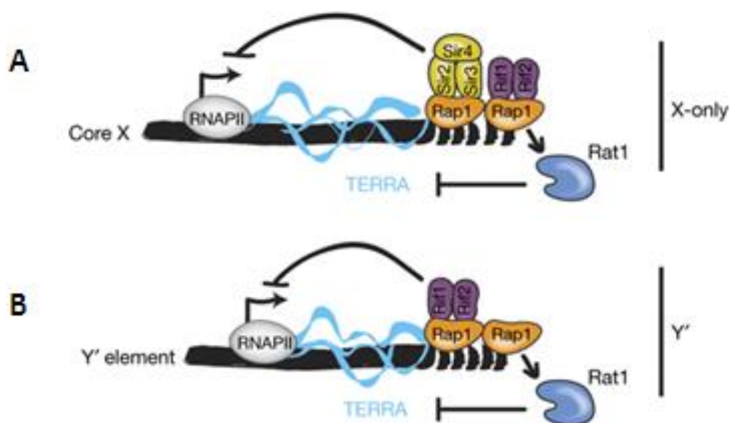

Figure 6: A model for Rap1-negative regulation of TERRA at X-only telomeres and Y′ telomeres. A: Rap1 associated with telomeric repeats (dashed line) affects TERRA levels at X-only telomeres through both Rat1-mediated degradation (probably through Rif1 and Rif2) and transcriptional silencing through the Sir2/3/4 complex. B: at Y′-containing telomeres, TERRA is regulated by Rap1 through the Rif1 and Rif2 proteins (degradation independent), as well as by the nuclear 5′–3′ exonuclease Rat1. RNAPII, RNA polymerase II; TERRA, Telomeric repeat-containing RNA. (Source: [78])

Rap1 is involved in regulation of TERRA because it promotes Rat1 exonuclease-mediated degradation. Furthermore it promotes a degradation-independent mechanism that is dependent on Rif and Sir proteins. Rif1 plays a major role than Rif2 in repressing TERRA at X and Y′ telomeres. Sir proteins mediate repression at X-only telomeres [78]. In yeast, telomeric binding of Rif1 (unlike Rap1 or Rif2) peaks at G1 phase [79], when TERRA levels are also high.

In mammalian cells, TERRA can basepair with the RNA template hTR and also bind with telomerase hTERT. In *in vitro* analysis, TERRA was found to be a telomerase ligand and natural direct inhibitor of human telomerase [78]. Thus TERRA should be differentially regulated according to the length of the telomere.

R-loops at telomeres

When the RNA polymerase traverses DNA during transcription it creates torsion and compacts the DNA ahead (positive supercoiling) while relaxing the DNA behind it (negative supercoiling). The topology is controlled by specific topoisomerases. If the topology maintanence is disturbed, negative supercoiling-induced relaxed DNA can basepair with the nascent RNA forming RNA-DNA hybrid or R-loops. Any step that is involved in keeping the nascent RNA from base pairing with DNA is important to prevent R-loops. R loops formation can impair DNA replication and cause genomic stability (for a review, see [80]).

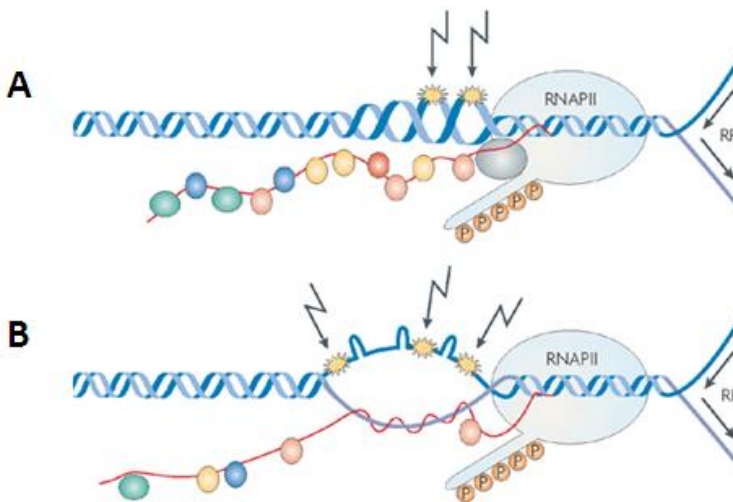

Figure 7: A: During transcription by RNA Pol II, the local negative supercoiling behind transcription bubble might facilitate transient ssDNA formation (represented by stars). B: Defective mechanisms in controlling transcription bubble movement, ssDNA formation or mRNA particle complex might lead to hybridization of RNA with the template DNA forming a transient R-loop which if left unrepaired might cause genomic instability (Source: [81])

Cells have RNAse enzymes, which cleave the RNA strand of the RNA–DNA hybrids. In humans, mutations in genes encoding the subunits of the RNase H2 complex cause Aicardi-Goutières Syndrome, a congenital immune-mediated neurodevelopmental disorder, and is generally fatal within the first few years [82]. In yeast, *RNH201* encodes the catalytic subunit of RNase H2p. RNases H

cleaves the RNA strand of the R-loop and it is known to prevent R-loop-associated problems [83]. Prolonged replication pausing due to increased transcription or R-loops will lead to topological stress-driven fork reversal [84–86], which can be avoided by creation of a DSB [87]. Such a beneficial mechanism if occurs at telomeric replication/transcription site, might lead to loss of telomeric DNA.

DNA damage checkpoint proteins

At any given point of time, DNA is subjected to many kinds of endogenous and exogenous damage agents. This will lead to formation of damaged DNA like single-strand break, double-strand break (DSB), etc. DNA damage should be repaired before chromosome segregation takes place in the successive cellular divisions. Even though DSB intermediates occur during meiosis and immune cells V(D)J recombination [88,89], a single unrepaired DSB can be highly deleterious for genomic stability. Budding yeast cells suffering a single unrepairable DSB exhibit a long, but transient, arrest in G2. With two unrepairable DSBs cells can become permanently arrested. The cells can escape this G2 arrest and this ability depends on the amount of the ssDNA created at broken chromosome ends [90].

Generation of accidental DSBs signals and activates the DNA damage checkpoint pathway. The DNA damage checkpoint response and the mechanisms leading to checkpoint activation are evolutionary conserved in all eukaryotes. Checkpoint activation controls cell cycle progression so that the repair of DNA lesions could be efficiently executed. Depending upon the phase of cell cycle when the damage occurs, the damaged DNA can be repaired either by Non-Homologous end joining (NHEJ), Homologous recombination (HR) or microhomology-mediated end joining (MMEJ) (See figure 8).

As most of mammalian somatic cells are predominantly in the G0/G1 phase, NHEJ is the predominant, simplest but error-prone repair mechanism to repair DSB by ligation of the two broken ends. Due to the high CDK activity in the G2 cell cycle phase, 5’ to 3’ resection occurs at the DSB and so it can be repaired by homologous recombination [88]. (for a review on DNA damage repair, see [91]).

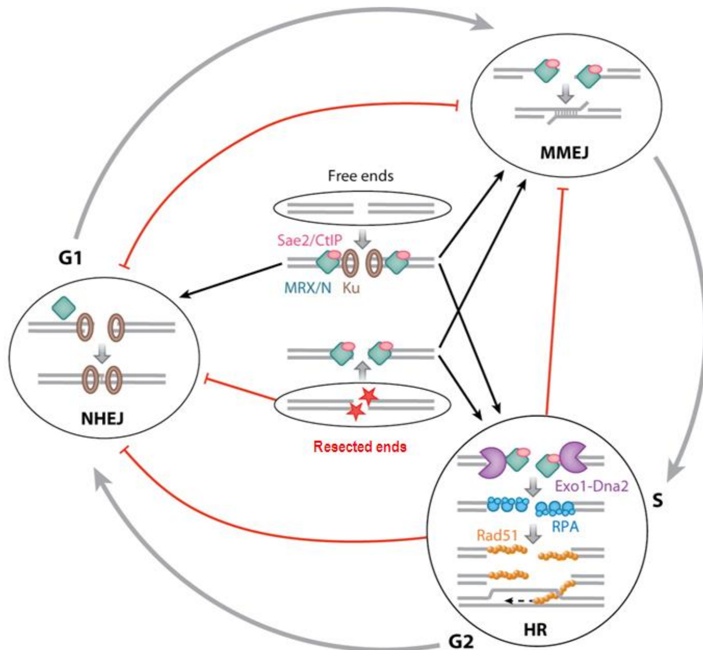

Figure 8: Regulation of repair pathway choice. The three modes of DSB repair are outlined. The choice of the repair depends on the cell cycle stage and the nature of the damage. (Source: [91]).

In budding yeast, the first set of proteins that are recruited at the break site independently of each other are the highly conserved Mre11, Rad50 and Xrs2 (MRX) complex and Ku70/80 heterodimer [92,93]. Mre11 belongs to the lambda phosphatase family of phosphoesterases and exhibits manganese-dependent nuclease activities *in vitro*, including 3′-5′ dsDNA exonuclease activity and an ssDNA endonuclease activity that acts on ssDNA/double-stranded DNA (dsDNA) transitions and hairpin loops [94].

Ku70/80 heterodimer recruits other proteins (Dnl4-Lif1/XRCC4 and Nej1/XLF) involved in NHEJ [93,95] and suppresses HR by inhibiting DNA end resection [96,97]. The Xrs2 subunit of the MRX complex physically interacts with Lif1 and this interaction is critical for NHEJ; also in humans, NBS1 and XRCC4, orthologs of Xrs2 and Lif1 respectively, interact in a two hybrid assay [98,99]. Structural analysis of human Ku complex indicates that Ku encircles duplex DNA through a preformed ring, which limits the sliding of Ku onto DNA from DNA break site [100].

In the G1 cell cycle phase, NHEJ is major mechanism that repairs the break. When DNA damage occurs in S or G2 cell cycle phase, the DSB is resected in a MRX complex/Sae2-dependent manner in 5′–3′ direction. Generation of 3′-ended ssDNA tails inhibit NHEJ and channel DSB repair to HR (for a review, see [101,102]). MRX and Sae2 are highly conserved proteins. The MRX complex in fission yeast and mammals is called MRN and is composed by Mre11, Rad50 and Nbs1 subunits (MRN). As for Sae2, Ctp1/Nip1 in fission yeast and CtIP in humans are all essential for promoting DNA end resection (for a review, see [103]). In mice, MRE11 nuclease deficient mutant causes early embryonic lethality and dramatic genomic instability [104].

In yeast, MRX recruits Tel1 through the C terminus of Xrs2 [105]. Tel1 is a conserved phosphatidylinositol 3-kinase-related kinase (PIKK) and a homolog of human ataxia-telangiectasia mutated (ATM). Once recruited, MRX and Tel1 contribute to generate 3′-ended ssDNA [106]. MRX cooperates with Exo1 nuclease to produce long ssDNA tracts at DSB ends [107]. The generated ssDNA tracts will be bound by the RPA complex [108]. At 5′ end a DSB, Rad24 binds to RPA [109]. Rad24 is related to subunits of the Replication factor C, which is involved in DNA replication. Rad24 loads a PCNA-like complex composed of Rad17-Mec3-Ddc1 (Rad9–Rad1–Hus1 or 9-1-1 complex in humans), which acts as sliding clamp. RPA and Rad24 cooperate to recruit Mec1 (PIKK; ATR in humans) [108]. Mec1 recruits and phosphorylates Ddc2 [110]. Dpb11 mediates recruitment of Rad9 (adaptor protein), by acting as a scaffold between Rad9 and Mec1-Ddc2 [111]. Mec1 and to a lesser extent Tel1 phosphorylate Rad9. Rad9 stimulates Mec1 and Tel1 kinase to phosphorylate and activate Chk1 kinase [112,113]. Chk1 regulates the phosphorylation and abundance of Pds1 (Securin) to prevent anaphase entry [114]. Rad9 also physically interacts with Rad53 (checkpoint effector kinase; Chk2 in humans), facilitating Rad53 in trans autophosphorylation and subsequent release of activated Rad53 [115–117]. Activation of Rad53 prevents both anaphase and mitotic exit in the presence of DNA damage. Rad53 exerts its role in checkpoint control through regulation of the Polo kinase Cdc5 [112]. In human cells, RPA recruits ATR to sites of DNA damage and for ATR-mediated Chk1 activation [118] (for a review on human checkpoint response, see [119]).

DNA damage checkpoint proteins at telomeres

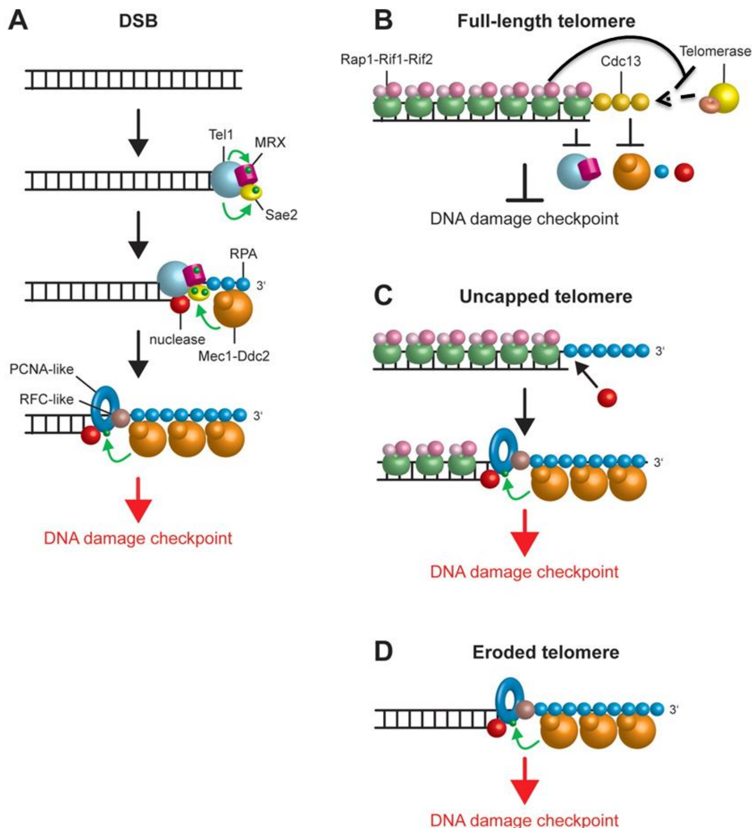

Figure 9: DNA damage response to DSBs and telomeres in budding yeast. (A) Intrachromosomal DSBs trigger a DNA damage checkpoint response. When a DSB occurs, the MRX complex and other factors are recruited to the unprocessed break. DSB recognition by MRX allows checkpoint activation by recruiting Tel1 which phosphorylates Sae2 and leads to further processing of DSB by exonucleases to generate 3′-ended ssDNA tails. The ssDNA tails will be coated by RPA, which allow the loading of Mec1–Ddc2 and subsequent Mec1-dependent checkpoint activation. Mec1 activation is also supported by independent loading of the PCNA-like Ddc1–Rad17–Mec3 complex by Rad24-RFC. (B) Full-length telomeres are protected from checkpoint activation. The presence of ssDNA- and dsDNA-binding proteins on functional telomere regulates recruitment of MRX, RPA, nucleases, telomerase, and checkpoint proteins. (C,D) Telomeres lose protection after loss of telomeric ssDNA- and dsDNA-binding proteins (uncapped telomere) or telomerase (eroded telomere). (C) In the absence of the ssDNA-binding protein Cdc13, telomerase recruitment is

impaired, and nucleases can act to generate ssDNA tail. Like at processed DSB, RPA binding can lead to activation of Mec1-dependent DNA damage checkpoint response. (D) RPA-bound ssDNA accumulates at telomeres also after telomere erosion due to telomerase loss. Green arrows indicate phosphorylation events (Source: [120]).

The first observation that damaged telomeres activate a DNA damage checkpoint response came from studies in *cdc13-1* mutant. At higher temperature, *cdc13*-1 cells accumulate ssDNA at telomeres and telomere proximal regions. Like the ssDNA generated at the DSB ends, the telomeric ssDNA leads to checkpoint activation [31]. Rap1 loss results in frequent NHEJ-dependent fusions between telomeres by the Lig4 (ligase IV), yKu70/80 and MRX proteins [65]. Similarly, telomeres of mammalian cells lacking TRF2 are subjected to DNA ligase IV-dependent NHEJ [121,122].

Yeast Ku binds to telomeric DNA and is involved in maintaining telomere length and protecting and silencing telomeres [123–126]. Ku binds the stem loop region of TLC1 RNA and this interaction is essential for the nuclear retention of TLC1 RNA, telomere elongation and telomerase-mediated healing of intra-chromosomal damage [127–130]. Cells lacking Ku have short telomeres and undergo checkpoint-mediated arrest at higher temperature and this arrest is associated with Exo1 dependent telomeric ssDNA generation that persist throughout the cell cycle [131]. Thus, like CST, Ku also plays a capping role at telomeres.

Ku interacts with Sir4 and contributes to telomeric anchoring at nuclear periphery and silencing [132,133]. Cells lacking Ku have normal replication program but the usual late firing origins near telomeres and subtelomeres are fired earlier and this effect is dependent on telomere length [134,135]. Mammalian Ku is found to interact with replication origin binding proteins and is important to load members of the pre-replicative complex (pre-RC) for efficient initiation of DNA replication [136,137]. The yeast MRX complex is also involved in telomere maintenance. MRX is required for generation of proper constitutive telomeric G-tails [4] and for the loading of Cdc13 on the telomeric ssDNA [138].

MRX can preferentially bind to short telomeres and is involved in the recruitment of the telomerase subunits Est1 and Est2; Cells lacking MRX have

short telomeres [56,126,138–140]. More than its nuclease activity, the structural integrity of MRX is important for telomere length maintenance in cycling cells [141]. Only in cells arrested in G2/M, the nuclease activity of Mre11 is required for telomere addition, suggesting a cell cycle-specific role of MRX at telomeres [70].

MRX seems to have also an protective role at telomeres, as it inhibits generation of telomeric ssDNA in cells lacking Ku [131] and *cdc13-1* mutant [142]. Mammalian MRN physically interacts with TRF2 and is recruited to normal telomeres [143]. Also in shelterin lacking cells, MRN is recruited to dysfunctional telomeres [144] and is required to remove the 3' telomeric overhang to promote chromosome fusions. MRN is also required to protect newly replicated leading strand telomeres from NHEJ [145,146] (for a review see [147]). Yeast RPA is also involved in telomere length regulation. Rfa2p binds to telomeres and is enriched at telomeres in S phase. *rfa2* mutant has short telomeres due to reduced recruitment of Est1p, but not of Est2p and Cdc13p, at telomeres [148].

Tel1 kinase is also involved in telomere maintenance. Like Ku, MRX and Tel1 are also involved in nuclear retention of TLC1 RNA [128]. Cells lacking *TEL1* have short but stable telomeres due to reduced Est1p and Est2 recruitment at telomeres, possibly owing to defective nuclear retention of TLC1 RNA [140,149]. Like at DSBs, telomeric Tel1 binding is also dependent on Xrs2 and is required for the recruitment of telomerase at short telomeres thereby leading to a preferential elongation of short telomeres [150,151]. The kinase activity of Tel1 is important for its lengthening activity [152,153] and Tel1 mutants with increased kinase activity have longer telomeres [154], but precise knowledge on telomeric Tel1 target is lacking [155]. In fission yeast, Tel1 (ATM) and Rad3 (ATR) phosphorylate the telomere protein Ccq1 and promote Ccq1 interaction with Est1 for telomere maintenance [156,157].

Mec1 is also involved in telomere maintenance, as *mec1* mutants have short telomeres [158] and cells lacking both *TEL1* or MRX and *MEC1* undergo telomere shortening and exhibit senescence phenotypes characteristic of cells lacking telomerase [139,158]. Cells lacking telomerase undergo Mrc1 dependent checkpoint activation [159,160]. In the survivors arising from telomerase lacking cells or in cells lacking yKu, Mec1 binding to telomeres increases [161]. Thus, In contrast to Tel1, Mec1 associates with short,

functionally compromised telomeres. Mec1, RPA, Mec3 and Rad24 are also involved in telomeric recombination in post senescence survivors [162].

Rif1 and Rif2 inhibit Tel1 recruitment at telomeres. Rif2 competes with Tel1 for binding to the C terminus of Xrs2 and in the absence of Tel1, Rap1 inhibits MRX association at telomeres [163]. In the absence of Rif2, Tel1 can bind equally well to short and wild type length telomeres [56]. Similarly, mammalian ATM is recruited to shelterin lacking dysfunctional telomeres [144]. In mammalian telomeres, TRF2 represses ATM, whereas POT1 prevents activation of ATR [164].

Although telomeres are bound by the checkpoint proteins, the checkpoint response is not activated and DNA repair/recombination processes such as NHEJ and HR are inhibited (for a review see, [165,166]). The transient telomeric ssDNA generated during replication is bound by Cdc13 and this binding has been proposed to inhibit RPA binding [167]. In fission yeast, the lack of essential epigenetic markers for checkpoint signal amplification and cell cycle arrest could be one of the mechanisms by which telomeres avoid complete checkpoint response [168]. In fact in mouse model, ATR suppresses telomere fragility and recombination [169]. In case of plants, ATR regulates DNA damage response by inhibiting chromosomal fusions and transcription of DNA repair genes and also by promoting programmed cell death in stem cells [170].

Chromatin dynamics and nuclear organization are important for gene regulation, DNA replication and also for the maintenance of genome stability. The nucleus contains spatially and functionally distinct subcompartments for specific purposes. Generally, chromatin near the nuclear envelop is transcriptionally inactive and late-replicating. However, recent evidences suggest that there might be exceptions because during stress response active genes are associated with nuclear pores [171]. In telomerase positive cells, budding yeast telomeres are normally clustered into 3–6 highly dynamic foci, which can fuse, disappear and reappear. The anchoring of the 32 telomeres takes place in a nonrandom manner, dependent upon the genomic size of the chromosome arm and other factors.

Spatial dynamics of DSBs and telomeres:

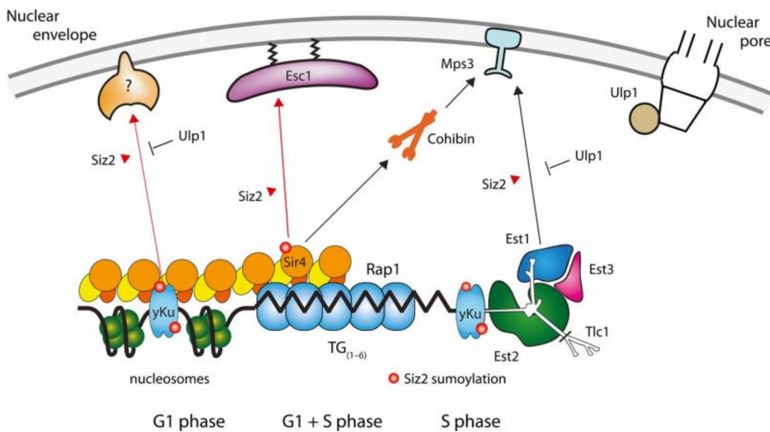

Figure 10: Telomere-tethering mechanisms. Model of the redundant pathways that tether yeast telomeres to the nuclear envelope. Parallel mechanisms lead to yeast telomere attachment at the nuclear envelope. Telomere tethering mechanisms varies across the cell cycle and is also regulated by SUMOylation by Siz2 SUMO ligase and desumoylation by Ulp1. Sir4-PAD domain binds the Esc1 C terminus, as well as Yku80 and Mps3. Yku80 binds telomerase, which also associates with Mps3 in S phase through Est1. There is an unidentified anchor for yeast Ku in G1 phase that is neither Esc1 nor Mps3 dependent (Source: [171])

Redundant pathways involving Sir4, the yKu heterodimer, Est1 regulate telomere anchoring in cell cycle dependent manner. Sir4 can be recruited to Esc1 and Mps3, whereas Est1 can be recruited to Mps3 [171]. Siz2 dependent sumoylation of Sir4 and yKu is essential for telomere anchoring. Deletion of *SIZ2* leads to telomerase-dependent telomere elongation. This indicates that SUMOylation-dependent anchoring of telomeres at the nuclear envelope antagonizes elongation by telomerase [172].

Telomere anchoring is essential to inhibit unwanted recombination events. Disruption of telomerase-Mps3 interaction causes hyperrecombination between short telomeres of strains lacking Tel1 kinase. Both in budding and fission yeast, telomere anchoring plays a key role in meiosis. Telomere-promoted rapid meiotic prophase chromosome movements physically move the chromatin and this rapid movement helps homology searching by

increasing the interaction between homologous and heterologous chromosomes [173].

Unlike yeast, telomeres of mammalian cells are randomly positioned throughout the nucleus [174]. In telomerase negative cancer cells, telomeres are highly mobile and can associate with each other. SUMOylation of yeast telomeric proteins leads to anchoring of telomeres at nuclear envelope, whereas SUMOylation of mammalian telomeric proteins triggers the formation of promyelocytic leukemia (PML) bodies around the telomeres. These PML body will be later enriched with repair and recombination factors and undergo ALT mediated telomere lengthening followed by disassembly of the PML body and release of the telomeres [175].

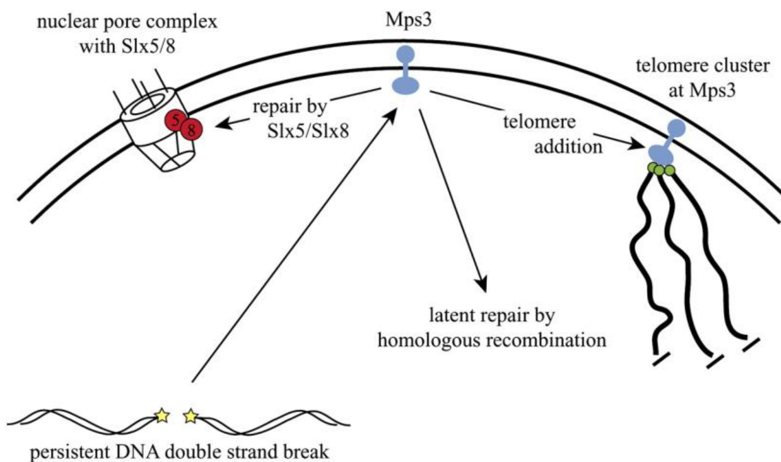

Figure 11: Slowly repaired or persistent DSB are recruited to nuclear envelop. The recruitment serves to decide if the DSB can be repaired by canonical HR or alternative methods like *de novo* telomere healing. Nuclear pore-associated Slx5/Slx8 ubiquitylates and directs the associated proteins at break site to proteosome mediated degradation to mediate efficient repair (Source: [176]).

In yeast, Irrepairable DSB and collapsed replication forks are also localized to the nuclear envelope in a Mec1/Tel1-dependent manner (See figure 11). Nuclear pore-associated Slx5/Slx8 ubiquitylates and degrades the protein associated at break site to mediate efficient repair.

If the DSB is left unrepaired, Cdc13 is recruited to the DSB and can be localized to nuclear membrane bound Est2. Cdc13 recruitment is dependent on Mre11 and Rad51-dependent manner and this binding is negatively regulated by Mec1 dependent phosphorylation of Cdc13. Cdc13-coated DSB will be be recruited to Mps3 bound telomerase complex to initiate *de novo* telomere healing process [177]. Such a repair mechanism, will lead to gross chromosomal instability and aneuploidy (or tumorigenesis in humans). It has been observed that Cdc13 can be recruited to DSB, even if the DSB is not repaired by *de novo t*elomere healing process.

Critically short telomeres or eroded telomeres like persistent DSBs are shifted to the nuclear pore for repair by telomerase independent alternative pathways [178]. So regulation of the spatial dynamics of telomeres and DSB are important for overall genome stability.

Single strand generation at DNA double strand break:

Repair of DNA double-strand breaks (DSBs) by NHEJ or HR requires processing of broken ends. In yeast, MRX along with Sae2 initiate 5’-3’ nucleolytic degradation of the DSB ends [179,180]. The resulting DNA ends are further processed by Exo1, Sgs1 helicase and Dna2 for extensive resection [181,182]. Cyclin-dependent kinase (CDK) activity plays a key role in the regulation and processing of Double strand breaks.

In yeast, G1-arrested cells can repair the DSB only by NHEJ [183], as Sae2 phosphorylation by the Clb–CDK complex is required for efficient 5' to 3' resection of the DSB ends [184,185]. Extensive resection is essential to generate long ssDNA which can be bound by RPA, Ddc2, Mec1 to completely activate checkpoint response and enable repair by recombination [182,184,186].

G1 cells lacking Ku, Lif1 or Lig4 are subjected to MRX dependent resection, whereas DSB processing in G2 is not influenced by the absence of Yku [97]. In the absence of Ku, MRX requirement is bypassed and resection is executed by Exo1 [187]. This indicates that Ku complex is a rate-limiting factor for the initiation of resection in G1 by competing with MRX and Exo1 for end binding. CDK1 phosphorylation of Dna2 drives nuclear import of Dna2 and the reduced nuclear import of Dna2 in G1 phase might be another reason for the reduced resection in *ku* mutant cells [188].

Similarly in fission yeast, MRN interacts with Ctp1 (functional ortholog of budding yeast Sae2) and promotes resection of the DSBs [189,190]. Moreover, Mre11 nuclease and Ctp1 are required to dissociate the MRN complex and the Ku70-Ku80 complex from the DSBs and to promote resection by Exo1 for efficient RPA localization [191]. In humans, CtIP (orthologous to fission yeast Ctp1) physically and functionally interacts with the MRN complex and promotes resection [192]. Very recently it was discovered that MRN-CtIP interaction is dependent upon physical interaction of MRN with CDK2 kinase [193]. *In vitro,* human Exo1 and BLM helicase physically interact and this interaction is stimulated by MRN, RPA; BLM also interacts with DNA2 to resect DNA and initiate DNA repair [194,195] and this is consistent with the *in vitro* data where BLM and hExo1 seem to act in parallel pathway to promote resection [186] (for a review, see [91]).

3' G-rich single strand overhang generation at telomeres

Replication of telomeres by leading strand machinery should lead to creation of blunt ended telomeres and so without any telomeric G tail; whereas replication by lagging strand machinery will be followed by last RNA primer removal and so Telomeric ssDNA might be present [196]. And so there must be 50% of chromosomes with overhangs. But throughout the cell cycle, in budding yeast majority of the telomeres have telomeric overhangs of 12-14bp [4] and similarly in humans, most (>80% of) telomeres have long G-rich overhangs of about 130–210 bases in length [197]. This indicates the possibility of both lagging and leading daughter telomeres with overhang structures.

In yeast cells lacking telomerase, when short linear plasmid containing telomeric DNA are introduced; the linear plasmids acquire TG_{1-3} tails on both ends of individual replicated daughter molecules [198]. Telomeric ssDNA of length 12-14bp are present throughout the cell cycle and increases to 50-100nt in late S phase, this overhang length is maintained both in the presence and absence of telomerase TLC RNA [4,199]. Based on the observation from linear plasmids and the appearance of telomeric ssDNA even in the absence of telomerase activity strengthens the idea that other mechanism might act at telomeres to generate overhangs even in leading strand. Since G-strand

overhangs serve as substrate to telomerase, they are important to maintain telomere length homeostasis.

Yeast cells acquire telomeric TG_{1-3} ss overhang in cell cycle regulated manner [4,199]. The formation of the telomeric G tail and elongation requires the passage of the replication fork at telomeres [14,200]. Cdk1 activity is required for the generation of the long telomeric 3′ overhang in late S phase [70]. Passage of replication fork at telomeres, might require the release of telomere bound proteins and lead to a transient state of telomeric deprotection. At this stage, CDK1-dependent 5′ resection might take place to generate telomeric overhangs.

Telomeres must be protected from uncontrolled nucleolytic activities. Telomere shortening caused by telomerase deletion increases the amount of telomeric ssDNA in predominantly Exo1 nuclease dependent manner [201] and triggers a DNA damage and other stress related responses [202]. In yku null cells, telomeres are shorter with accumulation of telomeric ssDNA and checkpoint-mediated cell cycle arrest at elevated temperatures. The generation of telomeric ssDNA in *yku70Δ* occurs in cell cycle independent manner and due to action of Exo1 nuclease at telomeres [124,131,203]. In *cdc13-1* mutant, telomeres undergo Exo1 dependent 5′ C strand degradation and cell cycle arrest at restrictive temperature [31,204]. In contrast to yku lacking cells, *cdc13-1* mutant or cells lacking *CDC13* or *STN1*, the overhang generation is cell cycle dependent occurring only in G2/M, but not in G1 of the cell cycle and requires the completion of S phase and Cdk1 kinase activity [205]. This observation supports the hypothesis that requirement of replication fork passage might lead to transient unprotected state at telomeres. Also, normal human telomeres are recognized as DNA damage in G2 phase of cell cycle [206].

At *de novo* telomeres, MRX complex is involved in telomeric ss Gtail generation at telomeres [138]. MRX is required for generation of proper constitutive telomeric G-tails in linear plasmids [135]. Previous work from our lab discovered that similar to DSB processing, Sae2, Exo1, Sgs1, Dna2 are all involved in generation of telomeric overhangs (See figure 12) [207]. Again, extensive resection of DSB requiring Cdk1 phosphorylation of Sae2, it was also important for telomere overhang formation and telomere elongation. A very interesting finding from this work came from Sae2-S267D variant mimicking

constitutive phosphorylation which did not totally bypass the need of Cdk1 for single-stranded telomeric DNA generation. This hinted that additional Cdk1 targets might be involved in positive or negative regulators of telomere degradation [207].

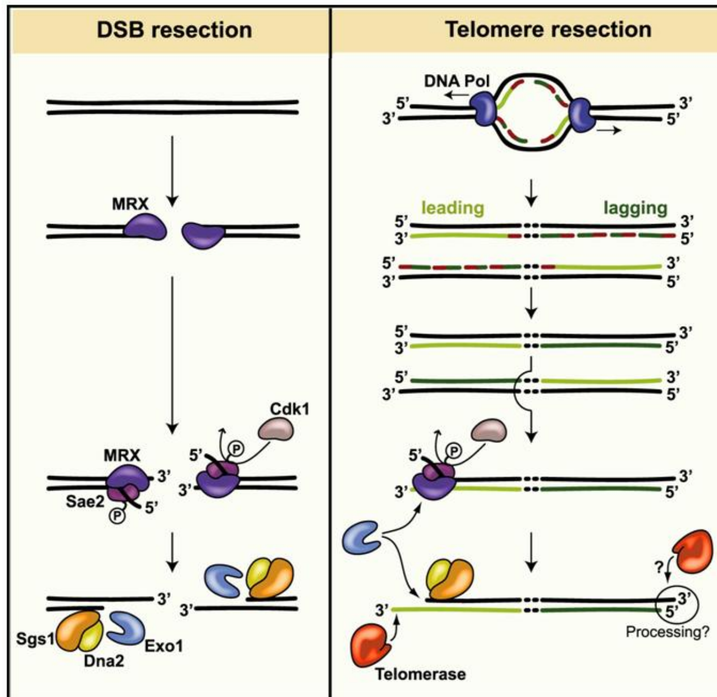

Figure 12: Similar mechanisms are involved in DNA End Processing at DSBs and Telomeres. Upon phosphorylation of Sae2 by Cdk1, MRX and Sae2 trigger initial resection. Extended overhangs are generated by Sgs1-Dna2. Exo1 can also contribute to overhang formation. Telomere shortening occurs at the leading strand telomere due to end processing. (Source: [208])

As mentioned before, Ku and Cdc13 are important for telomere protection from degradation and lack of Rap1 or Rif2 lead to telomere-telomere fusion due to NHEJ. However, the precise roles of telomere binding proteins in telomere protection at molecular level were still unknown. This Ph.D thesis was aimed at studying how yeast telomeric proteins might protect telomeres from degradation using *de novo* telomere assay and native *in gel* hybridization techniques in different cell cycle phases.

Results

Shelterin-Like Proteins and Yku Inhibit Nucleolytic Processing of *Saccharomyces cerevisiae* Telomeres

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Abstract

Eukaryotic cells distinguish their chromosome ends from accidental DNA double-strand breaks (DSBs) by packaging them into protective structures called telomeres that prevent DNA repair/recombination activities. Here we investigate the role of key telomeric proteins in protecting budding yeast telomeres from degradation. We show that the *Saccharomyces cerevisiae* shelterin-like proteins Rif1, Rif2, and Rap1 inhibit nucleolytic processing at both *de novo* and native telomeres during G1 and G2 cell cycle phases, with Rif2 and Rap1 showing the strongest effects. Also Yku prevents telomere resection in G1, independently of its role in non-homologous end joining. Yku and the shelterin-like proteins have additive effects in inhibiting DNA degradation at G1 *de novo* telomeres, where Yku plays the major role in preventing initiation, whereas Rif1, Rif2, and Rap1 act primarily by limiting extensive resection. In fact, exonucleolytic degradation of a *de novo* telomere is more efficient in *yku70Δ* than in *rif2Δ* G1 cells, but generation of ssDNA in Yku-lacking cells is limited to DNA regions close to the telomere tip. This limited processing is due to the inhibitory action of Rap1, Rif1, and Rif2, as their inactivation allows extensive telomere resection not only in wild-type but also in *yku70Δ* G1 cells. Finally, Rap1 and Rif2 prevent telomere degradation by inhibiting MRX access to telomeres, which are also protected from the Exo1 nuclease by Yku. Thus, chromosome end degradation is controlled by telomeric proteins that specifically inhibit the action of different nucleases.

Author Summary

Telomeres are specialized nucleoprotein complexes that distinguish the natural ends of linear chromosomes from intrachromosomal double-strand breaks. In fact, telomeres are protected from DNA damage checkpoints, homologous recombination, or end-to-end fusions that normally promote repair of intrachromosomal DNA breaks. When chromosome end protection fails, dysfunctional telomeres are targeted by the DNA repair and recombination apparatus, whose outcomes range from the generation of chromosomal abnormalities, general hallmarks for human cancer cells, to permanent cell cycle arrest and cell death. While several studies address the consequences of telomere dysfunctions, the mechanisms by which telomere protection is achieved remain to be determined. Here, we investigate this issue by analyzing the role of evolutionarily conserved telomeric proteins in

protecting budding yeast telomeres from degradation. We demonstrate that the key telomeric proteins Yku, Rap1, Rif1, and Rif2 inhibit telomere degradation by specifically preventing the action of different nucleases. As these proteins are functionally conserved between budding yeast and mammalian cells, they might also play critical roles in preventing telomere degradation in humans.

Introduction

Intrachromosomal double-strand breaks (DSBs) elicit a DNA damage response, which comprises DNA repair pathways and surveillance mechanisms called DNA damage checkpoints. By contrast, telomeres are by definition stable and inert natural ends of linear chromosomes, as they are protected from checkpoints, as well as from homologous recombination (HR) or end-to-end fusions that normally promote repair of intrachromosomal DSBs (reviewed in [1]). Telomere basic structure is conserved among eukaryotes and consists of short tandem DNA repeats, which are G-rich in the strand containing the 3′ end (G-strand).

Although telomere ends are apparently shielded from being recognized as DSBs, they share important similarities with intrachromosomal DSBs. In fact, DSBs are resected to generate 3′-ended single-stranded DNA (ssDNA) tails, which channel their repair into HR. Similarly, the tips of human, mouse, ciliate, yeast and plant telomeres terminate with 3′ overhangs due to the protrusion of the G-strand over its complementary C-strand. Furthermore, several proteins such as the MRX complex, Sae2, Sgs1, Exo1 and Dna2 are required for generation of ssDNA at both telomeres and intrachromosomal DSBs, with Sae2 and MRX belonging to the same pathway, while the helicase Sgs1 acts in conjunction with the nuclease Dna2 [2-4]. Finally, both DSB and telomere resection is promoted by the activity of cyclin-dependent protein kinase Cdk1 [5-7], which phosphorylates Sae2 Ser267 [4] .

It is well known that ssDNA accumulation at DSBs invokes an ATR/Mec1-dependent DNA damage response when it exceeds a certain threshold [9]. Noteworthy, the single-stranded G-tails of budding yeast telomeres are short (about 10–15 nucleotides) for most of the cell cycle, and their length increases transiently at the time of telomere replication in late S phase [10]. As the nuclease requirements at DSBs and telomeres are similar [4], this finding

suggests an inherent resistance of telomeric ends to exonuclease attack, which could contribute to avoid telomeres from being sensed as DNA damage. One report suggests that an elongating telomere formed at a TG-flanked DSB actually exerts an “anticheckpoint” effect on the non-TG-containing side of the break [11], though the origin of this checkpoint attenuation has been questioned [12].

In budding yeast, telomere protection is achieved through single- and double-stranded DNA binding proteins. In particular, the heterodimeric Yku complex (Yku70-Yku80) contributes to protect telomeres, as Yku lack causes shortened telomeres and Exo1-dependent accumulation of telomeric ssDNA [13-16], as well as checkpoint-mediated cell cycle arrest at elevated temperatures [15-17]. Furthermore, Cdc13 inactivation leads to C-rich strand degradation, with subsequent accumulation of long ssDNA regions that extend into non-telomeric sequences [18-20]. Finally, the Rap1 protein, together with its interactors Rif1 and Rif2, binds telomeric double-stranded DNA repeats and inhibits both telomere fusions by non-homologous end joining (NHEJ) [21] and telomerase-dependent telomere elongation [22-23]. The Rap1 C-terminal domain is sufficient for interaction with Rif1 and Rif2 [24-26] and is responsible for Rap1-mediated inhibition of both NHEJ and telomere elongation. In fact, deletion of Rap1 C-terminus causes both NHEJ-dependent telomeric fusions, due to the lack of Rif2 and Sir4 at telomeres [21], and an increase in telomere length, which is similar to the one observed when both Rif1 and Rif2 are lacking [26].

Proteins negatively regulating telomerase and NHEJ are found at telomeres also in other eukaryotes, such as fission yeast [27] and mammals, where they form a complex called shelterin that functionally recapitulates the Rap1-Rif1-Rif2 complex (reviewed in [28]).

Several studies address the consequences of telomere dysfunctions, while the mechanisms by which telomere protection is achieved remain to be determined. Here, we investigate this issue by analyzing the role of key telomeric proteins in protecting budding yeast telomeres from degradation. By using an inducible short telomere assay, we show that loss of Rif1 or Rif2, as well as deletion of Rap1 C-terminus, promotes C-rich strand degradation at an HO-derived telomere in G1 and enhances it in G2. The lack of Rap1 C-terminus

or Rif2 shows the strongest effect at the induced short telomere and also causes ssDNA accumulation at native telomeres in cycling cells.

Moreover, Yku prevents telomere resection in G1 at both native and HO-induced telomeres independently of its role in NHEJ. Resection of the HO-induced telomere in G1-arrested *yku70Δ* cells is restricted to the DNA regions closest to the telomeric tips, likely due to the action of Rap1, Rif1 and Rif2, whose inactivation extends telomere processing in *yku70Δ* G1 cells. Finally, ssDNA generation at both native and HO-induced telomeres requires Exo1 in *yku70Δ* G1 cells, whereas it depends primarily on MRX in both *rap1ΔC and rif2Δ* cells, where recruitment of the MRX subunit Mre11 to the HO-induced telomere is enhanced.

Thus, while Yku protects telomeres from Exo1 action, the shelterin-like proteins prevent telomere degradation by inhibiting MRX loading onto telomeric ends.

Results

Rap1, Rif1, and Rif2 inhibit 3′ single-stranded overhang generation at a *de novo* telomere in both G1 and G2

Nucleolytic degradation of telomeric ends is inhibited in G1, when Cdk1 (Cdc28/Clb in yeast) activity is low, whereas it occurs in G2/M cells, where Cdk1 activity is high [6], [7]. We investigated whether the shelterin-like proteins Rif1, Rif2 and Rap1 regulated 3′ overhang generation at *Saccharomyces cerevisiae* telomeres by examining the effects of their inactivation on telomeric ssDNA formation in both G1 and G2. We used an inducible short telomere assay (Figure 1A) [11], [29] that allows generation of a single short telomere without affecting the length of the other telomeres in the same cell. In this system, galactose-induced HO endonuclease generates a single DSB at an HO cleavage site adjacent to an 81-base pair TG repeat sequence that is inserted at the *ADH4* locus, 15 kb from the left telomere of chromosome VII (Figure 1A). After HO galactose-induction, the fragment distal to the break is lost, and, over time, the short telomeric “seed” sequence is elongated by telomerase[11],[29]. Length changes of either the 5′ C-strand or the 3′ G-strand of the newly created HO-induced telomere can be followed by using two single-stranded riboprobes (probes A and B in Figure 1) that detect

the 5′ C-strand or the 3′ G-strand, respectively, by hybridizing to a DNA region spanning 212 bp from the HO site (Figure 1A).

HO was induced by galactose addition in G1-arrested *rap1ΔC*, *rif1Δ* and *rif2Δ* cells (Figure 1B), the latter lacking the Rap1 C-terminus (residues 670–807) that is sufficient for both telomere length regulation and Rap1 interaction with Rif1 and Rif2 [25-26]. When the 5′ C-strand was analyzed with its complementary probe A in EcoRV and RsaI double-digested genomic DNA (Figure 1C), the predicted EcoRV-HO band (166 bp; cut C-strand) corresponding to the 5′ C-rich strand of the HO-induced telomere was detected in all cell cultures about 2 hours after HO induction.

Consistent with the requirement of Cdk1 activity for telomere resection [6], [7], the C-strand signal was stable in G1-arrested wild type cells (Figure 1C and 1D). By contrast, it progressively decreased in both *rap1ΔC and rif2Δ* G1-arrested cells (Figure 1C and 1D), indicating that C-strand resection in these two mutants had proceeded beyond the hybridization region. C-strand degradation at the HO-derived telomere occurred also in *rif1Δ* cells, although less efficiently than in *rap1ΔC and rif2Δ* cells (Figure 1C and 1D). The decrease of single-stranded 5′ C-strand signal in all these mutants was due to DNA degradation and not to elongation by the coordinated action of telomerase and lagging strand DNA synthesis, as we observed a similar decrease also in *rif1Δ*, *rap1ΔC and rif2Δ* G1 cells lacking the catalytic subunit of telomerase (data not shown).

The 3′ G-strand of the HO-induced telomere was analyzed in the same DNA samples by using the G-strand complementary probe B (Figure 1E and 1F). Because EcoRV and RsaI do not cleave ssDNA, the 166 nt EcoRV-HO 3′ G-strand fragment is converted into slower migrating r1 and r2 DNA fragments as 5′ to 3′ resection proceeds beyond the EcoRV up to the two RsaI restriction sites located 304 and 346 bp, respectively, from the HO cutting site (Figure 1A). The amount of the predicted EcoRV-HO fragment (cut G-strand), which was constant in G1-arrested wild type cells, decreased over time in *rif1Δ*, *rap1ΔC* and *rif2Δ* cells that also showed r1 3′-ended resection products (Figure 1E and 1F), indicating that resection had proceeded beyond the EcoRV site towards the RsaI site located 304 bp from the HO cut. Again, the amount of the resection products was higher in *rap1ΔC and rif2Δ* cells

than in *rif1Δ* cells (Figure 1E and 1F), indicating a stronger role for Rap1 and Rif2 in protecting telomeres from degradation in G1.

Figure1: Rap1, Rif1, and Rif2 inhibit resection at a de novo telomere in G1.

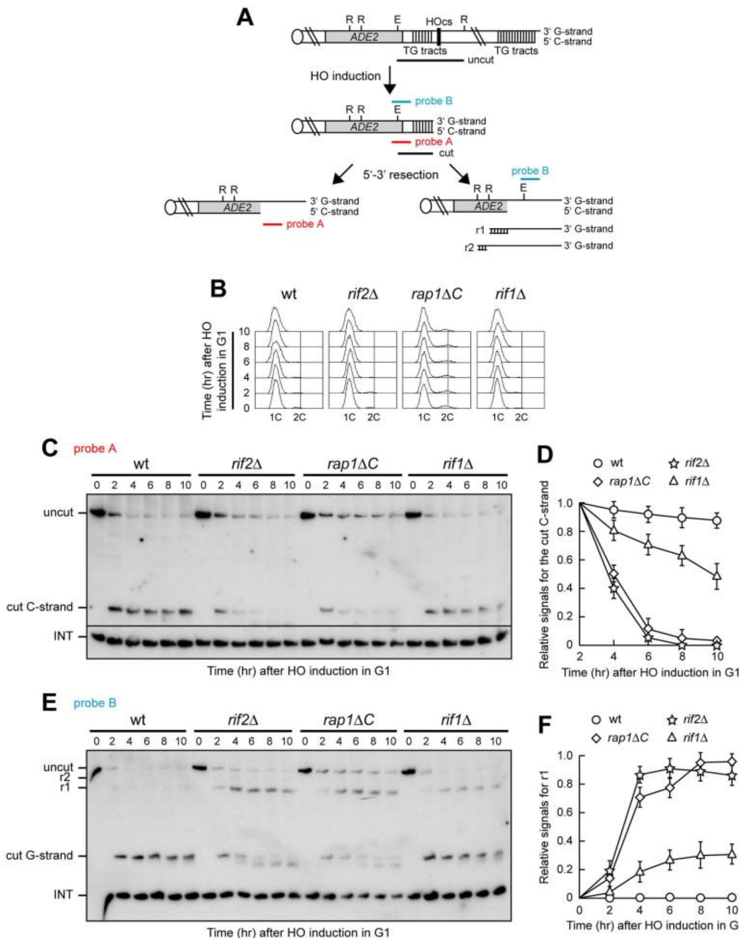

Figure 1: (A) The HO-induced telomere system. Galactose-induced HO endonuclease generates a single DSB at an HO cleavage site (HOcs) adjacent to an 81-bp TG repeat sequence (TG tracts) that is inserted at the ADH4 locus on chromosome VII. RsaI- and EcoRV-digested genomic DNA was hybridized with two single-stranded riboprobes, which anneal to either the 5′ C-strand (probe A) or the 3′ G-strand (probe B) to a site located 212 bp from the HO cutting site. Both probes reveal an uncut 390 nt DNA fragment (uncut), which is converted by HO cleavage into a 166 nt fragment (cut) that can be detected by both probe A (5′ C-strand) and probe B (3′ G-strand). Degradation of the 5′ C-strand leads to disappearance of the probe A signal as resection proceeds beyond the hybridization region. Furthermore, it eliminates the cutting sites for the EcoRV (E) and RsaI (R) restriction enzymes, thus converting the 3′ cut G-strand into

longer r1 (304 nt) and r2 (346 nt) DNA fragments detected by probe B. Both probes also detects a 138 nt fragment from the *ade2*-101 locus on Chr. XV (INT), which serves as internal loading control. (B–F) HO expression was induced at time zero by galactose addition to α-factor-arrested wild type (YLL2599) and otherwise isogenic *rif2Δ*, *rap1ΔC* and *rif1Δ* cell cultures that were then kept arrested in G1. (B) FACS analysis of DNA content. (C) RsaI- and EcoRV-digested genomic DNA was hybridized with probe A. Degradation of the 5′ C-strand leads to the disappearance of the 166 nt signal (cut C-strand) generated by this probe. (D) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (C). (E) The same RsaI- and EcoRV-digested genomic DNA analyzed in (C) was hybridized with probe B. Degradation of the 5′ C-strand leads to the conversion of the 3′ cut G-strand 166 nt fragment into the slower migrating r1 DNA fragment described in (A). (F) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (E).

Consistent with previous observations [29], 3′ G-strand length of the HO-induced telomere decreased by ~10 nucleotides in both *rap1ΔC and rif2Δ* cells (Figure 1E). This very limited G-strand degradation was not specifically caused by the lack of Rif2 or Rap1, as it was detectable after HO induction also in G2-arrested wild type cells undergoing telomere resection (Figure 2B). A similar phenomenon has been described at intrachromosomal DSBs, where both the 5′ and the 3′ strands disappear with time in wild type cells after HO cleavage, and the 5′ strand is processed faster than the 3′ strand [9].

Telomere protection by the shelterin-like proteins occurred also outside G1, as shown by the analysis of 3′ single-stranded G-tail generation at the HO-induced telomere in G2-arrested *rif1Δ*, *rap1ΔC* and *rif2Δ* cells (Figure 2). As expected, r1 resection products were detectable in G2-arrested wild type cells, but their amount in these cells was significantly lower than in *rif2Δ*, *rap1ΔC* and *rif1Δ* cells (Figure 2B and 2C). Both *rap1ΔC and rif2Δ* G2 cells showed also some r2 resection products (Figure 2B), indicating that they allowed resection to proceed beyond the first RsaI site. The ~10 nucleotides decrease in length of the 3′ G-strand occurring in G2-arrested wild type cells was not detectable in *rap1ΔC and rif2Δ* G2 cells (Figure 2B), likely because the 3′ G-strand in these two mutants was converted into longer r1 resection products much more efficiently than in wild type cells. Thus, Rif1, Rif2 and Rap1 inhibit degradation of the HO-induced telomere in both G1 and G2, with Rif2 and Rap1 playing the major role.

Figure 2. Rap1, Rif1, and Rif2 inhibit resection at a de novo telomere in G2.

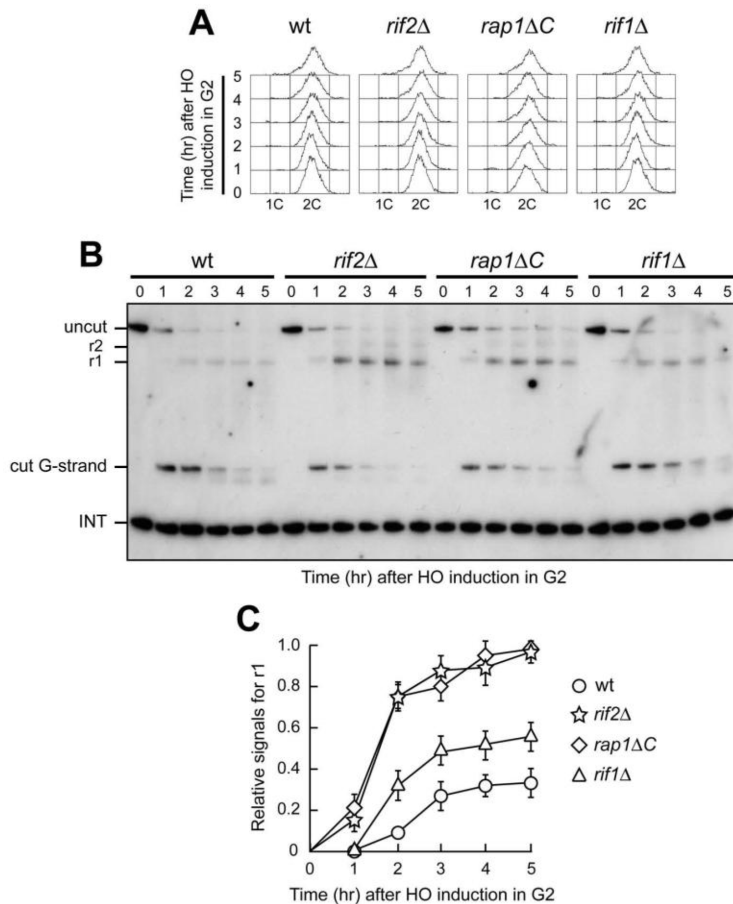

Figure 2: HO expression was induced at time zero by galactose addition to nocodazole-arrested wild type (YLL2599) and otherwise isogenic *rif2Δ*, *rap1ΔC* and *rif1Δ* cell cultures that were then kept arrested in G2. (A) FACS analysis of DNA content. (B) RsaI- and EcoRV-digested genomic DNA was hybridized with probe B as in Figure 1E. (C) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (B).

Yku inhibits 3′ single-stranded overhang generation at a *de novo* telomere in G1

Yku lack accelerates 5′-to-3′ nucleolytic degradation of intrachromosomal DSBs in yeast cells with low Cdk1 activity [30]. This effect is partially due to NHEJ defects that might increase the time available to the resection machinery, as DSB processing is also increased in G1-arrested cells lacking the NHEJ DNA ligase IV (Dnl4/Lig4), although to a lesser extent than in *yku70Δ* cells [30].

We investigated the possible role of Yku and/or Dnl4 in preventing telomere resection by analyzing the effect of their loss on the kinetics of 5′ C-strand degradation at the HO-induced telomere in both G1 and G2. We also evaluated how the lack of Yku and Dnl4 influenced 5′-strand degradation at an HO-induced DSB lacking the terminal TG repeats (Figure 3G) [11], in order to highlight possible differences in the regulation of DNA degradation at DSBs versus telomeres. Similar to what was found at intrachromosomal DSBs [30], Yku absence did not enhance processing of the HO-induced telomere in G2, as G2-arrested wild type and *yku70Δ* cells (Figure 3A) displayed very similar kinetics of 5′ C-strand degradation (Figure 3B and 3C). By contrast, the amount of 5′ C-strand of the HO-induced telomere decreased in G1-arrested *yku70Δ* cells, while it remained constant in both wild type and *dnl4Δ* cells under the same conditions (Figure 3D–3F). As expected [30], the 5′-strand at the HO-induced DSB lacking the TG repeats (Figure 3G) was degraded much more efficiently in both G1-arrested *yku70Δ* and *dnl4Δ* cells than in wild type, with *yku70Δ* cells showing the strongest effect (Figure 3H–3L). Thus, Dnl4 does not block telomere resection in G1, whereas Yku does, indicating that the role of Yku in telomere protection is not related to its NHEJ function. This finding also highlights differences in the regulation of nucleolytic processing at DSBs versus telomeres.

Figure 3: Yku inhibits resection at a de novo telomere specifically in G1.

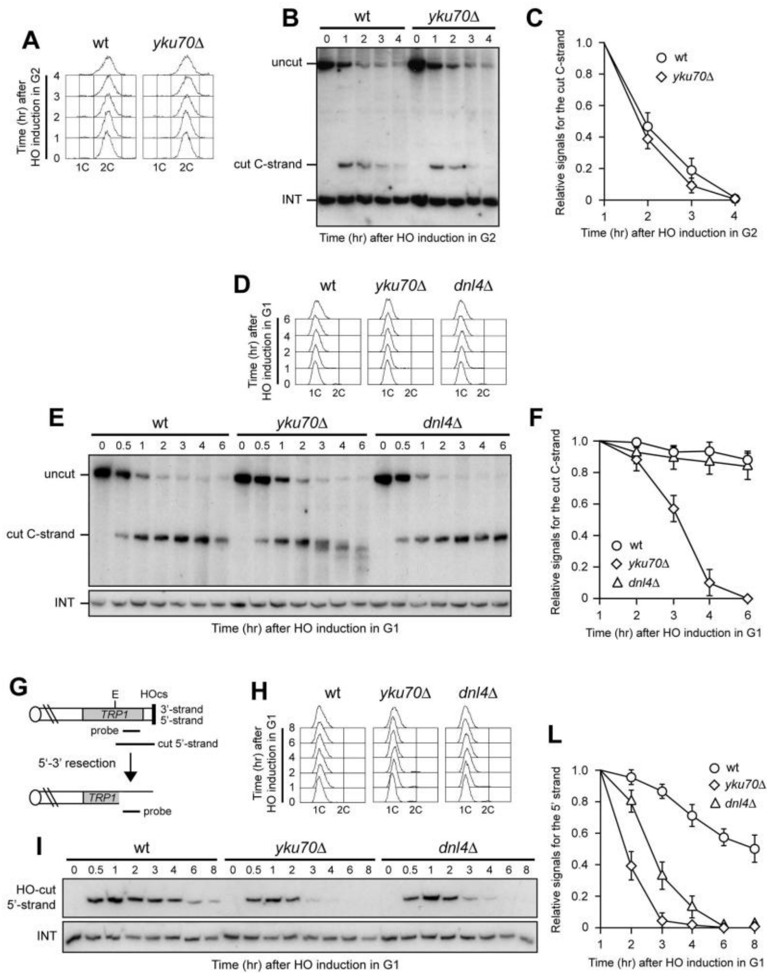

Figure 3: (A–C) HO expression was induced at time zero by galactose addition to nocodazole-arrested wild type (YLL2599) and otherwise isogenic *yku70Δ* cell cultures that were then kept arrested in G2. (A) FACS analysis of DNA content. (B) RsaI- and EcoRV-digested genomic DNA was hybridized with probe A as described in Figure 1C. (C) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (B). (D–F) HO expression was induced at time zero by galactose addition to α-factor-arrested wild type (YLL2599) and otherwise isogenic *yku70Δ* and *dnl4Δ*cell cultures that were then kept arrested in G1. (D) FACS analysis of DNA content.

(E) RsaI-digested genomic DNA was hybridized with the single-stranded riboprobe A described in Figure 1A, which anneals to the 5′ C-strand and reveals an uncut 460 nt DNA fragment (uncut). After HO cleavage, this fragment is converted into a 304 nt fragment (cut) detected by the same probe (cut C-strand). (F) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (E). (G) The system used to generate an HO-induced DSB. Hybridization of EcoRV-digested genomic DNA with a probe that anneals to the 5′ strand to a site located 215 nt from the HO cutting site reveals a 430 nt HO-cut 5′-strand fragment. Loss of the 5′ strand beyond the hybridization region leads to disappearance of the signal generated by the probe. (H–L) HO expression was induced at time zero by galactose addition to α-factor-arrested wild type (YLL2600) and otherwise isogenic *yku70Δ* and *dnl4Δ* cells, all carrying the system in (G). Cells were then kept arrested in G1. (H) FACS analysis of DNA content. (I) EcoRV-digested genomic DNA was hybridized with the probe indicated in (G). The INT band, corresponding to a chromosome IV sequence, serves as internal loading control. (L) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (I).

Rif1, Rif2, and Rap1 limit resection at a *de novo* telomere in *yku70Δ* G1 cells

Interestingly, G1-arrested *yku70Δ* cells converted the 5′ C-strand fragment of the HO-induced telomere into discrete smaller DNA fragments (Figure 3E), suggesting that C-strand degradation under these conditions is limited to the terminal part. In order to confirm this observation, we monitored the 3′ G-strand of the HO-induced telomere in *yku70Δ* cells. As shown in Figure 4A and 4B, the 3′ cut G-strand was not converted into the longer resection products r1 and r2 in G1-arrested *yku70Δ* cells. Therefore, exonucleolytic degradation did not proceed beyond the EcoRV site located 166 bp from the HO site.

Thus, other proteins might limit resection of the HO-induced telomere in G1 even in the absence of Yku70, and the shelterin-like proteins appear to exert this effect. In fact, 3′-ended r1 resection products were clearly detectable in G1-arrested *yku70Δ rif2Δ*, *yku70Δ rap1ΔC* and, although to a lesser extent, *yku70Δ rif1Δ* cells (Figure 4A and 4B). Furthermore, the smaller C-strand fragments that accumulated in G1-arrested *yku70Δ* cells were only slightly detectable in similarly treated *yku70Δ rif2Δ*(Figure 4C) and *yku70Δ rap1ΔC* cells (data not shown), indicating that 5′ C-strand degradation in these cells had proceeded beyond 166 bp from the HO site. Thus, Rap1, Rif2 and, to a lesser extent, Rif1 limit telomeric ssDNA generation in G1 cells lacking Yku.

Figure 4. Rif2 and Rap1 inactivation enhances resection at a de novo telomere in yku70Δ cells.

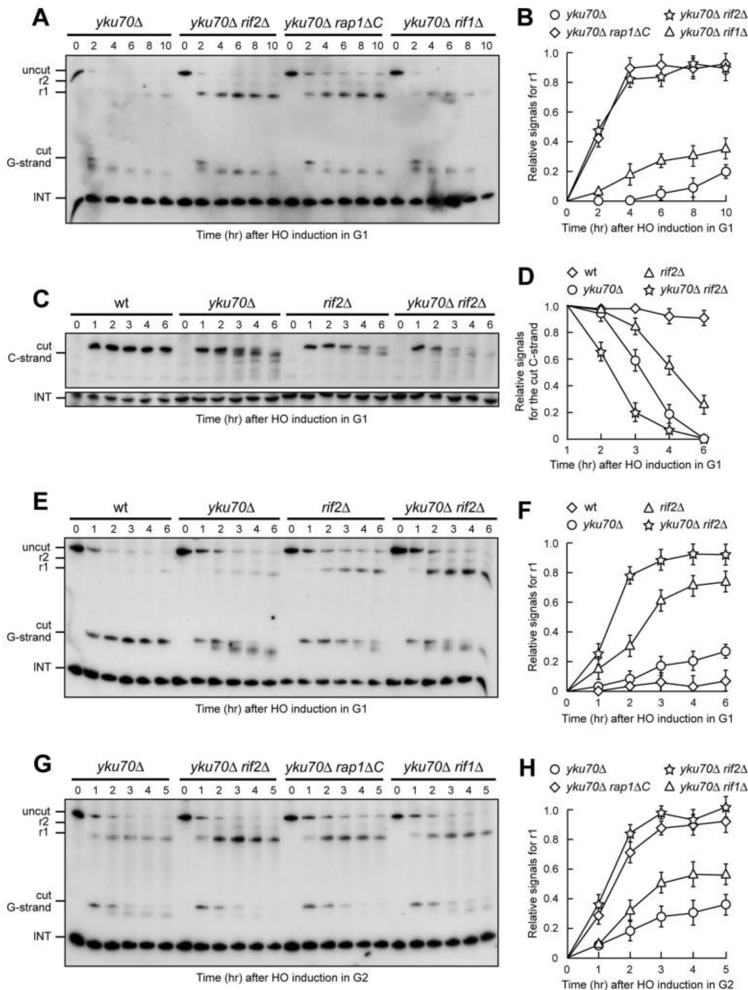

Figure 4 : (A–F) HO expression was induced at time zero by galactose addition to α-factor-arrested cells with the indicated genotypes that were then kept arrested in G1. (A) RsaI- and EcoRV-digested genomic DNA was hybridized with probe B as in Figure 1E. (B) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (A). (C) RsaI-digested genomic DNA was hybridized with probe A as in Figure 3E. (D) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (C). (E) RsaI- and EcoRV-digested genomic DNA was hybridized with probe B as in Figure 1E. (F) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (E). (G,H) HO expression was induced at time zero by galactose addition to nocodazole-arrested cells with the indicated genotypes that were then kept arrested in G2.

(G) RsaI- and EcoRV-digested genomic DNA was hybridized with probe B as in Figure 1E. (H) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (G).

Notably, although telomere resection in *yku70Δ* G1 cells was confined to the telomere tip, the 166 nt 5′ C-strand signal decreased faster in *yku70Δ* than in *rif2Δ* G1 cells (Figure 4C and 4D). Furthermore, the ~10 nucleotides decrease in length of the 3′ G-strand was more efficient in *yku70Δ* than in *rif2Δ* G1-arrested cells (Figure 4E). Thus, more resection events are initiated at G1 telomeres in the absence of Yku than in the absence of Rif2. These findings, together with the observation that the shelterin-like proteins still inhibit extensive resection in Yku-lacking cells, suggest that Yku has a major role in preventing initiation of telomere processing, while the shelterin-like proteins are primarily responsible for limiting extensive resection. Accordingly, the concomitant lack of Yku70 and Rif2 showed additive effects on *de novo* telomere degradation in G1. In fact, both C-strand degradation (Figure 4C and 4D) and generation of r1 resection products (Figure 4E and 4F) occurred more efficiently in G1-arrested *yku70Δ rif2Δ* double mutant cells than in either *yku70Δ* or *rif2Δ* single mutants.

Similar to what we observed after inactivation of Rap1, Rif1 or Rif2 in G2 cells with functional Yku (Figure 2), r1 amounts were higher in *yku70Δ rif2Δ*, *yku70Δ rap1C* and *yku70Δ rif1Δ* cells than in*yku70Δ* single mutant cells after galactose addition in G2 (Figure 4G and 4H), indicating that Rap1, Rif2 and Rif1 inactivation promotes telomere processing in G2 also in the absence of Yku70. The finding that the r1 resection products accumulated with similar kinetics in G2-arrested wild type (Figure 2C) and *yku70Δ* single mutant cells (Figure 4H) further confirms that Yku70 loss does not affect telomere resection in G2.

Yku70, Rif2, and Rap1 inhibit G-strand overhang generation at native telomeres

The above findings prompted us to investigate whether the key role of Yku, Rif2 and Rap1 in preventing ssDNA generation at *de novo* telomeres could be extended to native telomeres. As Yku inhibits HO-induced telomere processing specifically in G1, we asked whether Yku70 loss could cause deprotection of native telomeres in G1 cells. To this end, we took advantage of previous data [15] showing that incubation at 37°C of *yku70Δ* cells causes checkpoint-

dependent cell cycle arrest and accumulation of telomeric ssDNA as measured by ssDNA quantitative amplification (QAOS). Thus, we incubated G1-arrested wild type and *yku70Δ* cells at either 23°C or 37°C for 4 hours in the presence of α-factor (Figure 5B). Genomic DNA was then analyzed by non-denaturing in gel hybridization with a C-rich radiolabeled oligonucleotide detecting the G-rich single-stranded telomere overhangs [31]. As expected, no telomeric ssDNA signals were detectable in G1-arrested wild type cells at either 23°C or 37°C (Figure 5A). In contrast, single-stranded G tail signals appeared in G1-arrested *yku70Δ* cells even at 23°C, and their intensity increased after incubation at 37°C (Figure 5A), thus highlighting an important role of Yku in protecting native telomeres in G1.

Also Rif2 and Rap1 turned out to inhibit exonucleolytic degradation at native telomeres (Figure 5C). Their role in this process was analyzed in cycling cells, because both proteins protect the HO-induced telomere from degradation in both G1 and G2 cells (Figure 1 and Figure 2). Single-stranded G tails were not detectable in wild type cycling cells, whereas they accumulated in both *rap1ΔC and rif2Δ* cells, which showed longer native telomeres than wild type, as expected (Figure 5C).

It is well known that a Mec1-dependent DNA damage response is invoked when accumulation of ssDNA at DSBs reaches a certain threshold [9]. We found that G1-arrested *yku70Δ* cells incubated at either 23°C or 37°C in the presence of α-factor did not show Rad53 electrophoretic mobility shifts that signal Mec1-dependent Rad53 phosphorylation and subsequent checkpoint activation (Figure 5D). Thus, Yku inactivation in G1 does not cause checkpoint activation. By contrast, and consistent with previous data (15), Rad53 phosphorylation was induced when exponentially growing *yku70Δ* cells were incubated at 37°C (Figure 5D).

We did not observe Rad53 phosphorylation even when G1-arrested *rap1ΔC and rif2Δ* cells were released into the cell cycle (Figure 5E), although they accumulated higher amounts of telomeric ssDNA at the HO-induced telomere than *yku70Δ* G1 cells. This lack of checkpoint activation might be due to either limited C-strand resection or general inability to phosphorylate Rad53. We then combined the *rif2Δ* allele with the temperature sensitive *cdc13-1* allele, which is well known to cause C-rich strand degradation and activation of the DNA damage checkpoint after incubation at 37°C [18]–[20] (Figure 5F).

Figure 5. Analysis of single-stranded overhangs at native telomeres.

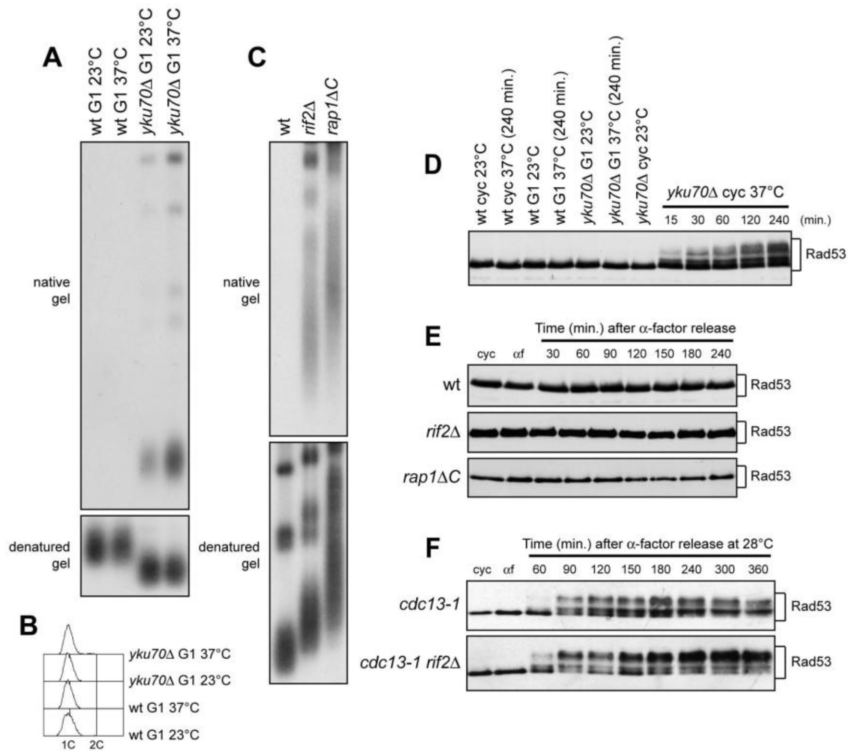

Figure 5 : (A,B) G1-arrested (G1) wild type (YLL2599) and otherwise isogenic *yku70Δ* cell cultures were incubated at either 23°C or 37°C for 4 hours in the presence of α-factor. (A) Genomic DNA was digested with XhoI and single-stranded telomere overhangs were visualized by in-gel hybridization (native gel) using an end-labeled C-rich oligonucleotide [31]. The same DNA samples were separated on a 0.8% agarose gel, denatured and hybridized with the end-labeled C-rich oligonucleotide for loading and telomere length control (denatured gel). (B) FACS analysis of DNA content. (C) Genomic DNA prepared from wild type (YLL2599) and otherwise isogenic *rap1ΔC and rif2Δ* cell cultures, exponentially growing at 25°C, was digested with XhoI and the single-stranded telomere overhangs were visualized by in-gel hybridization as in (A). (D) Wild type (YLL2599) and otherwise isogenic *yku70Δ* cell cultures exponentially growing (cyc) at 23°C were incubated at 37°C for the indicated time points. G1-arrested wild type and *yku70Δ* cells (G1) were incubated at either 23°C or 37°C for 4 hours. Rad53 was visualized at the indicated times by western analysis with anti-Rad53 antibodies. (E) α-factor arrested wild type (YLL2599) and otherwise isogenic *rap1ΔC and rif2Δ* cell cultures were released into the cell cycle at 25°C. Rad53 was visualized as in (D). (F) α-factor-arrested *cdc13-1* and *cdc13-1 rif2Δ* cells were released into the cell cycle at 28°C. Rad53 was visualized as in (D).

When G1-arrested *cdc13-1 rif2Δ* cells were released into the cell cycle at 28°C (semi-permissive temperature for *cdc13-1*), they showed a higher amount of phosphorylated Rad53 than similarly treated *cdc13-1* single mutant cells (Figure 5F). Thus, loss of Rif2 (and possibly of Rap1) enhances the checkpoint response in the presence of partially unprotected telomeres, suggesting that the amount of telomeric ssDNA formation caused by the lack of shelterin-like proteins does not reach the threshold level for the checkpoint response.

Different nucleases are required for telomeric ssDNA generation in the absence of Yku or shelterin-like proteins

As the *yku70Δ*, *rap1ΔC and rif2Δ* alleles increased ssDNA generation at native telomeres, we asked which nucleolytic activities were involved in this process. The nuclease Exo1 turned out to be required in both cycling and G1-arrested *yku70Δ* cells. In fact, ssDNA at native telomeres was undetectable in DNA samples prepared from either G1-arrested or exponentially growing *yku70Δ mre11Δ* double mutant cells incubated at 37°C for 4 hours (Figure 6A and 6B). Under the same conditions, MRE11 deletion only slightly suppressed accumulation of telomeric ssDNA in G1-arrested *yku70Δ* cells (Figure 6A), and did not significantly influence it in cycling *yku70Δ* cells (Figure 6B), indicating that ssDNA generation at native telomeres in the absence of Yku depends primarily on Exo1.

Mre11 was instead required at *rap1ΔC and rif2Δ* native telomeres to generate G-rich ssDNA, which was almost completely absent in both *rif2Δ mre11Δ* (Figure 6C) and *rap1ΔC mre11Δ* cycling cells (Figure 6D). By contrast, EXO1 deletion did not affect the same process in *rap1ΔC and rif2Δ* cycling cells (Figure 6C and 6D). Thus, native telomere nucleolytic degradation that is normally inhibited by Rif2 and Rap1 is mainly Mre11-dependent.

The absence of Mre11 leads to telomere shortening in *yku70Δ*, *rap1ΔC and rif2Δ* cells (Figure 6), likely because it prevents loading of the Tel1 kinase, which in turn allows recruitment of the Est1 telomerase subunit by phosphorylating Cdc13 [32]–[34]. In order to rule out possible artefacts caused by telomere structure alterations, we analyzed the effects of the *mre11Δ* and *mre11Δ* alleles also at the newly created HO-induced telomere in G1 cells that cannot elongate this telomere due to the low Cdk1 activity.

Figure 6 Nuclease requirements for ssDNA generation at native telomeres.

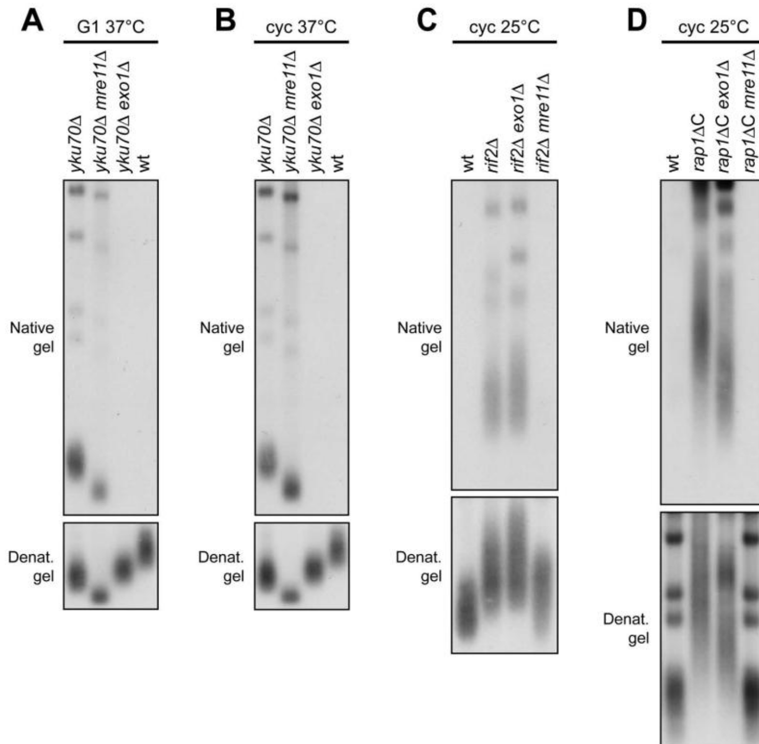

Figure 6: (A) G1-arrested cells were incubated at 37°C for 4 hours in the presence of α-factor. Genomic DNA was analyzed as in Figure 5A. (B) Exponentially growing cells were incubated at 37°C for 4 hours. Genomic DNA was analyzed as in Figure 5A. (C,D) Genomic DNA prepared from exponentially growing cells at 25°C was analyzed as in Figure 5A.

Similar to what we observed at native telomeres, 5′ C-strand degradation in G1-arrested *yku70Δ* cells was abolished in the absence of Exo1, whereas it occurred in *yku70Δ mre11Δ* cells (Figure 7A–7C). Conversely, the lack of Exo1 did not affect 5′ C-strand degradation in G1-arrested *rif2Δ* cells, where degradation of the same strand was instead abolished in the absence of Mre11 (Figure 7D–7F). Unfortunately, we were unable to synchronize *rap1ΔC*

mre11Δ and *rap1ΔC mre11Δ* cell cultures due to their growth defects (data not shown). Altogether, these data indicate that Exo1 is primarily responsible for telomeric DNA degradation in the absence of Yku70, whereas the same process is mainly Mre11-dependent in *rap1ΔC and rif2Δ* cells, suggesting that Yku and shelterin-like proteins specifically prevent the action of different nucleases.

Rif2 and Rap1 inhibit Mre11 association at a *de novo* telomere in G1

Our data indicate that Mre11 plays a key role in telomeric ssDNA generation in the absence of Rif2 or Rap1, and Rif2 has been shown to regulate MRX recruitment at telomeres in cycling cells by inhibiting Tel1 association at telomeric ends [35]. We then monitored Mre11 recruitment at the HO-induced telomere in G1-arrested wild type, *rap1ΔC and rif2Δ* cells carrying a fully functional MYC-tagged MRE11allele. Sheared chromatin from formaldehyde cross-linked cell samples taken at different time points after galactose addition was immunoprecipitated with anti-Myc antibodies. Quantitative real-time polymerase chain reaction (qPCR) was then used to monitor coimmunoprecipitation of a DNA fragment located 640 bp centromere-proximal to the HO site (TEL) and of a nontelomeric ARO1 fragment (CON). The TEL/CON ratio, which was used to measure Mre11 association with the HO-induced telomere, was much higher in both *rap1ΔC and rif2Δ* cells than in wild type (Figure 7G), indicating that Rif2 and Rap1 prevent Mre11 association at telomeric ends in G1. This finding, together with the observation that Mre11 is required to generate telomeric ssDNA in the absence of Rif2 or Rap1, suggests that Rif2 and Rap1 might inhibit telomere processing by preventing Mre11 binding.

Figure 7: Nuclease requirements for ssDNA generation at a de novo telomere.

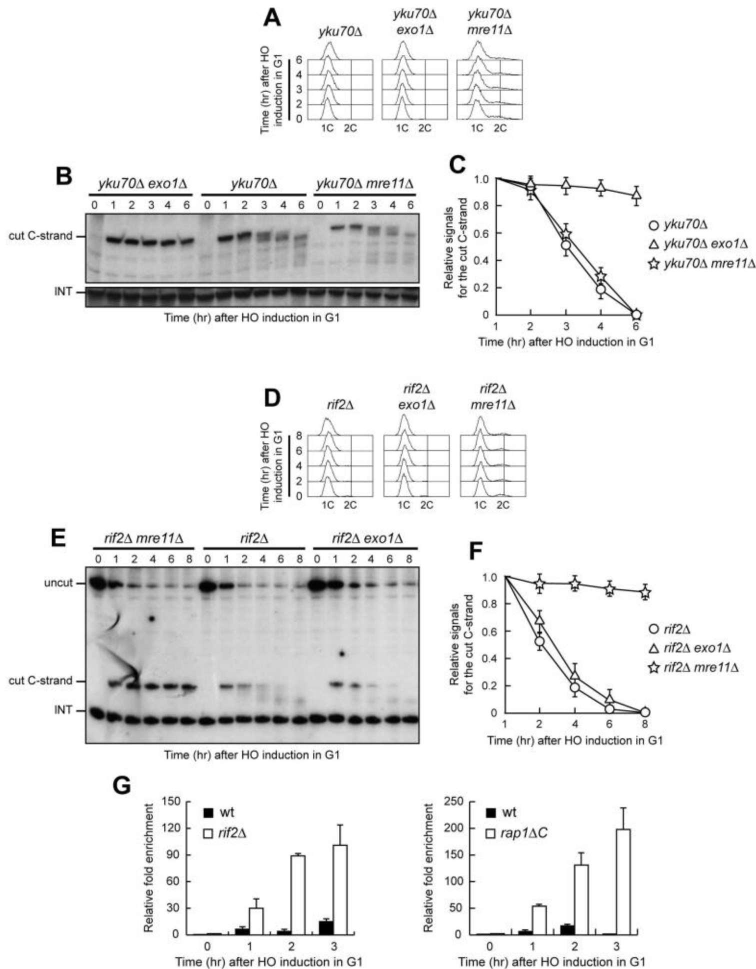

Figure 7: (A–C) HO expression was induced at time zero by galactose addition to α-factor-arrested *yku70Δ*,*yku70Δ mre11Δ* and *yku70Δ mre11Δ* cell cultures that were then kept arrested in G1. (A) FACS analysis of DNA content. (B) RsaI-digested genomic DNA was hybridized with probe A as in Figure 3E. (C) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (B). (D–F) HO expression was induced at time zero by galactose addition to α-factor-arrested *rif2Δ*, *rif2Δ mre11Δ* and *rif2Δ exo1Δ* cell cultures that were then kept arrested in G1. (D) FACS analysis of DNA content. (E) RsaI- and EcoRV-digested genomic DNA was hybridized with probe A as described in Figure 1C. (F) Densitometric analysis. Plotted values are the mean value ±SD from three independent experiments as in (E). (G) HO expression was induced at time zero by galactose addition to α-factor-arrested wild type, *rap1ΔC and rif2Δ* cells, all expressing a fully functional MRE11-MYC tagged

allele. Cells were then kept arrested in G1 and chromatin samples taken at different times after HO induction were immunoprecipitated with anti-Myc antibody. Coimmunoprecipitated DNA was analyzed by quantitative real-time PCR (qPCR) using primer pairs located at the nontelomeric ARO1 fragment of chromosome IV (CON) and 640 bp proximal to the HO site (TEL), respectively. Data are expressed as relative fold enrichment of TEL over CON signal after normalization to input signals for each primer set. The data presented are the mean of those obtained in three independent experiments. Error bars indicate s. d.

Discussion

Previous studies have shown that processing of S. cerevisiae telomeres is less efficient in G1 than in G2/M and Cdks are crucial for this difference [6], [7]. This work identifies the shelterin-like proteins Rap1, Rif1 and Rif2, as well as Yku, as other important players in the regulation of this process, where they inhibit nucleolytic telomere degradation. In particular, lack of Rif1, Rif2 or C-terminus of Rap1 promote C-rich strand degradation at an HO-derived telomere in G1 and enhance it in G2. Moreover, cycling cells devoid of Rif2 or Rap1 C-terminus display accumulation of ssDNA also at native telomeres. Thus, all these shelterin-like proteins inhibit nucleolytic degradation at telomeres, with Rap1 and Rif2 showing the strongest effects. Consistent with our finding, end processing and Mre11 binding have been shown to be reduced at an HO-induced telomere with 250 bp TG tracts compared to one with 81 bp TG tracts [36], which likely bind a smaller number of Rap1-Rif1-Rif2 complexes than the former [22-23]. Interestingly, ssDNA generation at both native and HO-induced telomeres is increased to the same extent in *rap1ΔC and rif2Δ* cells, suggesting that the effect exerted by Rap1 is likely mediated by Rif2. In fact, Rap1 recruits Rif2 to the TG tracts through its C-terminal domain [26]. On the other hand, also Rif1 is recruited by Rap1 to TG tracts [24-25], but Rif1 loss has a minor effect on C-strand resection, indicating different functions for Rif1 and Rif2 in inhibiting nucleolytic telomere processing. Similarly, Rif2, but not Rif1, prevents telomeric fusions by NHEJ [21].

Also Yku has a role in inhibiting telomere resection, but it acts specifically in G1. In fact, ssDNA generation at both HO-induced and native telomeres is increased in G1-arrested *yku70Δ* cells compared to wild type, whereas no significant differences are observed in G2/M. The Yku-mediated inhibitory effect on telomeric processing is independent on Yku role in NHEJ, as Dnl4 loss

does not promote ssDNA generation at the HO-induced telomere in G1, unlike at intrachromosomal DSBs [9,30]. This finding is consistent with the observation that NHEJ is inhibited at telomeres [37], possibly because its components are excluded from telomeric ends. Interestingly, resection at the HO-induced telomere in G1-arrested *yku70Δ* cells does not proceed beyond 166 bp from the HO site, suggesting that either the rate or the processivity of resection is reduced in G1 compared to G2/M in the absence of Yku. It is noteworthy that this limited processing is due to the inhibitory action of Rap1, Rif1 and Rif2, as their inactivation allows extensive resection not only in wild type but also in *yku70Δ* G1 cells.

Although C-strand degradation in the absence of Yku is restricted to the regions closest to the telomeric tip, this degradation is more efficient in G1-arrested *yku70Δ* cells than in *rif2Δ* cells. This observation, together with the finding that the shelterin-like proteins limit extensive resection in Yku-lacking cells, suggests that Yku is mainly involved in inhibiting initiation, whereas Rif1, Rif2 and Rap1 act primarily by limiting extensive resection. Consistent with the different inhibitory functions of Yku and shelterin-like proteins, the concomitant lack of Yku and Rif2 has additive effects on *de novo* telomere degradation in G1. In fact, both C-strand degradation and generation of r1 resection products occur more efficiently in G1 *yku70Δ rif2Δ* double mutant cells than in *rif2Δ* and *yku70Δ* single mutants.

It is worth pointing out that telomere processing in the absence of Yku, Rif2, Rap1 or Rif1 takes place in G1 independently of the low Cdk1 activity. As DSB resection is not completely abolished in G1 [5,30], the Cdk1 role might be simply to potentiate the resection machineries, thus explaining why Cdk1 requirement for telomere resection can be bypassed by inactivation of negative regulators of this process.

Interestingly, even the 3′ G strand of the HO-induced telomere decreases ~10 nucleotides in length and this limited degradation seems to correlate with the ability to initiate 5′-3′ processing. This phenomenon is reminiscent of the removal of the 3′ overhangs from uncapped telomeres by the human nucleotide excision repair endonuclease ERCC1/XPF [38]. Although the physiological significance of the 3′ G strand shortening is unknown, removal of these nucleotides might facilitate telomerase RNA annealing to its template.

The resection extent at the HO-induced telomere is higher in *rap1ΔC and rif2Δ* cycling cells than in *yku70Δ* G1 cells, but ssDNA at native telomeres does not elicit the DNA damage checkpoint in any of these mutant cells, suggesting that other mechanisms might prevent a DNA damage response at telomeres. One possibility is that the ssDNA accumulated in the absence of Yku or the shelterin-like proteins is still covered by Cdc13, which has been shown to inhibit Mec1 association to DNA ends [12]. Consistent with this hypothesis, the lack of Rif2 enhances checkpoint activation in cells crippled for Cdc13 activity (Figure 5F). Alternatively, or in addition, as Mec1 is the main responder to DSBs in yeast and its activation needs ssDNA [39], the amount of telomeric ssDNA in these cells may be insufficient to elicit a checkpoint response. In mammalian cells, loss of the shelterin protein TRF2 leads to ATM-dependent DNA damage response that does not require extensive degradation of the telomeric 5′ strand [40]. The knowledge that the ATM yeast ortholog, Tel1, has a very minor role in the checkpoint response to DSBs compared to Mec1 [41] might explain this difference between yeast and mammals in the response to telomere alterations.

The inhibitory actions of Yku and shelterin-like proteins seem to target different nucleases. In fact, Exo1 appears to be important for telomeric ssDNA generation at both native and HO-induced telomeres in *yku70Δ* G1 cells, suggesting that Yku might hide the telomeric ends from Exo1 association. By contrast, telomeric ssDNA generation in both *rap1ΔC and rif2Δ* cells depends primarily on Mre11, whose recruitment in G1 to the HO-induced telomere is enhanced in *rap1ΔC and rif2Δ* cells. Thus, while Yku protects telomeres towards Exo1 in G1, Rap1 and Rif2 likely prevent telomere processing by inhibiting loading of the MRX complex onto telomeric ends in both G1 and G2 (Figure 8). However, we cannot exclude that Yku might protect G1 telomeres also from MRX (Figure 8), as it has been observed at intrachromosomal DSB [30], because MRX action in *yku70Δ* G1 cells is anyhow inhibited by Rap1, Rif1 and Rif2. The nuclease responsible for telomere processing in the absence of the shelterin-like proteins might be MRX itself and/or the endonuclease Sae2, which was shown to act in concert with MRX in telomere processing [4]. As some MRX association at the HO-induced telomere can be detected in wild type G1 cells, Rap1 and Rif2 might impair 5′-end resection also by inhibiting MRX/Sae2 activity besides its association to DNA. In any case, telomere processing can take place in G2, likely because Cdk1 activity potentiates the

resection machinery and Yku does not exert its inhibitory effect in this cell cycle phase (Figure 8).

Figure 8. A working model for limiting DNA degradation at telomeres.

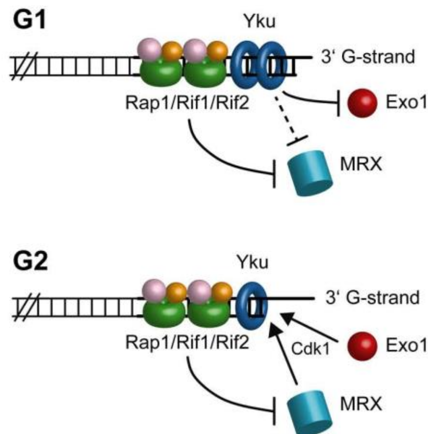

Figure 8: In G1, Yku protects telomeres from Exo1, while Rap1, Rif1 and Rif2 mainly act by preventing MRX access. As MRX action is still inhibited by Rap1, Rif1 and Rif2 in *yku70Δ* G1 cells, Yku might protect G1 telomeres also from MRX. In G2, only Rap1 and Rif2 still exert their inhibitory effects on telomere processing. Telomere resection can take place in G2 because Yku does not exert its inhibitory effect and Cdk1 activity potentiates nuclease actions.

It is noteworthy that Exo1 is a key factor for ssDNA generation at telomeres in Yku-lacking cells, while it plays only a minor role in doing so at intrachromosomal DSBs, where resection in *yku70Δ* mutant cells is primarily MRX-dependent [30] (our unpublished data). As Rif2 and Rap1 inhibit telomere degradation even in the absence of Yku, their presence at telomeres might block MRX access, thus explaining the different requirements of nuclease activities at DSBs versus telomeres in the absence of Yku.

Telomere protecting mechanisms are particularly important to prevent illegitimate repair/recombination, whose outcomes at telomeres can range

from the generation of chromosomal abnormalities, general hallmarks for human cancer cells, to permanent cell cycle arrest and cell death.

Altogether, this work increases our knowledge of this complex regulation, as it highlights a role of evolutionarily conserved proteins in protecting chromosome ends during different cell cycle phases by preventing the action of different nucleases.

Materials and Methods

Strains and plasmids

Strain genotypes are listed in the table. The strains used for monitoring telomere resection at the HO-induced telomere and HO-induced DSB were derivatives of strains UCC5913 and RMY169, respectively, kindly provided by D. Gottschling (Fred Hutchinson Cancer Research Center, USA) and T. Weinert (University of Arizona, USA). Strain RMY169 was created by replacing the *ADE2*-TG cassette of strain UCC5913 with the *TRP1* gene [11]. In order to allow an efficient and persistent G1 arrest, all strains carried the deletion of the *BAR1* gene, encoding a protease that degrades the mating pheromone α-factor. The *cdc13-1* mutant was kindly provided by D. Lydall (University of Newcastle, UK.). The plasmid pM585, carrying the rap1Δ670-807 allele, was kindly provided by D. Shore (University of Geneva, Switzerland). Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) supplemented with 2% glucose (YEPD) or 2% raffinose (YEP+raf) or 2% raffinose and 2% galactose (YEP+raf+gal). Unless otherwise stated, all the experiments were carried out at the temperature of 25°C.

Western blot analysis

Protein extracts were prepared by TCA precipitation as described in [42]. Rad53 was detected using anti-Rad53 polyclonal antibodies kindly provided by J. Diffley (Clare Hall, London, UK). Secondary antibodies were purchased from Amersham and proteins were visualized by an enhanced chemiluminescence system according to the manufacturer.

Saccharomyces cerevisiae strains used in this study

Strain	Relevant genotype
UCC5913[*]	*MATa-inc ade2-101 lys2-801 his3-Δ200 trp1-Δ63 ura3-52 leu2-Δ1::GAL1-HO-LEU2 VII-L::ADE2-TG(1-3)-HO site-LYS2*
RMY169[*]	*MATa-inc ade2-101 lys2-801 his3-Δ200 trp1-Δ63 ura3-52 leu2-Δ1::GAL1-HO-LEU2 VII-L::TRP1-HO site-LYS2*
YLL2554	UCC5913 *MRE11-18MYC::TRP1*
YLL2599	UCC5913 *bar1Δ::HPHMX*
YLL2600	RMY169 *bar1Δ::KANMX4*
YLL2606	RMY169 *yku70Δ::URA3 bar1Δ::HPHMX*
YLL2607	RMY169 *dnl4Δ::NATMX bar1Δ::HPHMX*
YLL2612	UCC5913 *yku70Δ::URA3 bar1Δ::HPHMX*
YLL2613	UCC5913 *dnl4Δ::NATMX bar1Δ::HPHMX*
YLL2646	UCC5913 *yku70Δ::URA3 rif1Δ::NATMX bar1Δ::HPHMX*
YLL2647	UCC5913 *yku70Δ::URA3 rif2Δ::NATMX bar1Δ::HPHMX*
YLL2649	UCC5913 *rif1Δ::NATMX bar1Δ::HPHMX*
YLL2650	UCC5913 *rif2Δ::NATMX bar1Δ::HPHMX*
YLL2651	UCC5913 *rap1Δ::KANMX4* [*CEN-HIS3-rap1Δ670-807*] *bar1Δ::HPHMX*
YLL2655	UCC5913 *yku70Δ::URA3 rap1Δ::KANMX4* [*CEN-HIS3-rap1Δ670-807*] *bar1Δ::HPHMX*
YLL2670	UCC5913 *MRE11-18MYC::TRP1 bar1Δ::HPHMX*
YLL2672	UCC5913 *MRE11-18MYC::TRP1 rap1Δ::KANMX4* [*CEN-HIS3-rap1Δ670-807*] *bar1Δ::HPHMX*
YLL2694	UCC5913 *MRE11-18MYC::TRP1 rif2Δ::NATMX*
YLL2725	UCC5913 *rif2Δ::NATMX mre11Δ::KANMX4 bar1Δ::HPHMX*
YLL2728	UCC5913 *yku70Δ::URA3 exo1Δ::NATMX bar1Δ::HPHMX*

YLL2730	UCC5913 *yku70Δ::URA3 mre11Δ::NATMX bar1Δ::HPHMX*
YLL2731	UCC5913 *rif2Δ::NATMX exo1Δ::URA3 bar1Δ::HPHMX*
YLL2733	UCC5913 *rap1Δ::KANMX4* [*CEN-HIS3-rap1Δ670-807*] *exo1Δ::URA3 bar1Δ::HPHMX*
YLL2736	UCC5913 *rap1Δ::KANMX4* [*CEN-HIS3-rap1Δ670-807*] *mre11Δ::NATMX bar1Δ::HPHMX*
K699	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-535*
DMP5108/ 19A	K699 *cdc13-1*
DMP5108/ 20A	K699 *cdc13-1 rif2Δ::KANMX4*

Plasmids are indicated by brackets. All strains are from this study except the strains

UCC5913: Diede SJ, Gottschling DE (2001) Exonuclease activity is required for sequence addition and Cdc13p loading at a *de novo* telomere. Curr Biol 11: 1336-1340.

RMY169: Michelson RJ, Rosenstein S, Weinert T (2005) A telomeric repeat sequence adjacent to a DNA double-stranded break produces an anticheckpoint. Genes Dev 19: 2546-2559.

Resection assay

Visualization of the single-stranded overhangs at native telomeres was done as described [31]. The same DNA samples were separated on a 0.8% agarose gel, denatured and hybridized with the end-labeled C-rich oligonucleotide for loading control. To monitor resection at the HO-derived telomeres, RsaI- and EcoRV-digested genomic DNA was subjected to denaturing polyacrilammide gel electrophoresis and then hybridized with the single-stranded riboprobes A or B, which anneal to the 5′ C-strand or the 3′ G-strand, respectively, to a site located 212 nt from the HO cutting site. Resection of the C-rich strand in Figure 3E and Figure 4C was monitored by hybridizing RsaI-digested genomic DNA with riboprobe A. To monitor resection of the 5′-strand at the HO-induced DSB, EcoRV-digested genomic DNA was hybridized with a single-stranded riboprobe, which anneal to the 5′-strand to a site located 215 nt from the HO cutting site. For quantitative analysis of C-strand and G-strand signals, the ratios between the intensities of ssDNA and loading control bands were calculated by using the NIH image program.

ChIP analysis

ChIP analysis was performed as described [43]. After exposure to formaldehyde, chromatin samples were immunoprecipitated with anti-Myc antibody. Quantification of immunoprecipitated DNA was achieved by qPCR on a Biorad MiniOpticon using primer pairs located at the nontelomeric ARO1fragment of chromosome IV (CON) and 640 bp centromere-proximal to the HO cutting site (TEL) and normalized to input signal for each primer set; data are expressed as the fold enrichment of TEL over the amount of CON in the immunoprecipitates.

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Author Contributions

Conceived and designed the experiments: DB MC MPL. Performed the experiments: DB MC SA MM. Analyzed the data: DB MC SA MM GL MPL. Wrote the paper: GL MPL.

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Rif1 Supports the Function of the CST Complex in Yeast Telomere Capping

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Abstract

Telomere integrity in budding yeast depends on the CST (Cdc13-Stn1-Ten1) and shelterin-like (Rap1-Rif1-Rif2) complexes, which are thought to act independently from each other. Here we show that a specific functional interaction indeed exists among components of the two complexes. In particular, unlike *RIF2* deletion, the lack of Rif1 is lethal for *stn1ΔC* cells and causes a dramatic reduction in viability of *cdc13-1* and *cdc13-5* mutants. This synthetic interaction between Rif1 and the CST complex occurs independently of *rif1*Δ-induced alterations in telomere length. Both *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells display very high amounts of telomeric single-stranded DNA and DNA damage checkpoint activation, indicating that severe defects in telomere integrity cause their loss of viability. In agreement with this hypothesis, both DNA damage checkpoint activation and lethality in *cdc13 rif1*Δ cells are partially counteracted by the lack of the Exo1 nuclease, which is involved in telomeric single-stranded DNA generation. The functional interaction between Rif1 and the CST complex is specific, because *RIF1*deletion does not enhance checkpoint activation in case of CST-independent telomere capping deficiencies, such as those caused by the absence of Yku or telomerase. Thus, these data highlight a novel role for Rif1 in assisting the essential telomere protection function of the CST complex.

Author Summary

Protection of chromosome ends is crucial for maintaining chromosome stability and genome integrity, and its failure leads to genome rearrangements that may facilitate carcinogenesis. This protection is achieved by the packaging of chromosome ends into protective structures called telomeres that prevent DNA repair/recombination activities. Telomeric DNA is bound and stabilized by two protein complexes named CST and shelterin, which are present in a wide range of multicellular organisms. Whether structural and functional connections exist between these two capping complexes is an important issue in telomere biology. Here, we investigate this topic by analyzing the consequences of disabling the two *Saccharomyces cerevisiae* shelterin-like components, Rif1 and Rif2, in different hypomorphic mutants defective in CST components. We demonstrate that Rif1 plays a previously unanticipated role in assisting the essential telomere protection function of the CST complex, indicating a tight coupling between CST and Rif1. As CST complexes have been

recently identified also in other organisms, including humans, which all rely on shelterin for telomere protection, this functional link between CST and shelterin might be an evolutionarily conserved common feature to ensure telomere integrity.

Introduction

Telomeres, the specialized nucleoprotein complexes at the ends of eukaryotic chromosomes, are essential for genome integrity. They protects chromosome ends from fusions, DNA degradation and recognition as DNA double-strand breaks (DSBs) that would otherwise lead to chromosome instability and cell death (reviewed in [1]). Telomeric DNA in the budding yeast *Saccharomyces cerevisiae*, as well as in nearly all other eukaryotes examined to date, comprise short TG-rich repeated sequences ending in a short single-stranded 3′ overhang (G tail) that corresponds to the strand bearing the TG-rich repeats. The addition of telomeric repeats depends on the action of telomerase, a specialized reverse transcriptase that extends the TG-rich strand of chromosome ends. Recruitment/activation of this enzyme requires the Cdc13 protein that binds to the telomeric TG-rich single-stranded DNA (ssDNA) [2-6]. The direct interaction between Cdc13 and the Est1 regulatory subunit of telomerase is essential for telomerase recruitment, and it is disrupted by the *cdc13-2* mutation that leads to gradual telomere erosion and accompanying senescence [2,4,7].

The average length of *S. cerevisiae* telomeric 3′ overhangs is 12–14 nucleotides, although it can increase to ∼50 nucleotides during the late S/G2 phase of the cell cycle [8-10]. While single-stranded telomeric G-tails can arise after removal of the last RNA primer during lagging-strand replication, the blunt ends of the leading-strand telomere must be converted into 3′ overhangs by resection of the 5′ strand. This 5′ to 3′ nucleolytic degradation involves several proteins, such as the MRX complex, the nucleases Exo1 and Dna2 and the helicase Sgs1 [10,11]. Cyclin-dependent kinase activity (Cdk1 in *S. cerevisiae*) is also required for generation of the extended single-stranded overhangs in late S phase [12,13]. As Cdk1 activity is low in G1, telomere resection can occur only during S/G2 [8], coinciding with the time frame in which G-tails are lengthened and can serve to recruit telomerase.

Keeping the G tail in check is crucial to ensure telomere stability, and studies in budding yeast have shown that Cdc13 prevents inappropriate generation of ssDNA at telomeric ends [2,14,15]. This essential capping function depends on Cdc13 interaction with the Stn1 and Ten1 proteins to form the so-called CST (Cdc13-Stn1-Ten1) complex. This complex binds to telomeric ssDNA repeats and exhibits structural similarities with the heterotrimeric ssDNA binding complex Replication protein A (RPA) [16], suggesting that CST is a telomere-specific version of RPA. Loss of Cdc13 function through either the*cdc13-1* temperature sensitive allele or the *cdc13-td* conditional degron allele results in telomere C-strand degradation, leading to activation of the DNA damage checkpoint [13,14,17,18]. Similarly, temperature sensitive mutations in either *STN1* or *TEN1* genes cause telomere degradation and checkpoint-mediated cell cycle arrest [19–21]. Interestingly, Stn1 interacts with Pol12 [22], a subunit of the DNA polymerase α (polα)-primase complex with putative regulatory functions, while Cdc13 interacts with the polα catalytic subunit of the same complex [7], suggesting that CST function might be tightly coupled to the priming of telomeric C strand synthesis. In any case, it is so far unknown whether the excess of telomeric ssDNA in *cst* mutants arises because the CST complex prevents the access of nuclease/helicase activities to telomeric ends and/or because it promotes polα-primase-dependent C strand synthesis.

In addition to the capping function, a role for the CST complex in repressing telomerase activity has been unveiled by the identification of *cdc13*, *stn1* and *ten1* alleles with increased telomere length. [2,21,23,24]. The repressing effect of Cdc13 appears to operate through an interaction between this protein and the C-terminal domain of Stn1 [25,26], which has been proposed to negatively regulate telomerase by competing with Est1 for binding to Cdc13 [4,24].

A second pathway involved in maintaining the identity of *S. cerevisiae* telomeres relies on a complex formed by the Rap1, Rif1 and Rif2 proteins. Although only Rap1 is the only shelterin subunit conserved in budding yeast, the Rap1-Rif1-Rif2 complex functionally recapitulates the shelterin complex acting at mammalian telomeres (reviewed in [27]). Rap1 is known to recruit its interacting partners Rif1 and Rif2 to telomeric double-stranded DNA via its C-terminal domain [28-30].

This complex negatively regulates telomere length, as the lack of either Rif1 or Rif2 causes telomere lengthening, which is dramatically increased when both proteins are absent [30]. The finding that telomere length in *rif1*Δ*rif2*Δ double mutant is similar to that observed in *RAP1* C-terminus deletion mutants [30] suggests that Rap1-dependent telomerase inhibition is predominantly mediated by the Rif proteins. However, Rif proteins have been shown to regulate telomere length even when the Rap1 C-terminus is absent [31], suggesting that they can be brought to telomeres independently of Rap1.

In addition to negatively regulate telomere length, Rap1 and Rif2 inhibit both nucleolytic processing and non homologous end joining (NHEJ) at telomeres [32-34]. Telomeric ssDNA generation in both rif2Δ and rap1ΔC cells requires the MRX complex [33], and the finding that MRX association at telomeres is enhanced in *rif2Δ* and *rap1ΔC* cells [33,35] suggests that Rap1 and Rif2 likely prevent MRX action by inhibiting MRX recruitment onto telomeric ends. Interestingly, the checkpoint response is not elicited after inactivation of Rap1 or Rif2, suggesting that either the accumulated telomeric ssDNA is insufficient for triggering checkpoint activation or this ssDNA is still covered by Cdc13, which can inhibit the association of the checkpoint kinase Mec1 to telomeres [36]. Notably, Rif1 is not involved in preventing telomeric fusions by NHEJ [32] and its lack causes only a slight increase in ssDNA generation at a de novo telomere [33]. These findings, together with the observation that Rif1 prevents telomerase action independently of Rif2, indicate that Rif1 and Rif2 play different functions at telomeres.

As both CST and the shelterin-like complex contribute to telomere protection, we asked whether and how these two capping complexes are functionally connected. We found that the viability of cells with defective CST complex requires Rif1, but not Rif2. In fact, *RIF1* deletion increases the temperature sensitivity of *cdc13-1* cells and impairs viability of *cdc13-5* cells at any temperature. Furthermore, the *rif1*Δ and *stn1ΔC* alleles are synthetically lethal. By contrast, the lack of Rif2 has no effects in the presence of the same *cdc13* and *stn1* alleles. We also show that *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells accumulate telomeric ssDNA that causes hyperactivation of the DNA damage checkpoint, indicating that loss of Rif1 exacerbates telomere integrity defects in *cdc13* mutants. By contrast, deletion of *RIF1* does not enhance

either cell lethality or checkpoint activation in *yku70*Δ or *est2*Δ telomere capping mutants. Thus, Rif1 is required for cell viability specifically when CST activity is reduced, highlighting a functional link between Rif1 and CST.

Results

Rif1, but not Rif2, is required for cell viability when Cdc13 or Stn1 activities are reduced

Yeast cells harbouring the *cdc13-1* temperature-sensitive allele of the gene encoding the essential telomeric protein Cdc13 are viable at permissive temperature (20–25°C), but die at restrictive temperature (26–37°C), likely due to accumulation of ssDNA at telomeres caused by the loss of Cdc13 capping functions [14]. As also the shelterin-like complex contributes to the maintenance of telomere integrity, we investigated its possible functional connections with Cdc13 by disabling either Rif1 or Rif2 in *cdc13-1* cells. Deletion of *RIF2* did not affect *cdc13-1* cell viability in YEPD medium at any tested temperature (Figure 1A). By contrast, *cdc13-1 rif1*Δ cells showed a maximum permissive temperature for growth of 20°C and were unable to grow at 25°C, where *cdc13-1* single mutant cells could grow at almost wild type rate (Figure 1A). The enhanced temperature-sensitivity of *cdc13-1 rif1*Δ cells was due to the lack of *RIF1*, because the presence of wild type *RIF1* on a centromeric plasmid allowed *cdc13-1 rif1*Δ cells to grow at 25°C (Figure 1B). The synthetic effect of the *cdc13-1 rif1*Δ combination was not uncovered during a previous genome wide search for gene deletions enhancing the temperature-sensitivity of *cdc13-1* cells [37], likely because that screening was done at 20°C, a temperature at which*cdc13-1 rif1*Δ double mutants do not show severe growth defects (Figure 1A). Our data above indicate that Rif1, but not Rif2, is required to support cell viability when Cdc13 protective function is partially compromised.

If the lack of Rif1 in *cdc13-1* cells increased the temperature-sensitivity by exacerbating the telomere end protection defects of these cells, Rif1 overexpression might suppress the temperature sensitivity caused by the *cdc13-1* allele. Indeed, high copy number plasmids carrying wild type RIF1, which had no effect on wild type cell viability, improved the ability of *cdc13-1* cells to form colonies on synthetic selective medium at the semi-permissive temperature of 26–27°C (Figure 1C).

Figure 1 Synthetic effects between the rif1Δ and cdc13-1 mutations.

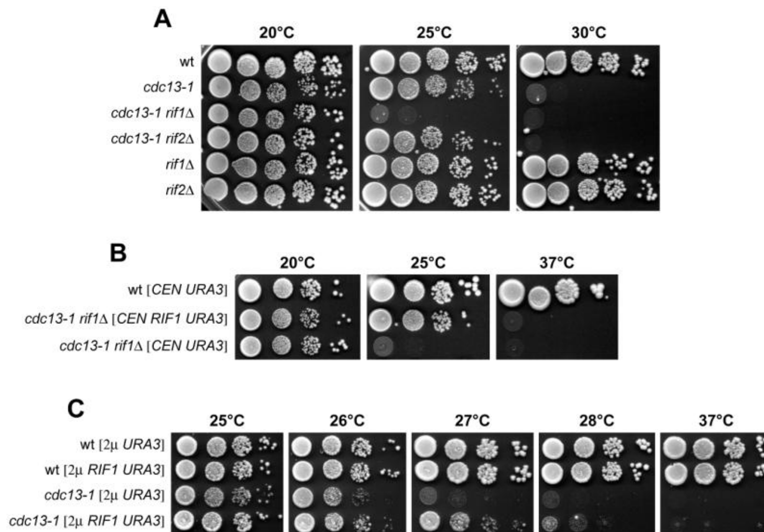

Figure 1: (A) Strains with the indicated genotypes were grown overnight in YEPD at 20°C. Serial 10-fold dilutions were spotted onto YEPD plates and incubated at the indicated temperatures for 2–4 days. (B–C) Strains containing the indicated plasmids were grown overnight at 20°C in synthetic liquid medium lacking uracil. Serial 10-fold dilutions were spotted onto plates lacking uracil that were incubated at the indicated temperatures for 3–5 days.

The function of Cdc13 in telomere protection is mediated by its direct interactions with Stn1 and Ten1, leading to formation of the CST complex (reviewed in [38]). In addition to the capping function, the CST complex is implicated in repression of telomerase action [2,21,23,24]. This CST-dependent negative regulation of telomerase can be separated from CST capping function, as yeast cells either carrying the *cdc13-5* allele or lacking the Stn1 C-terminus (residues 282–494) (*stn1ΔC*) display extensive telomere elongation but no or minimal growth defects [24,26]. We evaluated the specificity of the genetic interaction between *rif1Δ* and *cdc13-1* by analysing the consequences of deleting *RIF1* and *RIF2* in *cdc13-5* or *stn1ΔC* cells.

Deletion of *RIF1* turned out to reduce cell viability of *cdc13-5* mutant cells at any temperatures, while deletion of *RIF2* did not (Figure 2A). Furthermore, meiotic tetrad dissection of *stn1ΔC/STN1 rif1Δ/RIF1* diploid cells did not allow the recovery of viable *stn1ΔC rif1Δ* double mutant spores (Figure 2B), indicating that *rif1Δ* and *stn1ΔC* were synthetic lethal.

By contrast, viable *rif2*Δ *stn1*Δ*C* spores were found with the expected frequency after tetrad dissection of*stn1*Δ*C*/*STN1 rif2*Δ/*RIF2* diploid cells (Figure 2C). The observed synthetic phenotypes suggest that both*stn1*Δ*C* and *cdc13-5* cells have capping deficiencies and that the lack of Rif1 enhances their protection defects.

Consistent with this hypothesis, *cdc13-5* and *stn1*Δ*C* mutants were shown to accumulate telomeric ssDNA, although the amount of this ssDNA was not enough to invoke a DNA damage response [24], [25]. We conclude that Rif1, but not Rif2, is required to support cell viability when a partial inactivation of CST capping function occurs.

A Cdc13 specific function that is not shared by the other subunits of the CST complex is its requirement for recruitment/activation of telomerase at chromosome ends[2-6]. Cdc13-mediated telomerase recruitment is disrupted by the *cdc13-2* mutation, which leads to progressive telomere shortening and senescence phenotype [4]. We therefore asked whether *RIF1* deletion influences viability and/or senescence progression of *cdc13-2* cells. Viable *cdc13-2 rif1*Δ spores were recovered after tetrad dissection of *cdc13-2/CDC13 rif1*Δ/*RIF1* diploid cells (data not shown), indicating that the lack of Rif1 does not affect the overall viability of *cdc13-2* cells. When spores from the dissection plate were streaked on YEPD plates for 4 successive times, the decline in growth of *cdc13-2* and *cdc13-2 rif1*Δ spores occurred with similar kinetics (Figure 2D), indicating that *RIF1* deletion did not accelerate the senescence phenotype of *cdc13-2* cells specifically defective in telomerase recruitment. Taken together, these genetic interactions indicate that Rif1, but not Rif2, has a role in assisting the essential function of the CST complex in telomere protection.

The CST complex functionally and physically interacts with the polα-primase complex [7,21,22,25] which is essential for telomeric C-strand synthesis during telomere elongation. Thus, we analyzed the genetic interactions between *rif1*Δ and temperature sensitive alleles affecting DNA primase (*pri2-1)*[39] or polα (*cdc17-1* and *pol1-1*) [40-41]. Both *cdc17-1 rif1*Δ and *pol1-1 rif1*Δ cells were viable, but their temperature-sensitivity was greatly enhanced compared to *cdc17-1* and *pol1-1* single mutants (Figure 2E). Similarly, the maximal permissive temperature of the *pri2-1 rif1*Δ double mutant was reduced relative to that of *pri2-1* single mutant cells (Figure 2F). Moreover both *pol1-1*

rif1Δ and *pri2-1 rif1Δ* cells showed growth defects even at the permissive temperature of 25°C (Figure 2E and 2F). Thus, Rif1, like CST, functionally interacts with the polα-primase complex.

Figure 2: RIF1 deletion affects viability of both cdc13-5 and stn1ΔCcells.

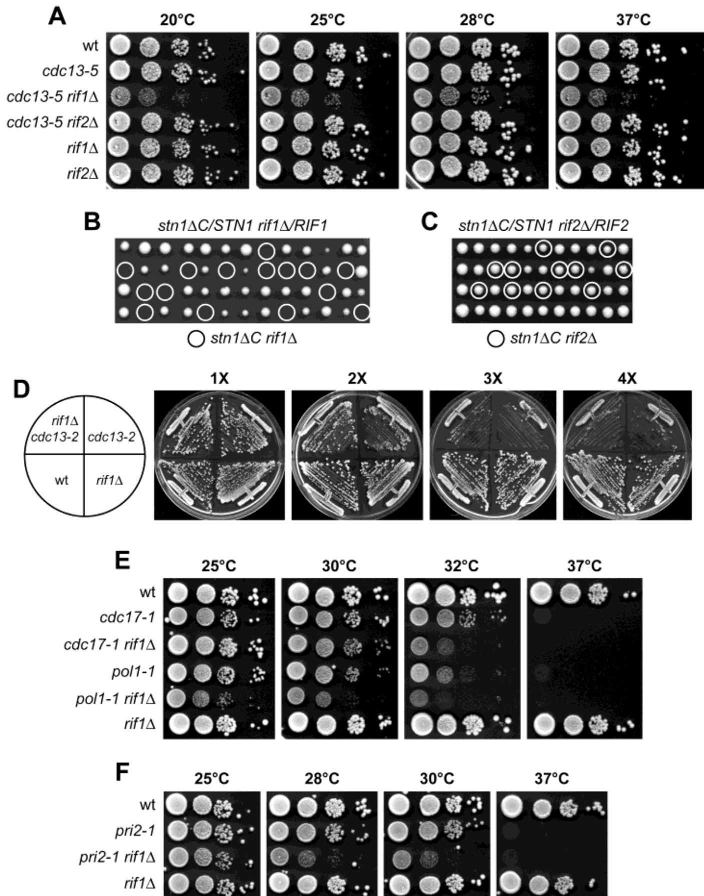

Figure 2: (A) Strains with the indicated genotypes were grown overnight in YEPD at 25°C. Serial 10-fold dilutions were spotted onto YEPD plates and incubated at the indicated temperatures for 2–4 days. (B–C) Viability of spores derived from diploids heterozygous for the indicated mutations. Spores with the indicated double mutant genotypes are circled. (D) Meiotic tetrads from a *CDC13/cdc13-2 RIF1/rif1Δ* diploid strain were dissected on YEPD plates. After ~25 generations on the dissection plate, spore clones from 20 tetrads were subjected to genotyping and concomitantly to four successive streak-outs (1X to 4X), corresponding to ~25, ~50, ~75 and ~100 generations of growth, respectively. All tetratype tetrads behaved as the one shown in this panel. (E–F) Cell cultures were grown overnight in YEPD at 25°C. Serial 10-fold dilutions were spotted onto YEPD plates that were incubated at the indicated temperatures for 2–3 days.

The lack of Rif1 enhances the DNA damage checkpoint response in *cdc13* mutant cells

The synthetic effects of combining *rif1*Δ with *cdc13* and *stn1* mutations suggest that Rif1 might normally assist the Cdc13 and Stn1 proteins in carrying out their essential telomere protection functions. It is known that *cdc13-1* cells undergo checkpoint-dependent metaphase arrest when incubated at the restrictive temperature [14]. Failure to turn on the checkpoint allows *cdc13-1* cells to form colonies at 28°C [42,43], indicating that checkpoint activation can partially account for the loss of viability of *cdc13-1* cells. We then asked whether the enhanced temperature sensitivity of *cdc13-1 rif1*Δ cells compared to *cdc13-1* cells might be due to upregulation of the DNA damage checkpoint response. Deletion of the checkpoint gene *RAD9*, which partially suppressed the temperature sensitivity of *cdc13-1*mutant cells, slightly improved the ability of *cdc13-1 rif1*Δ cells to grow at 23–25°C (Figure 3A), indicating that the synthetic interaction between Rif1 and Cdc13 can be partially alleviated by checkpoint inactivation. Furthermore, when wild type, *rif1*Δ, *cdc13-1* and *cdc13-1 rif1*Δ cell cultures were arrested in G1 with α-factor at 20°C (permissive temperature) and then released from G1 arrest at 25°C (non-permissive temperature for *cdc13-1 rif1*Δ cells), they all replicated DNA and budded with similar kinetics after release (Figure 3B and 3C). However, most *cdc13-1 rif1*Δ cells then arrested in metaphase as large budded cells with a single nucleus, while wild type, *cdc13-1* and *rif1*Δ cells divided nuclei after 75–90 minutes (Figure 3D).

To assess whether the cell cycle arrest of *cdc13-1 rif1*Δ cells was due to DNA damage checkpoint activation, we examined the Rad53 checkpoint kinase, whose phosphorylation is necessary for checkpoint activation and can be detected as changes in Rad53 electrophoretic mobility. Rad53 was phosphorylated in *cdc13-1 rif1*Δ cells that were released from G1 arrest at 25°C, whereas no Rad53 phosphorylation was seen in any of the other similarly treated cell cultures (Figure 3E).

Figure 3 Metaphase arrest and checkpoint activation in cdc13 rif1Δ cells.

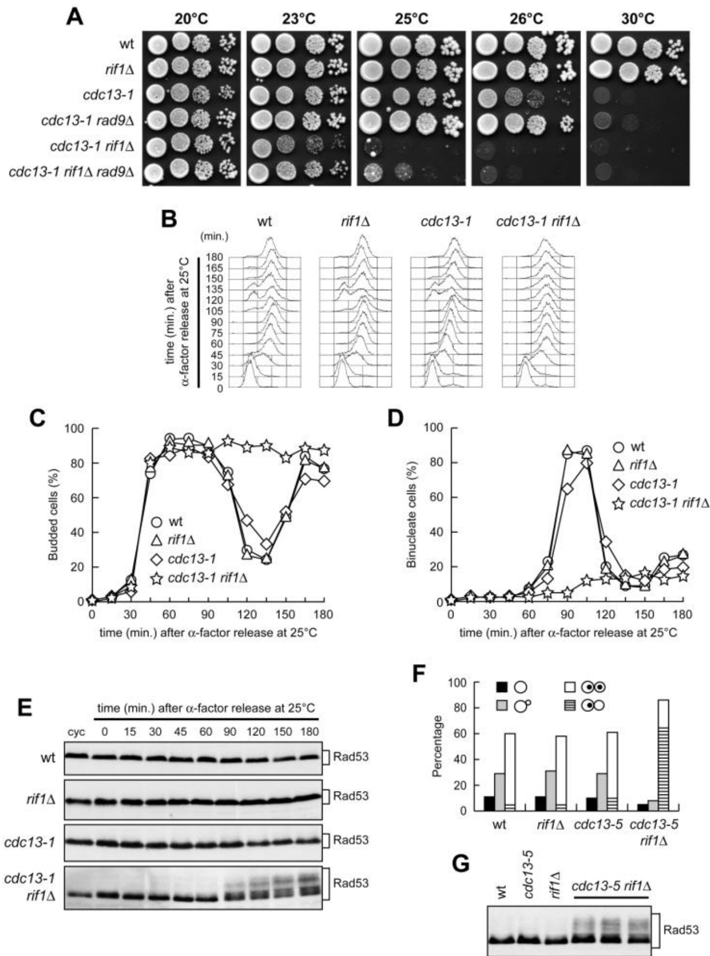

Figure 3: (A) Strains with the indicated genotypes were grown overnight in YEPD at 20°C. Serial 10-fold dilutions were spotted onto YEPD plates and incubated at the indicated temperatures for 2–4 days. (B–E) Cell cultures exponentially growing at 20°C in YEPD were arrested in G1 with α-factor and then released from G1 arrest in YEPD at 25°C (time zero). Samples were taken at the indicated times after release from α-factor for FACS analysis of DNA content (B), for determining the kinetics of bud emergence (C) and nuclear division (D), and for western blot analysis of Rad53 using anti-Rad53 antibodies (E). cyc, cycling cells. (F–G) Cell cultures were grown exponentially in YEPD at 25°C. (F) The frequency of cells with no, small or large buds was determined by analyzing a total of 200 cells for each strain. The percentage of large budded cells with one or two nuclei was evaluated by fluorescence microscopy. (G) Rad53 in cell cultures exponentially growing at 25°C was visualized as in panel E. Three independent *cdc13-5 rif1Δ* strains were analyzed.

RIF1 deletion caused a checkpoint-mediated G2/M cell cycle arrest also in *cdc13-5* cells. In fact, exponentially growing *cdc13-5 rif1*Δ cell cultures at 25°C contained a higher percentage of large budded cells with a single nucleus than *rif1*Δ or *cdc13-5* cell cultures under the same conditions (Figure 3F). Furthermore, Rad53 phosphorylation was detected in these *cdc13-5 rif1*Δ cells, but not in the *rif1*Δ and*cdc13-5* cell cultures (Figure 3G). Thus, the lack of Rif1 results in DNA damage checkpoint activation in both *cdc13-1* and *cdc13-5* cells under conditions that do not activate the checkpoint when Rif1 is present.

The synthetic interaction between Rif1 and CST is independent of *rif1Δ*-induced telomere overelongation

The lack of Rif1 is known to cause telomere overelongation [29]. Thus, we examined telomere length in*cdc13-1 rif1Δ* double mutant cells. The length of duplex telomeric DNA was examined after transferring at 25°C cell cultures exponentially growing at 20°C, followed by Southern blot analysis with a TG-rich probe of XhoI-digested genomic DNA prepared at different times after shift at 25°C (Figure 4A). As expected [29], *rif1Δ* mutant cells had longer telomeres than wild type and *cdc13-1* cells (Figure 4A). Telomeres in *cdc13-1 rif1Δ* double mutant cells either at 20°C or after incubation at 25°C were longer than those of wild type and *cdc13-1* cells, but undistinguishable from those of *rif1Δ* cells (Figure 4A). Not only RIF1 deletion, but also the *cdc13-5* mutation is known to cause telomere overelongation [24] (Figure 4B). Interestingly, when telomere length was analyzed in *cdc13-5 rif1Δ* double mutant cells grown at 25°C, telomeres were longer in *cdc13-5 rif1Δ* double mutant cells than in *cdc13-5* and *rif1Δ* single mutants (Figure 4B), indicating that the *cdc13-5* mutation exacerbates the telomere overelongation defect caused by the lack of Rif1.

The finding that telomeres in *cdc13-1 rif1*Δ double mutant cells at 25°C were longer than those of *cdc13-1* cells, but undistinguishable from those of *cdc13-1 rif1Δ* cells grown at 20°C (Figure 4A) suggests that the growth defects of *cdc13-1 rif1Δ* cells at 25°C are not due to *rif1Δ*-induced telomere overelongation. Telomere lengthening in *rif1Δ* mutant cells is telomerase-dependent [44] and requires the action of the checkpoint kinase Tel1 that facilitates telomerase recruitment [45,46].

Figure 4. Native telomere length in cdc13 rif1Δ cells.

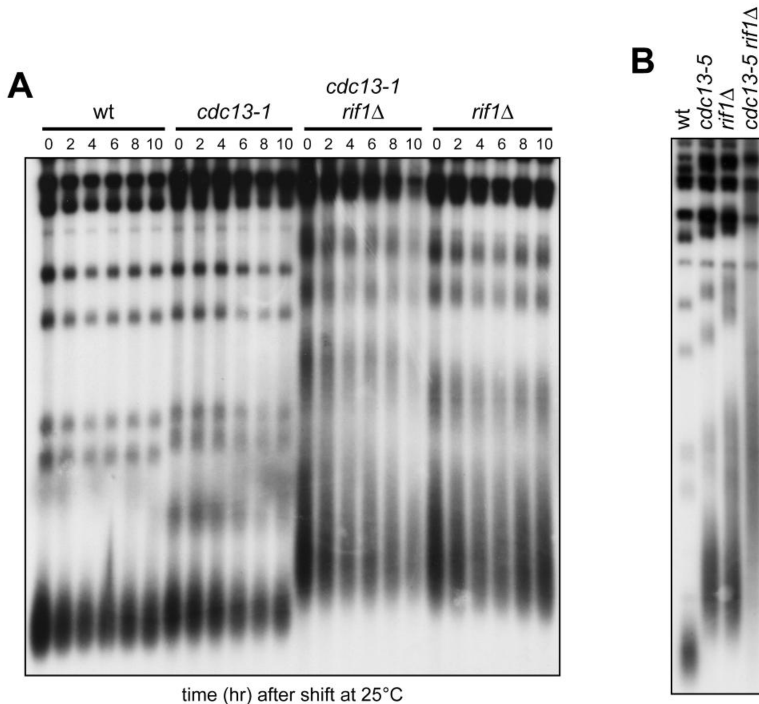

Figure 4: (A) Cells with the indicated genotypes exponentially growing in YEPD at 20°C were shifted at 25°C at time 0. Cells were collected at the indicated time points after shift and XhoI-cut genomic DNA was subjected to Southern blot analysis using a radiolabeled poly(GT) telomere-specific probe. (B) XhoI-cut genomic DNA extracted from cells with the indicated genotypes exponentially growing at 25°C was subjected to Southern blot analysis as in panel A.

To provide additional evidences that loss of viability in *cdc13 rif1Δ* mutants occurs independently of *rif1Δ*-induced alterations in telomere length, we asked whether *RIF1* deletion was still deleterious in *cdc13-1*, *cdc13-5* and *stn1ΔC* cells in a context where telomeres cannot be elongated due to the lack of Tel1 [45]. We found that *TEL1*deletion did not alleviate the growth defects of *cdc13-1 rif1*Δ cells (Figure 5A). Rather, *cdc13-1 tel1Δ* and *cdc13-1 rif1Δ tel1Δ* cells showed an enhanced temperature sensitivity compared to *cdc13-1* and *cdc13-1 rif1Δ* cells, respectively, presumably due to the

combined effects of loss of a telomere elongation mechanism and inability to protect telomeres from shortening activities. Furthermore, the growth defects of *cdc13-5 rif1*Δ double mutant cells were similar to those of *cdc13-5 rif1*Δ *tel1*Δ triple mutant cells (Figure 5B).

Finally, viable *stn1ΔC rif1Δ tel1Δ* mutant spores could not be recovered after meiotic tetrad dissection of *stn1ΔC/STN1 rif1Δ/RIF1 tel1Δ/TEL1* diploid cells (data not shown), indicating that *stn1ΔC* and *rif1*Δ were synthetic lethal even in the absence of Tel1.

Figure 5: Effect of deleting TEL1 or RIF2 on growth of cdc13 rif1Δcells.

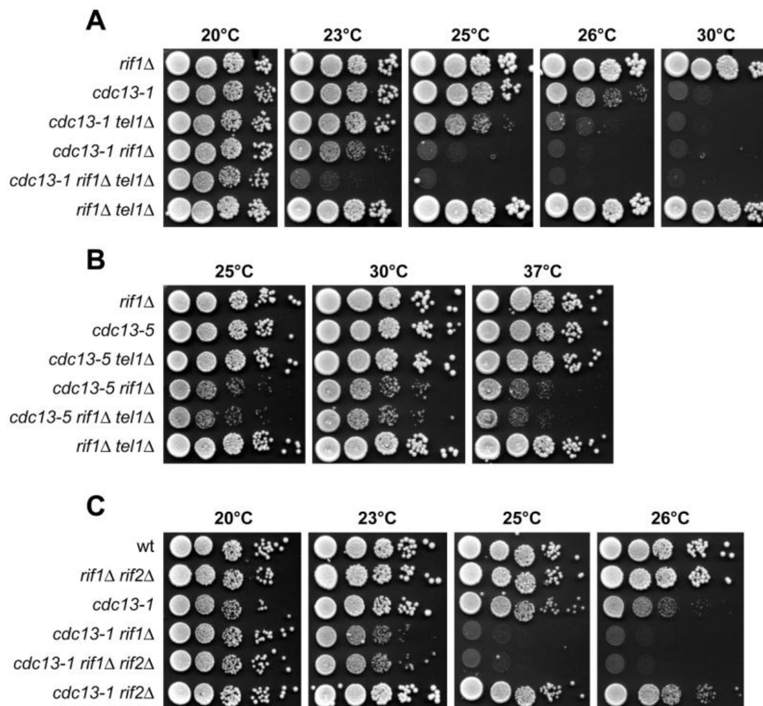

Figure 5: Cells with the indicated genotypes were grown overnight in YEPD at 20°C (A and C) or 25°C (B). Serial 10-fold dilutions were spotted onto YEPD plates that were then incubated at the indicated temperatures for 2–4 days.

As telomere lengthening is dramatically increased when both Rif1 and Rif2 are absent [30], we also investigated whether the absence of Rif2 exacerbates *cdc13-1 rif1*Δ growth defects. As shown in Figure 5C, *cdc13-1 rif1*Δ *rif2*Δ cells formed colonies at the maximum temperature of 20°C and behaved similarly to *cdc13-1 rif1*Δ cells. We therefore conclude that the synthetic interaction between *rif1Δ* and *cdc13* alleles is not due to *rif1*Δ-induced alterations in telomere length, but it is a direct consequence of Rif1 loss.

The lack of Rif1 causes generation of telomeric ssDNA in *cdc13* cells

It is known that *cdc13-1* cells at 37°C accumulate telomeric ssDNA that triggers checkpoint-mediated cell cycle arrest [14]. Thus, we investigated whether *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells contained aberrant levels of single-stranded TG sequences at their telomeres that could be responsible for loss of viability in *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells at 25°C. The integrity of chromosome ends was analyzed by an in-gel hybridization procedure [9], probing for the presence of single-stranded TG sequences. Both *cdc13-1* and *rif1*Δ single mutants either grown at 20°C (Figure 6A, lanes 2 and 4) or incubated at 25°C for 3 hours (Figure 6A, lanes 6 and 8) showed only a very slight increase in single-stranded TG sequences compared to wild type (Figure 6A, lanes 1 and 5).

By contrast, *cdc13-1 rif1*Δ double mutant cells contained higher amounts of telomeric ssDNA than *cdc13-1* and *rif1*Δ cells already at 20°C (Figure 6A, lane 3) and the amount of this ssDNA increased dramatically when *cdc13-1 rif1*Δ cells were incubated at 25°C for 3 hours (Figure 6A, lane 7). A similar telomere deprotection defect was observed also for *cdc13-5 rif1*Δ cells grown at 25°C (Figure 6A, lane 11), which displayed an increased amount of telomeric ssDNA compared to similarly treated wild type and *cdc13-5* cells (Figure 6A, lanes 9 and 10).

Figure 6: RIF1 deletion enhances ssDNA formation at native telomeres of cdc13 cells.

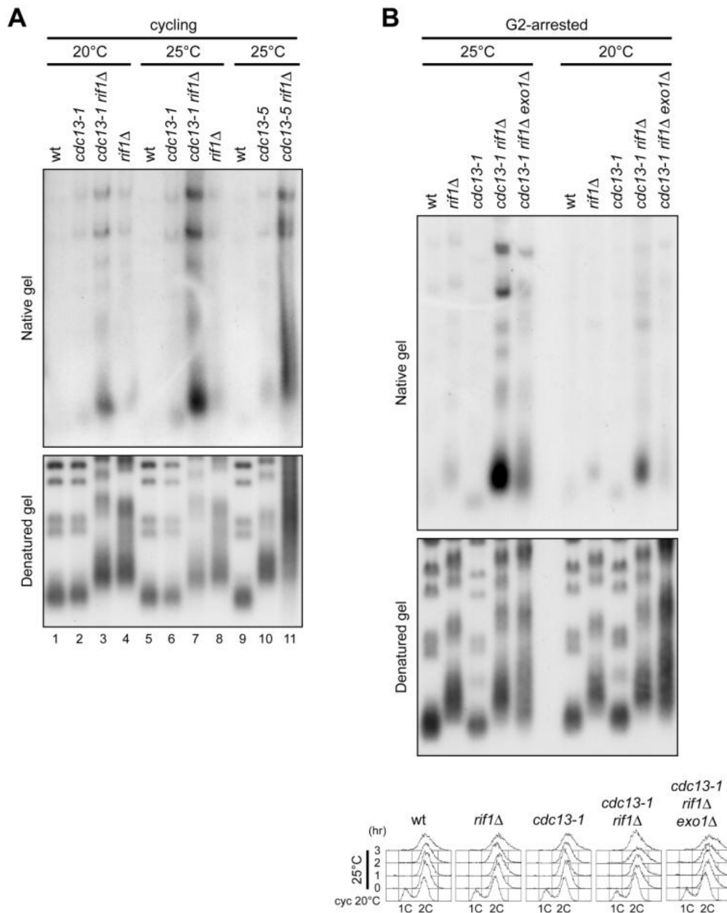

Figure 6: (A) Wild type and otherwise isogenic *cdc13-1*, *cdc13-1 rif1*Δ and *rif1*Δ cell cultures exponentially growing at 20°C (lanes 1–4) were incubated at 25°C for 3 hours (lanes 5–8). Wild type and otherwise isogenic *cdc13-5* and *cdc13-5 rif1*Δ cell cultures were grown exponentially at 25°C (lanes 9–11). Genomic DNA was digested with XhoI and single-stranded telomere overhangs were visualized by in-gel hybridization (native gel) using an end-labelled C-rich oligonucleotide. The same DNA samples were separated on a 0.8% agarose gel, denatured and hybridized with the end-labeled C-rich oligonucleotide for loading and telomere length control (denatured gel). (B) Cell cultures were arrested in G2 with nocodazole at 20°C (right) and then transferred at 25°C in the presence of nocodazole for 3 hours (left), followed by analysis of single-stranded telomere overhangs (top) as in panel A, and FACS analysis of DNA content (bottom).

Because the length of single-stranded G overhangs increases during S phase [8], the strong telomeric ssDNA signals observed in *cdc13-1 rif1*Δ cell cultures at 25°C (Figure 6A) might be due to an enrichment of S/G2 cells. We ruled out this possibility by monitoring the levels of single-stranded TG sequences in *cdc13-1 rif1*Δ cell cultures that were arrested in G2 with nocodazole at 20°C and then transferred to 25°C in the presence of nocodazole for 3 hours (Figure 6B). Similarly to what we observed in exponentially growing cell cultures, G2-arrested *cdc13-1 rif1*Δ cells at 20°C displayed increased amounts of ssDNA compared to each single mutant under the same conditions, and incubation at 25°C led to further increase of this ssDNA (Figure 6B). Taken together, these findings indicate that the lack of Rif1 causes a severe defect in telomere protection when Cdc13 activity is partially compromised.

The lack of Exo1 counteracts DNA damage checkpoint activation and telomeric ssDNA accumulation in *cdc13 rif1*Δ cells

If telomeric ssDNA accumulation contributes to checkpoint activation in *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells, then mutations reducing ssDNA generation should alleviate the arrest and relieve the lethality caused by the lack of Rif1 in *cdc13-1* and *cdc13-5* background. Because the Exo1 nuclease contributes to generate telomeric ssDNA in *cdc13-1* cells [47], we examined the effect of deleting *EXO1*in *cdc13 rif1*Δ cells. When G2-arrested cell cultures at 20°C were transferred to 25°C for 3 hours, *cdc13-1 rif1*Δ *exo1*Δ triple mutant cells contained significantly lower amounts of telomeric ssDNA than *cdc13-1 rif1*Δ cells (Figure 6B). A similar behaviour of the triple mutant was detectable even when G2-arrested cultures where kept at 20°C, although the quantity of telomeric ssDNA accumulated by *cdc13-1 rif1*Δ cells at this temperature was lower than at 25°C (Figure 6B). Furthermore, *EXO1* deletion partially suppressed both the temperature-sensitivity of *cdc13-1 rif1*Δ cells (Figure 7A) and the loss of viability of*cdc13-5 rif1*Δ cells (Figure 7B), further supporting the hypothesis that reduced viability in these strains was due to defects in telomere protection.

Figure 7: EXO1 deletion partially suppresses cell lethality and checkpoint activation in cdc13 rif1Δ cells.

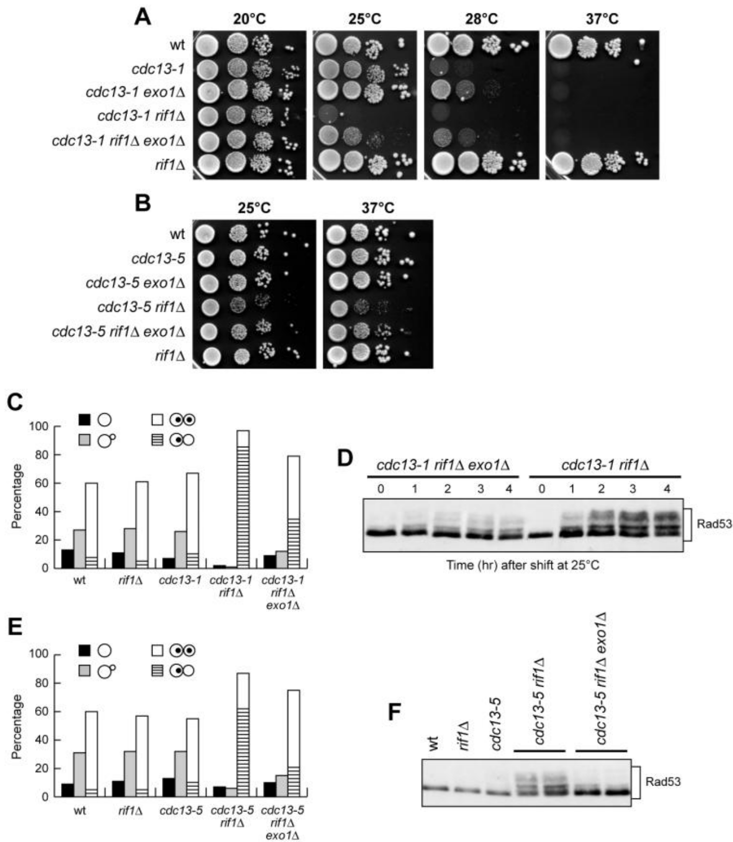

Figure 7: (A–B) Serial 10-fold dilutions of cell cultures grown overnight in YEPD at 20°C (A) and 25°C (B) were spotted onto YEPD plates and incubated at the indicated temperatures for 2–4 days. (C–D) Cell cultures exponentially growing in YEPD at 20°C were shifted to 25°C. (C) The frequencies of cells with no, small or large buds and of large budded cells with one or two nuclei were determined after 3 hours at 25°C as in Figure 3F, by analyzing a total of 200 cells for each strain. (D) Western blot analysis with anti-Rad53 antibodies of total protein extracts prepared at the indicated times. This experiment was repeated three times with similar results. (E–F) Cultures of cells with the indicated genotypes, exponentially growing at 25°C in YEPD, were analyzed as in panels C (E) and D (F), respectively. Two independent *cdc13-5 rif1*Δ and *cdc13-5 rif1*Δ *exo1*Δ strains were analyzed for Rad53 phosphorylation.

Exo1-mediated suppression of the *cdc13 rif1Δ* growth defects correlated with alleviation of checkpoint-mediated cell cycle arrest. In fact, when cell cultures exponentially growing at 20°C were incubated at 25°C for 3 hours, the amount of both metaphase-arrested cells and Rad53 phosphorylation was reproducibly lower in *cdc13-1 rif1Δ exo1Δ* cells than in *cdc13-1 rif1Δ* cells (Figure 7C and 7D). Similar results were obtained also with *cdc13-5 rif1Δ exo1Δ* cells growing at 25°C, which accumulated less metaphase-arrested cells and phosphorylated Rad53 than similarly treated *cdc13-5 rif1Δ* cells (Figure 7E and 7F). Thus, both cell lethality and checkpoint-mediated cell cycle arrest in cdc13 *rif1Δ* cells appear to be caused, at least partially, by Exo1-dependent telomere DNA degradation.

The lack of Rif1 does not enhance the checkpoint response to CST-independent capping deficiencies or to an irreparable DSB

The lack of Rif1 might increase the lethality of cells with reduced CST activity just because it causes a telomere deprotection defect that exacerbates the inherent telomere capping defects of *cdc13* or *stn1*mutants. If this hypothesis were correct, *RIF1* deletion should affect viability also of other non-CST mutants defective in end protection. Alternatively, Rif1-CST functional interaction might be specific, thus reflecting a functional connection between Rif1 and CST. To distinguish between these two possibilities, we analyzed the effects of deleting *RIF1* in Yku70 lacking cells, which display Exo1-dependent accumulation of telomeric ssDNA, as well as checkpoint-mediated cell cycle arrest at elevated temperatures (37°C) [47,51]. Loss of Yku in *est2Δ* cells, which lack the telomerase catalytic subunit, leads to synthetic lethality, presumably due to the combined effects of telomere shortening and capping defects [48-50,52]. As expected [53], *yku70*Δ cells were viable at 25°C and 30°C, but they were unable to form colonies at 37°C (Figure 8A). Similarly, *yku70*Δ *rif1*Δ double mutant cells grew well at 25°C and 30°C (Figure 8A) and did not show Rad53 phosphorylation when grown at 25°C (Figure 8B, time 0).

Furthermore, similar amounts of phosphorylated Rad53 were detected in both *yku70*Δ and *yku70*Δ *rif1*Δ cell cultures that were kept at 37°C for 4 hours (Figure 8B), indicating that loss of Rif1 does not enhance the telomere protection defects already present in *yku70*Δ cells. Consistent with a previous observation [54], *RIF1* deletion partially suppressed the temperature sensitivity (Figure 8A) and the telomere length defect (data not shown) caused by the lack of Yku70, suggesting that the elongated state of the telomeres could be the reason why *yku70*Δ *rif1*Δ cells can proliferate at 37°C.

Checkpoint activation can also be induced during telomere erosion caused by insufficient telomerase activity [55,56]. Thus, we asked whether *RIF1* deletion accelerated senescence progression and/or upregulated checkpoint activation in cells lacking the telomerase catalytic subunit Est2. Meiotic tetrads were dissected from a diploid strain heterozygous for the *est2*Δ and *rif1*Δ alleles, which are recessive and therefore do not affect telomere length in the diploid. After 2 days of incubation at 25°C (approximately 25 generations), spore clones from the dissection plate were both streaked for 4 successive times (Figure 8C) and propagated in YEPD liquid medium to prepare protein extracts for Rad53 phosphorylation analysis at different time points (Figure 8D). Similar to what was previously observed[44], *RIF1* deletion did not accelerate senescence progression in *est2*Δ cells, as *est2*Δ *rif1*Δ clones showed a decline in growth similar to that of *est2*Δ clones (Figure 8C). Furthermore, *est2*Δ and *est2*Δ *rif1*Δ cell cultures showed similar patterns of Rad53 phosphorylation with increasing number of generations (Figure 8D). Thus, the lack of Rif1 does not enhance either DNA damage checkpoint activation or senescence progression during telomere erosion caused by the lack of telomerase.

Finally, because the telomerase machinery is known to be recruited to an unrepaired DSB [57], we ruled out the possibility of a general role for Rif1 in inhibiting checkpoint activation by examining activation/deactivation of the checkpoint induced by an unrepaired DSB. To this end, we used JKM139 derivative strains, where a single DSB can be generated at the *MAT* locus by expressing the site-specific HO endonuclease gene from a galactose-inducible promoter [58]. This DSB cannot be repaired by homologous recombination, because the homologous donor sequences *HML* or *HMR* are deleted.

Figure 8: RIF1 deletion does not influence the checkpoint response to CST-independent telomere capping deficiencies or to an irreparable DSB.

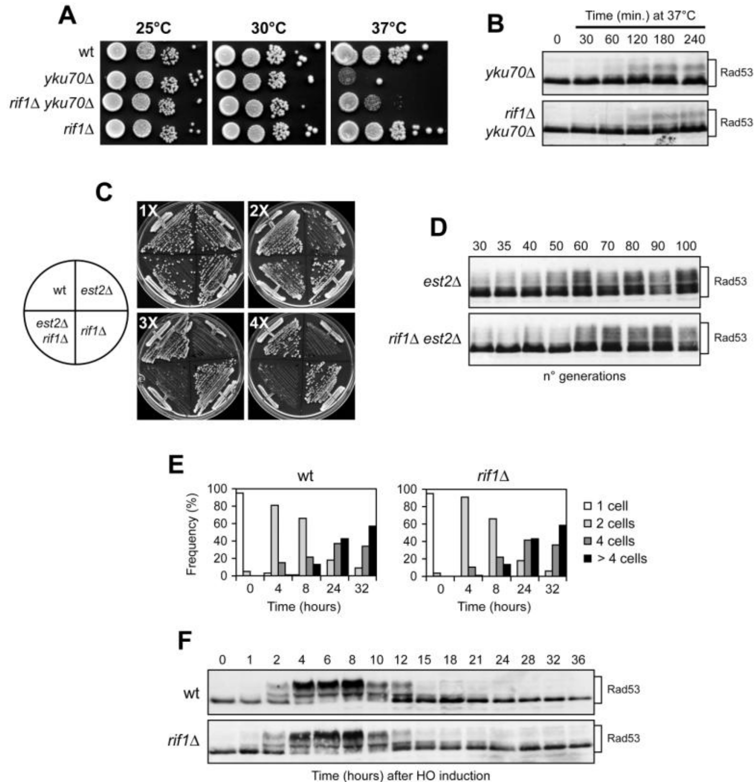

Figure 8: (A) Cell cultures with the indicated genotypes were grown overnight in YEPD at 25°C. Serial 10-fold dilutions were spotted onto YEPD plates and incubated at the indicated temperatures for 2–3 days. (B) Cell cultures exponentially growing in YEPD at 25°C were shifted to 37°C at time 0. Rad53 was visualized at the indicated time points as inFigure 3E. (C) Meiotic tetrads from a *EST2/est2ΔRIF1/rif1Δ* diploid strain were dissected on YEPD plates. After ~25 generations, spore clones from 20 tetrads were subjected to genotyping and to four successive streak-outs (1X to 4X), corresponding to ~25, ~50, ~75 and ~100 generations of growth, respectively. All tetratype tetrads behaved as the one shown in this panel. (D) After ~25 generations of growth on the dissection plates, *est2Δ* and *est2Δ rif1Δ* spore clones were propagated in liquid YEPD medium. At the indicated generations, protein extracts were subjected to western blot analysis with anti-Rad53 antibodies. (E–F) Checkpoint response to an irreparable DSB. (E) YEP+raf G1-arrested cell cultures of wild type JKM139 and its isogenic *rif1Δ* derivative strain were spotted on galactose-containing plates that were incubated at 28°C (time zero). At the indicated time points, 200 cells for each strain were analyzed to determine the frequency of single cells and of cells forming microcolonies of 2, 4 or more than 4 cells. (F) Galactose was added at time zero to cell cultures of the strains in panel E exponentially growing in YEP+raf. Protein extracts were subjected to western blot analysis with anti-Rad53 antibodies.

As shown in Figure 8E, when G1-arrested cell cultures were spotted on galactose containing plates, both wild type and *rif1*Δ JKM139 derivative cells overrode the checkpoint-mediated cell cycle arrest within 24–32 hours, producing microcolonies with 4 or more cells. Moreover, when galactose was added to exponentially growing cell cultures of the same strains, Rad53 phosphorylation became detectable as electrophoretic mobility shift in both wild type and *rif1*Δ cell cultures about 2 hours after HO induction, and it decreased in both cell cultures after 12–15 hours (Figure 8F), when most cells resumed cell cycle progression (data not shown). Thus, Rif1 does not affect the checkpoint response to an irreparable DSB. Altogether these data indicate that Rif1 supports specifically CST functions in telomere protection.

Discussion

Both shelterin and CST complexes are present in a wide range of unicellular and multicellular organisms, where they protect the integrity of chromosomes ends (reviewed in [38]). Thus, the understanding of their structural and functional connections is an important issue in telomere regulation. We have approached this topic by analysing the consequences of disabling the shelterin-like *S. cerevisiae* proteins Rif1 or Rif2 in different hypomorphic mutants defective in CST components. We provide evidence that Rif1, but not Rif2, is essential for cell viability when the CST complex is partially compromised. In fact, *RIF1* deletion exacerbates the temperature sensitivity of *cdc13-1* mutant cells that are primarily defective in Cdc13 telomere capping functions. Furthermore, cells carrying the *cdc13-5* or the *stn1ΔC* mutation, neither of which causes per se DNA damage checkpoint activation and growth defects [24], [26], grow very poorly or are unable to form colonies, respectively, when combined with the *rif1*Δ allele. By contrast, *RIF1* deletion does not affect either viability or senescence progression of *cdc13-2* cells, which are specifically defective in telomerase recruitment. This Cdc13 function is not shared by the other CST subunits, suggesting that Rif1 is specifically required to support the essential capping functions of the CST complex.

Cell lethality caused by the absence of Rif1 in both *cdc13-1* and *cdc13-5* cells appears to be due to severe telomere integrity defects. In fact, telomeres in

both *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ double mutant cells display an excess of ssDNA that leads to DNA damage checkpoint activation. Deleting the nuclease *EXO1* gene partially restores viability of *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells and reduces the level of telomeric ssDNA in *cdc13-1 rif1*Δ cells, indicating that cell lethality in *cdc13 rif1*Δ cells is partially due to Exo1-dependent telomere DNA degradation and subsequent activation of the DNA damage checkpoint.

Although Rif1 and Rif2 interact both with the C-terminus of Rap1 and with each other [29,30], our finding that only Rif1 is required for cell viability when Cdc13 or Stn1 capping activities are reduced indicates that Rif1 has a unique role in supporting CST capping function that is not shared by Rif2. Earlier studies are consistent with the idea that Rif1 and Rif2 regulate telomere metabolism by different mechanisms [30,31,35]. Furthermore, while the content of Rif2 is lower at shortened than at wild type telomeres, the level of Rif1 is similar at both, suggesting that these two proteins are distributed differently along a telomere [59]. Finally, inhibition of telomeric fusions requires Rif2, but not Rif1 [32].

Noteworthy, although *RIF1* deletion is known to cause telomere overelongation [29], the synthetic interaction between Rif1 and CST occurs independently of *rif1*Δ-induced alterations in telomere length. In fact, the lack of Tel1, which counteracts *rif1Δ*-induced telomere overelongation [45], does not alleviate the growth defects of *cdc13 rif1Δ* cells. Furthermore, deletion of *RIF2*, which enhances telomere elongation induced by the lack of Rif1 [30], does not exacerbate the synthetic phenotypes of *cdc13 rif1Δ* double mutant cells. Thus, loss of viability in *cdc13 rif1*Δ cells is not due to telomere overelongation caused by *RIF1* deletion, but it is a direct consequence of Rif1 loss.

By analyzing the effects of combining *RIF1* deletion with mutations that cause telomere deprotection without affecting CST functions, we found that the functional interaction between Rif1 and the CST complex is highly specific. In fact, the lack of Rif1 does not enhance the DNA damage checkpoint response in telomerase lacking cells, which are known to experience gradual telomere erosion leading to activation of the DNA damage checkpoint [55,56]. Furthermore, *RIF1* deletion does not upregulate DNA damage checkpoint activation in *yku70*Δ cells, which display Exo1-dependent accumulation of ssDNA and checkpoint-mediated cell cycle arrest at 37°C [47-51]. This is

consistent with previous observations that comparable signals for G strand overhangs can be detected on telomeres derived from *yku70*Δ and *yku70*Δ *rif1*Δ cells [54], indicating that *RIF1* deletion does not exacerbate the end protection defect due to the absence of Yku. By contrast, the lack of Rif1 partially suppresses both temperature-sensitivity and telomere shortening in *yku70*Δ cells (Figure 8A) [54], possibly because the restored telomere length helps to compensate for *yku70*Δ capping defects. Notably, although *RIF1* deletion leads to telomere overelongation in *cdc13-1* and *cdc13-5* mutants, this elongated telomere state does not help to increase viability in *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells.

The simplest interpretation of the specific genetic interactions we found between Rif1 and CST is that a functional connection exists between Rif1 and the CST complex, such that Rif1 plays a previously unanticipated role in assisting the CST complex in carrying out its essential telomere protection function. Indeed, this functional interaction is unexpected in light of Rif1 and CST localization along a telomere. In fact, while CST is present at the very ends of chromosomes, Rif1 is thought to be distributed centromere proximal on the duplex telomeric DNA [59]. However, as yeast telomeres have been proposed to fold back onto the subtelomeric regions to form a ∼3-kb region of core heterochromatin [60,61], this higher-order structure could place Rif1 and CST in close proximity, thus explaining their functional interaction.

The function of Rif1 in sustaining CST activity cannot be simply attributable to the Rif1-mediated suppression of ssDNA formation at telomeres, as *rif1*Δ cells show only a very slight increase in ssDNA at both native (Figure 6) and HO-induced telomeres [33] compared to wild type. Furthermore, although deletion of Rif2 leads to increased amounts of telomeric ssDNA [33], *cdc13-1 rif2*Δ, *cdc13-5 rif2*Δ and*stn1*Δ*C rif2*Δ double mutants are viable and do not display growth defects. Finally, other mutants defective in telomere capping or telomere elongation (*yku70*Δ and *est2*Δ) are perfectly viable in the absence of Rif1.

One possibility is that Rif1 physically interacts, directly or indirectly, with the CST complex. Indeed, human Stn1 was found to copurify with the shelterin subunit TPP1 [62], suggesting the existence of CST-shelterin complexes in mammals. Unfortunately, we were so far unable to coimmunoprecipitate Rif1 with Cdc13 or Stn1, and further analyses will be required to determine

whether Rif1 and the CST complex undergo stable or transient association during the cell cycle.

Indeed, not only 5′-3′ resection, but also incomplete synthesis of Okazaki fragments is expected to increase the size of the G tail during telomere replication. The yeast CST complex genetically and physically interacts with the polα-primase complex [7,22,25] and the human CST-like complex increases polα-primase processivity [63,64]. Furthermore, the lack of CST function in G1 and throughout most of S phase does not lead to an increase of telomeric ssDNA [13], suggesting that the essential function of CST is restricted to telomere replication in late S phase. Altogether, these observations suggest that CST may control overhang length not only by blocking the access of nucleases, but also by activating polα-primase-dependent C-strand synthesis that can compensate G tail lengthening activities. Based on the finding that Rif1 regulates telomerase action and functionally interacts with the polα-primase complex (Figure 2), it is tempting to propose that Rif1 favours CST ability to replenish the exposed ssDNA at telomeres through activation/recruitment of polα-primase, thus coupling telomerase-dependent elongation to the conventional DNA replication process.

The recent discoveries that human TPP1 interacts physically with Stn1 [62] and that CST-like complexes exist also in *S. pombe*, plants and mammals [65-68] raise the question of whether functional connections between the two capping complexes exist also in other organisms. As telomere protection is critical for preserving genetic stability and counteracting cancer development, to address this question will be an important future challenge.

Materials and Methods

Strains and plasmids

Strain genotypes are listed in supplementary (See table). Unless otherwise stated, the yeast strains used during this study were derivatives of W303 (*ho MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3 can1-100*). All gene disruptions were carried out by PCR-based methods. The *cdc13-1* mutant was kindly provided by D. Lydall (University of Newcastle, UK). The *cdc13-2* mutant was kindly provided by V. Lundblad (Salk Institute, La Jolla, USA). The *stn1ΔC* and *cdc13-5* alleles carried a stop codon following amino acids 282 and 694 respectively [24], [25], and were generated by PCR-based methods. Wild type and *cdc13-1* strains carrying either the 2 µ vector or 2 µ *RIF1* plasmid were constructed by transforming wild type and *cdc13-1* strains with plasmids YEplac195 (2 µ *URA3*) and pML435 (2 µ *RIF1 URA3*), respectively. The strains used for monitoring checkpoint activation in response to an irreparable DSB were derivatives of strain JKM139 (*MATa ho hmlΔ hmrΔ ade1 lys5 leu2-3,112 trp1::hisG ura3-52 ade3::GAL-HO*), kindly provided by J. Haber (Brandeis University, Waltham, MA, USA) [58]. To induce *HO* expression in JKM139 and its derivative strains, cells were grown in raffinose-containing yeast extract peptone (YEP) and then transferred to raffinose- and galactose-containing YEP.

Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) supplemented with 2% glucose (YEPD) or 2% raffinose (YEP+raf) or 2% raffinose and 2% galactose (YEP+raf+gal). Synthetic complete medium lacking uracil supplemented with 2% glucose was used to maintain the selective pressure for the 2 µ *URA3* plasmids.

Saccharomyces cerevisiae strains used in this study

Strain	Relevant genotype
W303	*MAT*a *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad5-535*
DMP5108/19A	W303 *cdc13-1*
YLL2794	W303 *cdc13-5::KANMX4*
cdc17-1	W303 *cdc17-1*
pol1-1	W303 *pol1-1*
pri2-1	W303 *pri2-1*
YLL1223	W303 *rif1Δ::HIS3*
DMP5178/1B	W303 *rif1Δ::KANMX4 tel1Δ::HIS3*
YLL1221	W303 *rif1Δ::KANMX4*
YLL1137	W303 *rif2Δ::HIS3*
YLL1134	W303 *rif2Δ::KANMX4*
YLL2804	W303 *stn1ΔC::KANMX4*
YLL939	W303 *yku70Δ:: KANMX4*
YLL2897	W303 + YEplac195 [2μ *URA3*]
YLL2898	W303 + pML435 [2μ *RIF1 URA3*]
YLL2899	W303 *cdc13-1* + YEplac195 [2μ *URA3*]
YLL2900	W303 *cdc13-1* + pML435 [2μ *RIF1 URA3*]
DMP5126/5C	W303 *cdc13-1 rif1Δ::HIS3*
DMP5181/2A	W303 *cdc13-1 tel1Δ::HIS3*
DMP1911/1A	W303 *cdc13-1 rad9Δ::URA3*

DMP5183/3B	W303 *cdc13-1 rif1Δ::HIS3 rad9Δ::URA3*
DMP5179/4B	W303 *cdc13-1 rif1Δ::HIS3 rif2Δ::KANMX4*
DMP5180/6C	W303 *cdc13-1 rif1Δ::KANMX4 tel1Δ::HIS3*
DMP5128/35D	W303 *cdc13-1 rif1Δ::KANMX4 exo1Δ::HIS3*
DMP5108/20A	W303 *cdc13-1 rif2Δ::KANMX4*
DMP5159/17D	W303 *cdc13-5::KANMX4 exo1Δ::LEU2*
DMP5137/1C	W303 *cdc13-5::KANMX4 rif1Δ::HIS3*
DMP5190/4C	W303 *cdc13-5::KANMX4 tel1Δ::HIS3*
DMP5189/3D	W303 *cdc13-5::KANMX4 rif1Δ::KANMX4 tel1Δ::HIS3*
DMP5160/15B	W303 *cdc13-5::KANMX4 rif1Δ::HIS3 exo1Δ::LEU2*
DMP5100/1A	W303 *cdc13-5::KANMX4 rif2Δ:: HIS3*
DMP5173/5C	W303 *cdc17-1 rif1Δ::HIS3*
DMP5172/8A	W303 *pol1-1 rif1Δ::HIS3*
DMP5171/3B	W303 *pri2-1 rif1Δ::HIS3*
DMP5157/2C	W303 *yku70Δ::KANMX4 rif1Δ::HIS3*
YVL2993	*MAT*a/α *CDC13/cdc13-2 ura3-52/ura3-52 lys2-801/lys2-801 trp1Δ1/trp1Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1*
YLL2701	*MAT*a/α *CDC13/cdc13-2 ura3-52/ura3-52 lys2-801/lys2-801 trp1Δ1/trp1Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 RIF1/rif1Δ::HIS3*
DMP5161/5B	W303 *MAT*a/α *EST2/est2Δ::KANMX4 RIF1/rif1Δ::HIS3*
JKM139	*MAT*a *ho hmlΔ::ADE1 hmrΔ::ADE1 ura3-52 leu2-3,112 trp1::hisG lys5 ade1 ade3::GAL::HO*
YLL2700	JKM139 *rif1Δ::HIS3*

Plasmids are indicated by brackets. All strains are from this study except the strains mentioned below.

cdc17-1: Carson MJ, Hartwell L (1985) *CDC17*: an essential gene that prevents telomere elongation in yeast. Cell 42: 249-257.

pol1-1: Pizzagalli A, Valsasnini P, Plevani P, Lucchini G (1988) DNA polymerase I gene of Saccharomyces cerevisiae: nucleotide sequence, mapping of a temperature-sensitive mutation, and protein homology with other DNA polymerases. Proc Natl Acad Sci USA 85: 3772-3776.

pri2-1: Longhese MP, Jovine L, Plevani P, Lucchini G (1993) Conditional mutations in the yeast DNA primase genes affect different aspects of DNA metabolism and interactions in the DNA polymerase α-primase complex. Genetics 133: 183-191.

YVL2993: Nugent CI, Hughes TR, Lue NF, Lundblad V (1996) Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. Science 274: 249-252.

JKM139: Lee SE, Moore A, Holmes JK, Umezu K, Kolodner RD., Haber JE (1998) Saccharomyces Ku70, Mre11/Rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage. Cell 94: 399–409.

Southern blot analysis of telomeres and in-gel hybridization

Genomic DNA was digested with *Xho*I. The resulting DNA fragments were separated by electrophoresis on 0.8% agarose gel and transferred to a GeneScreen nylon membrane (New England Nuclear, Boston), followed by hybridization with a ^{32}P-labelled poly(GT) probe and exposure to X-ray sensitive films. Standard hybridization conditions were used. Visualization of single-stranded overhangs at native telomeres was done by in-gel hybridization [9], using a single-stranded 22-mer CA oligonuleotide probe. The same DNA samples were separated on a 0.8% agarose gel, denatured and hybridized with an end-labeled C-rich oligonucleotide for loading control.

Other techniques

For western blot analysis, protein extracts were prepared by TCA precipitation. Rad53 was detected using anti-Rad53 polyclonal antibodies kindly provided by J. Diffley (Clare Hall, London, UK). Secondary antibodies were purchased from Amersham and proteins were visualized by an enhanced chemiluminescence system according to the manufacturer. Flow cytometric DNA analysis was determined on a Becton-Dickinson FACScan on cells stained with propidium iodide.

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Discussion

The DNA damage proteins function also at telomeres to regulate telomere length and stability. At the same time, telomeres should be differentiated from a DSB to avoid DNA damage checkpoint activation or harmful telomere-telomere fusions and HR which might lead to aneuploidy. Since ssDNA is a strong signal for checkpoint activation and triggers DSB repair by HR, formation of ssDNA at the telomeric tips should be strictly regulated. As telomeric repeats are bound by the shelterin-like proteins, one possibility is that these proteins might inhibit resection. To test this possibility, we studied resection at an HO-induced *de novo* telomere in strains lacking the shelterin-like proteins. We found that loss of Rif1 or Rif2, as well as deletion of Rap1 C-terminus, promotes C-rich strand degradation in G1 and enhances it in the G2 cell cycle phase. The Yku70/Yku80 capping protein complex also prevents telomere resection in G1 phase. Resection in G1-arrested *yku70Δ* cells is restricted to DNA regions closest to the telomeric tips, likely due to the inhibitory action of Rap1, Rif1 and Rif2, whose inactivation increases telomere processing in *yku70Δ* G1 cells. These data suggest that Yku is mainly involved in inhibiting initiation of resection, whereas Rif1, Rif2 and Rap1 act primarily by limiting extensive resection. Resection in the absence of Yku, Rif2, Rap1 or Rif1 takes place in G1 cell cycle phase independently of the low Cdk1 activity. This indicates that Cdk1 requirement for telomere resection can be bypassed by inactivation of negative regulators at telomeres.

As Rap1, Rif1, Rif2 and Yku proteins protect the *de novo* telomere from degradation in both G1 and G2 cells, we also investigated whether they exert the same action also at native telomeres by using nondenaturing *in gel* hybridization technique. As expected, no telomeric ssDNA signals were detectable in wild type cells either G1-arrested or cycling. In contrast, single-stranded G tail signals appeared in G1-arrested *yku70Δ* cells even at 23°C, and their intensity increased after incubation at 37°C, thus highlighting an important role of Yku in protecting native telomeres in G1. Rap1 and Rif2 are also essential to inhibit exonucleolytic degradation at native telomeres. We also identified the nucleases involved in generation of telomeric ssDNA. Exo1 nuclease is required to generate ssDNA at both native and HO-induced telomeres in *yku70Δ* G1 cells, whereas ssDNA generation depends primarily on MRX in both *rap1ΔC* and *rif2Δ* cells in which recruitment of the MRX subunit Mre11 to the HO-induced telomere is enhanced. Thus, we can

conclude that while Yku protects telomeres from Exo1 action, the shelterin-like proteins prevent telomere degradation by inhibiting MRX loading onto telomeric ends.

In our work, we observed that Rif1 has a minor role in telomere protection compared to Rif2 and Rap1. Thus, we tested whether the role of Rif1 in telomere capping can be masked by the CST complex. At that time, Diego Bonetti, a post-doctoral researcher in our lab, observed that *cdc13-1* was synthetically lethal with *rif1Δ*, indicating a strong functional interaction between Rif1 and Cdc13. This observation was quite surprising, because *rif1Δ* was not found during a previous genome wide search for gene deletions enhancing the temperature-sensitivity of *cdc13-1* cells [209]. However, that screening was done at 20°C, a temperature at which *cdc13-1 rif1*Δ double mutants do not show severe growth defects. We also found that, unlike *RIF2* deletion, the lack of Rif1 is lethal not only in *cdc13-1* cells, but also when combined with *stn1ΔC* cells and causes a dramatic reduction in viability of *cdc13-5* mutants. By contrast, *RIF1* deletion does not enhance checkpoint activation in case of CST-independent telomere capping deficiencies, such as those caused by the absence of Yku or telomerase, indicating that the functional interaction between Rif1 and the CST complex is specific. Both *cdc13-1 rif1*Δ and *cdc13-5 rif1*Δ cells display very high amounts of telomeric single-stranded DNA and DNA damage checkpoint activation, both of them partially suppressed by deletion of Exo1 nuclease. These observations indicate that the severe defects in telomere integrity in *cdc13-1 rif1*Δ cells are the cause for their loss of viability.

An hypothesis for the specific genetic interaction between cdc13-1 and rif1Δ.

Cdc13, together with the other subunits of the CST complex, is required for the Polα-Primase priming during replication of the lagging strand telomere. Interestingly, the fission yeast and human telomeric ssDNA binding protein Pot1 (the structural homologues of Cdc13) can bind the ssTERRA RNA molecules [210]. This observation raises the possibility that also Cdc13 might bind to TERRA. If these were the case, the binding of Cdc13 or Cdc13-1 to TERRA can titrate Cdc13 or Cdc13-1 from the lagging strand telomeric DNA and this could lead to a defective lagging strand replication and increased telomeric ssDNA, which causes checkpoint activation. Combining *cdc13-1* with *rif1Δ*, which is known to cause an increase of TERRA transcription, can increase the amount of TERRA-Cdc13-1 molecules that can lead to a severe defects in telomeric lagging strand replication. Thus the synthetic interaction between *cdc13-1* and *rif1Δ* might be due to defective telomeric lagging strand replication.

Interestingly, *cdc13-1 rif1Δ* cells are viable at 20°C, whereas they lose viability at 25°C. In mammalian cells, the G rich telomeric TERRA RNA can form intramolecular G4 structures that can inhibit the binding of Cdc13 to TERRA [77]. G4 structures are known to form more efficiently at lower temperature than at higher temperature *in vitro*. Like G4 DNA, the stability of TERRA G4 structures can be influenced by temperatures and salt concentrations, such as that TERRA might form G4 structures more efficiently at 20°C than at 25°C. If these were the case, then the amount of Cdc13-1 bound to TERRA will be reduced at 20°C compared to 25°C, even if there is an increased amount of TERRA due to lack of *RIF1* (see figure 13). If TERRA G4 structures are unstable at 25°C due to temperature-dependent intracellular changes, then more Cdc13-1 molecules will be bound to TERRA, and this will be further increased by the *rif1Δ*-induced TERRA transcription. This will lead to defective Cdc13 dependent lagging strand replication that impairs cell viability (see figure 14).

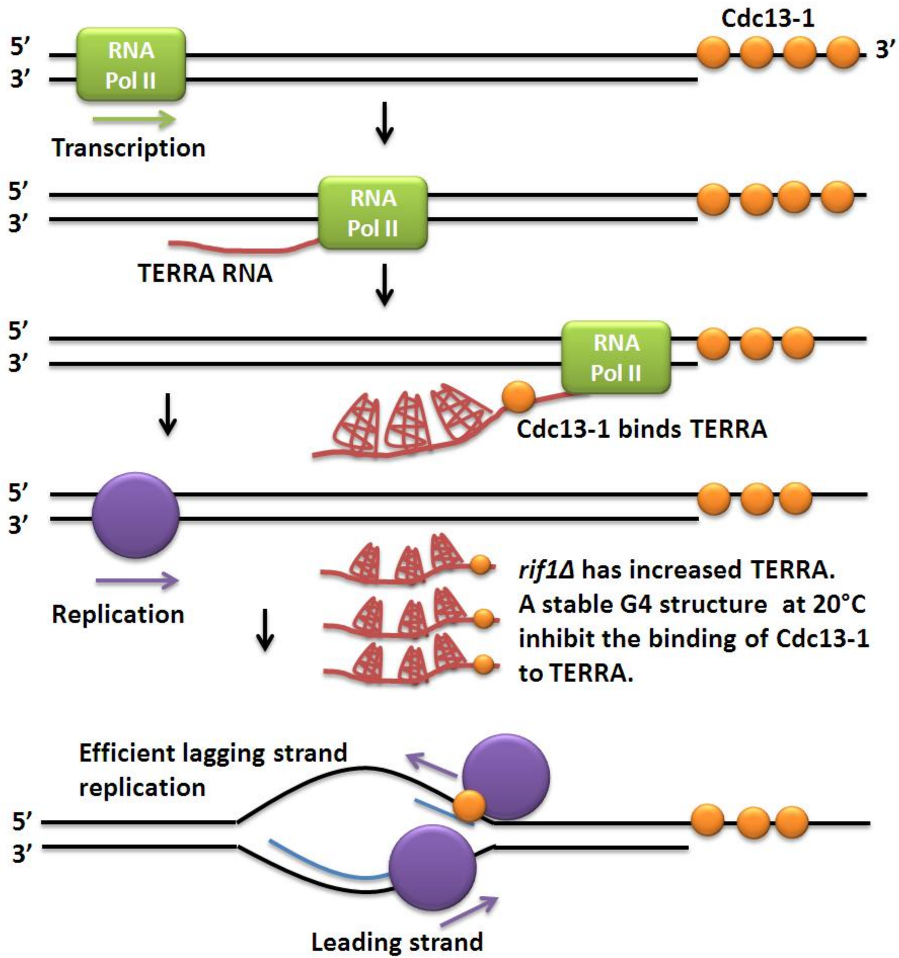

Figure 13: Model, part 1. Telomeric transcription leads to TERRA formation. TERRA can titrate the Cdc13 and Cdc13-1 molecules from the telomeric ssDNA overhangs. At 20°C, TERRA molecules might form G4 structures that inhibit the binding of Cdc13 and Cdc13-1. The lack of *RIF1* causes an increase in TERRA transcription. However, formation of G4 TERRA structures decreases the amount of Cdc13 or Cdc13-1 bound to TERRA, such that lagging strand synthesis can occur efficiently.

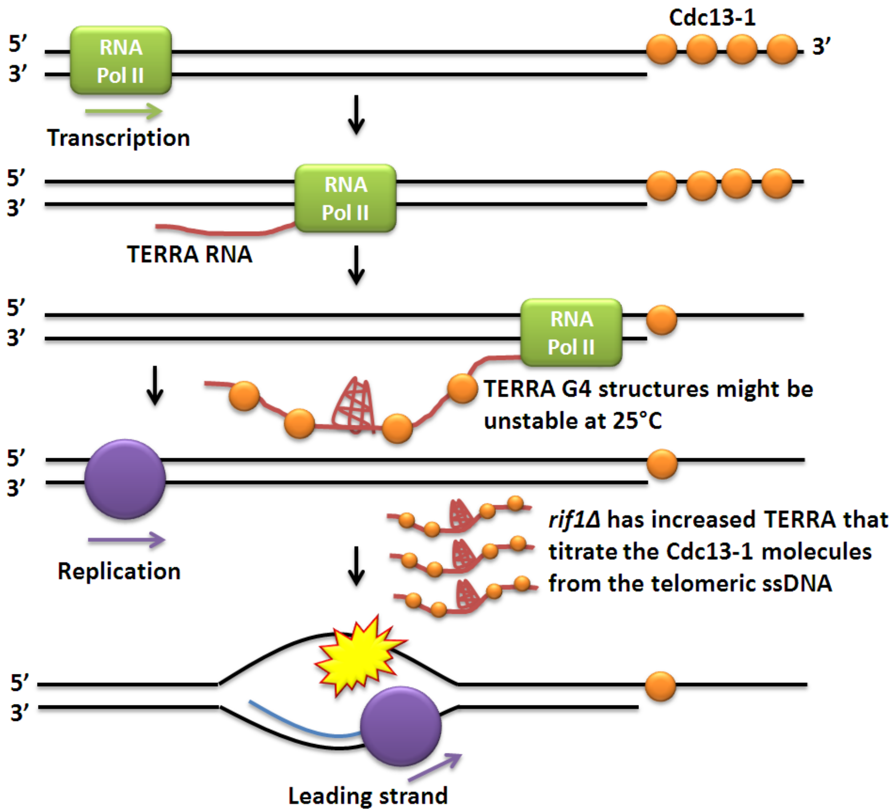

Figure 14: Model, part 2. At 25°C, the TERRA G4 structures might be unstable due to temperature mediated intracellular changes. The lack of *RIF1* increases TERRA formation and this leads to an increase amount of Cdc13-1 bound to TERRA that can titrate Cdc13-1 from the telomeric DNA. This can affect Cdc13-dependent priming of the lagging strand synthesis that causes increased telomeric ssDNA and checkpoint activation.

Future perspectives

In vitro studies

In vitro studies should be performed to determine if Cdc13 and its mutant versions (i.e. Cdc13-1) can bind TERRA and whether this binding can be influenced by temperatures or salt concentrations.

Telomere replication and transcription?

Complementary RNA molecules of TERRA, namely ARRET, comprising of subtelomeric sequence but devoid of telomeric repeats, have been reported. Their formation might be due to the presence of telomeric overhang with reduced 5’ C rich telomeric template for transcription [75]. ARRET can be formed only if transcription is initiated near the ends of telomeric 3’ G rich or if RNA Pol II transcription at the 5’ C rich strand could switch template and continue transcription. E. coli and mammalian RNA Pol II can switch DNA templates by means of end-to-end transposition without loss of the transcript [211]. Can such a mechanism occur at telomeres?

How exactly telomere replication and transcription are regulated? In wild type cells, it seems that transcription occurs before telomere replication. But does it hold true at telomeres of different length? Does it differ in mutants of telomeric genes? Can transcription occur after telomere replication? Stalled replication forks might have more time to be transcribed by RNA Pol II. This suggests the existence of a robust mechanism that inhibit transcription either physically or by forming particular structures.

TERRA as primer?

If a replication fork collides behind a transcription fork (Co-directional collision) at the leading strand, the stalled fork can be restarted by using mRNA as a primer [212]. TERRA is upregulated when nonsense-mediated mRNA decay (NMD) machinery is impaired. A defect in the NMD pathway in budding yeast cells leads to misregulation of mRNA decay of several proteins including telomeric proteins [213]. The NMD proteins can have a direct role at telomeres because in mammalian cells they can bind to telomeres and telomerase [214,215].

Telomeric G4

Apart from protective role, in *in vitro* primer extension assays to study telomere elongation, intermolecular G4 are highly stable and are excellent substrates when compared to intramolecular G4 for ciliate telomerase. From yeast to humans, telomerase could form dimers or multimers and this has been shown to regulate the catalytic activity of telomerase enzyme [216,217]. It should be determined if telomerase dimerization can elongate more than one telomere simultaneously.

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