Chapter

Telomerase in Space and Time: Regulation of Yeast Telomerase Function at Telomeres and DNA Breaks

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Abstract

A development of new strategies against telomerase-associated disorders, such as dyskeratosis congenita, aplastic anemia or cancer, relies on a detailed understanding of telomerase life cycle and the multiple layers of its regulation. *Saccharomyces cerevisiae* is a prime model to study telomerase function and it has already revealed many conserved pathways for telomerase biology. In this chapter, we review the current knowledge of the regulatory pathways that control telomerase function in budding yeast. In particular, we discuss the cell cycle-dependent assembly of telomerase and its recruitment to telomeres. We also focus on the mechanisms that target telomerase to short telomeres. Finally, we discuss possible pathways that inhibit telomerase function at DNA double-strand breaks, thus limiting deleterious *de novo* telomere addition events.

Keywords: telomerase, cell cycle, regulation, RNP biogenesis

1. Introduction

Eukaryotic chromosomal DNA must be completely duplicated for both daughter cells to receive a full complement of DNA during cell division. Given the inherent properties of the conventional replication machinery, a newly replicated lagging-strand DNA is always slightly shorter than the template parental strand at the ends of chromosomes [1, 2]. As a result, the lagging-strand chromosome end acquires a short 3′-overhang—a conserved feature of ends of linear chromosomes crucial for genome stability. At the same time, the leading-strand ends are initially generated as blunt-ended, and will therefore need to be resected to restore the 3′-overhang [3–5]. This process is repeated during each replication cycle and will inevitably lead to a progressive shortening of the chromosome ends and loss of vital genetic information [6, 7].

In order to meet these end-replication challenges, chromosome ends are capped with a stretch of noncoding DNA repeats, called telomeres. The actual length of these repeat stretches within one cell is not uniform, and slight length variations can occur without any consequences for cell viability and fitness [8]. Moreover, shortened tracts that reach a lower limit of functionality can be restored to a longer form by a specialized reverse transcriptase, called telomerase [9, 10]. This

ribonucleoprotein (RNP) enzyme synthesizes new telomeric repeats using its intrinsic protein catalytic subunit and a segment of its internal RNA as a template.

Given its essential task in maintaining genome stability, it is not surprising that telomerase function is tightly controlled at multiple levels. Disruption of this fine-tuned regulation leads to telomerase malfunction or its unregulated expression, which may contribute to serious genetic disorders such as dyskeratosis congenita, aplastic anemia and cancer [11].

Historically, budding yeast has been an extremely rich source of information on the biology of telomeres and telomerase. Therefore, in this review we discuss the intricate network of *Saccharomyces cerevisiae* regulatory mechanisms, that cooperate to ensure timely and sufficient telomerase activity at telomeres. In particular, we focus on the cell cycle-dependent regulation of telomerase assembly and its recruitment to telomeres. We also discuss the mechanisms that target telomerase to short telomeres, which pose a major threat to genome stability, and therefore must be dealt with immediately. Finally, we describe the mechanisms that limit telomerase function at DNA double-strand breaks (DSBs), thereby preventing deleterious *de novo* telomere addition events.

2. Interactions between telomerase components within the RNP complex

In budding yeast, the telomerase holoenzyme consists of the noncoding TLC1 RNA that contains a template for telomere synthesis, and several protein subunits that are bound onto the RNA [8] (**Figure 1A**). The catalytic activity of telomerase relies on the Est2 protein, which together with TLC1 is sufficient for the enzymatic activity of telomerase *in vitro* [12, 13]. The yeast Est2 protein, as well as the catalytic telomerase subunits from other organisms (e.g., human, mouse, fission yeast and ciliates), share sequence homology to the reverse transcriptase family of DNA polymerases, and therefore are collectively known as telomerase reverse transcriptases (TERTs) [12, 14–21]. In addition to Est2, several accessory telomerase subunits associate with the telomerase RNA to mediate telomerase function *in vivo*: Est1 is required for telomerase recruitment to telomeres via its association with the Cdc13 single-stranded telomeric DNA-binding protein; Est3 provides a regulatory function which so far is not very well understood; a set of essential Pop proteins, Pop1, Pop6 and Pop7, stabilizes the association of Est1 and Est2 with TLC1 [22–25]. Elimination of TLC1 or any of the three Est proteins leads to a progressive loss of telomeres and subsequent cellular senescence (an ever shorter telomere, *est*, phenotype), underscoring the importance of all these components for telomerase function *in vivo*. The same *est* phenotype is also observed if the interaction of Cdc13 with Est1 is abrogated, or Pop protein binding to TLC1 is disrupted [22, 25–27]. The TLC1 RNA also associates with the Yku70/80 heterodimer and the Sm_7 complex [28, 29]. In the context of telomerase function, Yku is important for its retention in the nucleus and recruitment to telomeres, while the Sm_7 complex participates in TLC1 maturation and promotes its stability [28, 30, 31].

Several distinct elements have been mapped onto the two-dimensional structure of the TLC1 RNA that was defined by phylogenetic analyses [32] (**Figure 1A**). At the heart of TLC1 lies a group of conserved core elements associated with the reverse transcriptase activity: the single-stranded template, the template boundary element and the pseudo-knot structure, to which the catalytic subunit Est2 is attached [32–34]. The core of the RNA is branched into three stem-loops [32]. A conserved three-way junction brings TLC1 3′- and 5′-ends to the same vicinity and also contains the Sm_7 binding motif. The second stem-loop structure holds the Yku complex [28]. The third arm contains the bulge-stem IVc, which supports Est1 binding at the base [35, 36] and the set of Pop proteins at the distal end [25, 37].

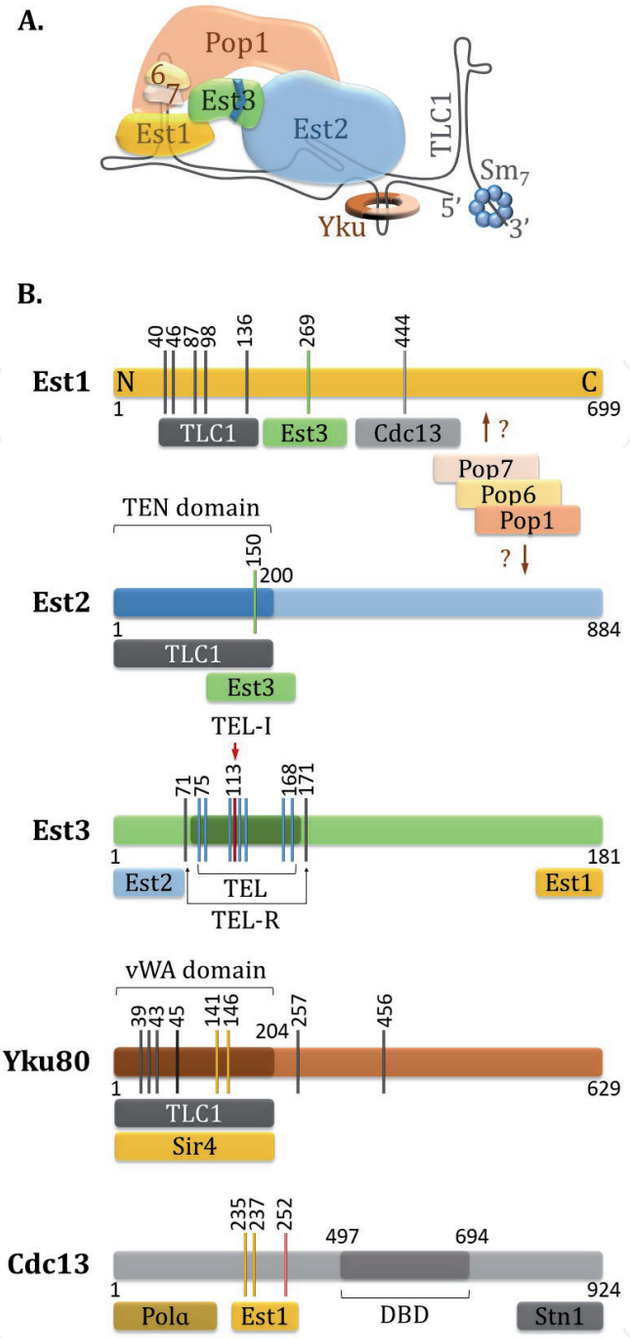

Figure 1.
Telomerase composition and interactions between telomerase components within the RNP. (A) The telomerase RNP consists of the TLC1 RNA, the Est2 catalytic protein and a set of accessory proteins: Est1, Est3, Pop1, 6 and 7, Yku70/80 and the Sm_7 complex (see the text for details). (B) Est1 associates stably with TLC1 in S-phase through the residue cluster at the Est1 N-terminus (dark gray lines). Est3 binds Est1 near the Est1-TLC1 interaction domain in late S-phase. Est1-Est3 binding is abolished by est1-R269E mutation (green line). Cdc13 associates with Est1 in late S-phase. This interaction is compromised in est1-60 (est1-K444E) mutants (light gray line) due to the disruption of a salt bridge between the Est1 and Cdc13 proteins. Pop1, 6 and 7 associate with Est1 and Est2 but the exact interaction surfaces are unknown. Est2 association with TLC1 occurs throughout the cell cycle and is mediated by the Est2 TEN domain. TEN is also required for Est2 binding to telomeric DNA and Est2-Est3 interaction in late S-phase. The Est2-N150D mutant protein (green line) does not bind Est3. Est3 binding to the RNP occurs in late S-phase and requires simultaneous Est3 interaction with Est1 and Est2. The Est3 TEL patch (blue lines) is a cluster of residues that promote Est3 association with telomerase. The Est3 TEL-I patch (Est3-S113Y) (red line) inhibits Est3 binding to the RNP. The Est3 separation-of-function patch (TEL-R) (gray lines) is essential for telomere maintenance but does not affect Est3 interaction with the known telomerase components. Yku80 as part of the Yku70/80 heterodimer associates with TLC1 throughout the cell cycle. This interaction is supported by the vWA domain of Yku80 (gray lines indicate important residues). yku80-135i mutant with a 5 amino acid insertion in the vWA domain (at Yku80-W45, black line) are defective in Yku80-TLC1 interaction. Sir4 also binds to the Yku80 vWA domain. This interaction is strengthened by Yku80-D141 and -E146 residues (yellow lines). Yku80-TLC1 and Yku80-Sir4 interactions can occur simultaneously. Cdc13 binds Est1 via two domains (yellow and pink lines, respectively). cdc13-2 (cdc13-E252K) mutation abolishes the salt bridge between Cdc13 and Est1. Cdc13 binds the 3′-single-stranded telomeric overhang via its DNA binding domain (DBD). Cdc13 also binds Pol1 and Stn1 via its N- and C-terminus, respectively.

A complex hierarchy of the protein subunits defines telomerase composition. Est1 and Est2 bind to separate regions of TLC1 RNA and do not interact directly [33, 34, 38]. Accordingly, Est1 and Est2 do not co-immunoprecipitate in the absence of TLC1 [38]. In addition, Est1 and Est2 binding to TLC1 is not interdependent: disruption of Est1 association with TLC1 does not affect Est2 binding, and vice versa, when Est2-TLC1 interaction is abolished, Est1 still maintains association with TLC1 [34, 35].

In contrast to Est1 and Est2, Est3 does not bind to TLC1 directly. Instead, it associates with telomerase via simultaneous binding to Est1 and Est2, bridging them together. On one end, Est3 interacts with the N-terminus of Est1 (**Figure 1B**). The Est1-Est3 interaction site is distinct from the Est1-TLC1 binding surface, which was also partially mapped to the protein N-terminus [39, 40]. The *est1-R269E* mutation results in the loss of Est1-Est3 interaction and telomere shortening [39, 40]. On the other side, Est3 associates with the TEN domain of Est2 [41–43]. Accordingly, the $est2^{ten}$ mutant displays dramatically reduced Est2-Est3 association and telomere shortening [39, 42]. The Est2-Est3 interaction surface overlaps with the Est2 site that binds telomeric DNA, raising a possibility that Est3 might regulate Est2 interaction with telomeres [44]. Notably, a combination of the *est1-R269E* and *est2-N150D* mutations, that attenuate Est3 interaction surfaces in Est1 and Est2, respectively, leads to an additive telomere defect comparable to *est3*Δ. This observation indicates that to function properly, Est3 must be bound to both Est1 and Est2, although only one interaction can be sufficient to support Est3 function [39].

However, it was shown that Est3 cannot interact with Est1 if Est2 is not bound to TLC1, and vice versa, Est3 will not bind Est2 if Est1 is not present in the complex [39]. This suggests that Est3 association with the telomerase RNP absolutely requires both Est1 and Est2 bound to TLC1. Hence, Est3 might have additional interaction surfaces with Est1 and Est2 that are not abolished in *est1-R269E* and $est2^{ten}$ mutations. This would explain their milder telomere phenotypes compared to the double mutant.

Interestingly, despite the relative abundance of Est3, only a small fraction of telomerase comprises Est3 during late S-phase, when telomeres are elongated [39, 45, 46]. This argues that Est3 association with the telomerase complex is restricted during the cell cycle. Indeed, an *est3-S113Y* mutation leads to an increased Est3 association with telomerase and elongated telomeres [39]. These data indicate that the area affected by *est3-S113Y* (dubbed TEL-I, for TEL inhibitory) might negatively regulate Est3 recruitment to the telomerase complex (**Figure 1B**). The TEL-I control site seems to affect Est3 binding to both Est1 and Est2, as the *est3-S113Y* mutation partially suppresses the telomere defect caused by Est1 and Est2 mutant proteins that do not interact with Est3.

In addition, Est3 contains a positive regulatory site (named TEL), which promotes Est3 association with Est2 [47]. In contrast to the TEL-I, mutating the TEL patch results in decreased association of Est3 with Est2 and telomere shortening. Notably, both patches are found in close proximity on the Est3 surface. Therefore, it was suggested that TEL and TEL-I might function as a toggle switch, mediating positive and negative regulation of Est3 binding, respectively [39]. Est3 association with the telomerase RNP occurs exclusively during late S-phase of the cell cycle [39, 48]. This is believed to be one of the mechanisms that restrict telomerase function during the cell cycle (discussed in detail in Section 3), thus, raising the possibility that the Est3 toggle switch might be designed to control its cell cycle-specific association with telomerase. Consistent with this idea, disruption of the TEL-I patch results in Est3 binding to telomerase in G1-phase of the cell cycle [39].

Notably, another Est3 regulatory element (the separation-of-function patch, TEL-R) was also identified in proximity to the TEL and TEL-I area [47] (**Figure 1B**).

Although it does not affect Est3 interaction with the telomerase RNP, it is essential for telomere maintenance. It is still unknown what the role of Est3 binding to Est1 and Est2 is; whether it induces a conformational change in telomerase that favors telomere extension or acts as a bridge to attract other yet-to-be-identified telomerase subunits. The existence of the Est3 separation-of-function patch is consistent with both ideas. For instance, it can be a subject of a post-translational modification, which would induce a conformational change in Est3 and affect the rest of telomerase components. Alternatively, it could be the site of a protein-protein interaction with another telomerase component. In both cases, disrupting this patch would abrogate telomere maintenance without affecting Est3 interaction with Est2 or Est1.

Recently, a set of novel telomerase components called Pop1, Pop6 and Pop7 was shown to associate with TLC1 at the P3-like domain of the stem IVc, in the vicinity to the Est1 binding site [25] (**Figure 1A**). These Pop 1/6/7 subunits of telomerase are shared with RNAseP and RNaseMRP, conserved RNP complexes, required for processing of tRNA, rRNA and mRNAs [49]. Notably, within RNAseP and MRP, binding of Pop proteins is also supported by the P3 domain, structurally similar to the one of telomerase. The Pop proteins are essential for telomerase *in vivo* function [25]. Similar to RNAseP/MRP, where Pop1, 6 and 7 stabilize the RNP structure, telomerase Pop subunits are implicated in stabilization of Est1 and Est2 on TLC1 [25, 37]. The Pop-mediated stabilization of telomerase proteins is likely conferred by achieving a precise TLC1 architecture [37]. Introduction of just two base pairs into the short stem between the Est1 and Pop binding sites results in a complete loss of Est1, whereas Pop proteins remained present. This effect was attributed to the rotation of the P3 domain, possibly disrupting interaction between the Pop proteins and Est1. In addition, these results show that the Pop complex binds to telomerase in an Est1-independent manner. Altogether, it suggests that both the functional and physical interaction between the Pop complex and Est1 occurs only in the context of the proper TLC1 architecture, which brings the proteins in close proximity to favor their interaction [37]. Whether the Pop complex also interacts with Est3 and affects its stable association with the telomerase complex, is still unknown and will be important to assess.

The unexpected discovery of the novel Pop telomerase components raises the possibility that the complete telomerase RNP composition is not yet solved and other components are waiting to be uncovered. Interestingly, a distinct set of mutations in the Est1 C-terminus leads to a short telomere phenotype, although all the Est1 interactions known so far remain intact (Est1-TLC1, Est1-Est3, Est1-Cdc13 or Est1-Pop1) [40]. Therefore, Est1 might mediate a novel protein-protein interaction that is important for telomerase function.

3. Regulation of telomerase assembly and disassembly during the cell cycle

In vivo, telomerase function is strictly cell cycle-regulated. As a consequence, telomere elongation only occurs within a narrow window of late S-phase, right after conventional replication is completed [45, 46, 50]. Multiple regulatory mechanisms, discussed throughout this review, ensure that telomerase is active only at the right time and place. One of such mechanisms is the cell cycle-dependent modification of telomerase composition (**Figure 2**). It operates via fine-tuning the abundance of the telomerase protein subunits and their association with the telomerase complex according to the stage of the cell cycle.

The Est2 catalytic subunit and the TLC1 RNA, in essence a minimal telomerase, can in principle associate with telomeres throughout the whole cell cycle [31, 51].

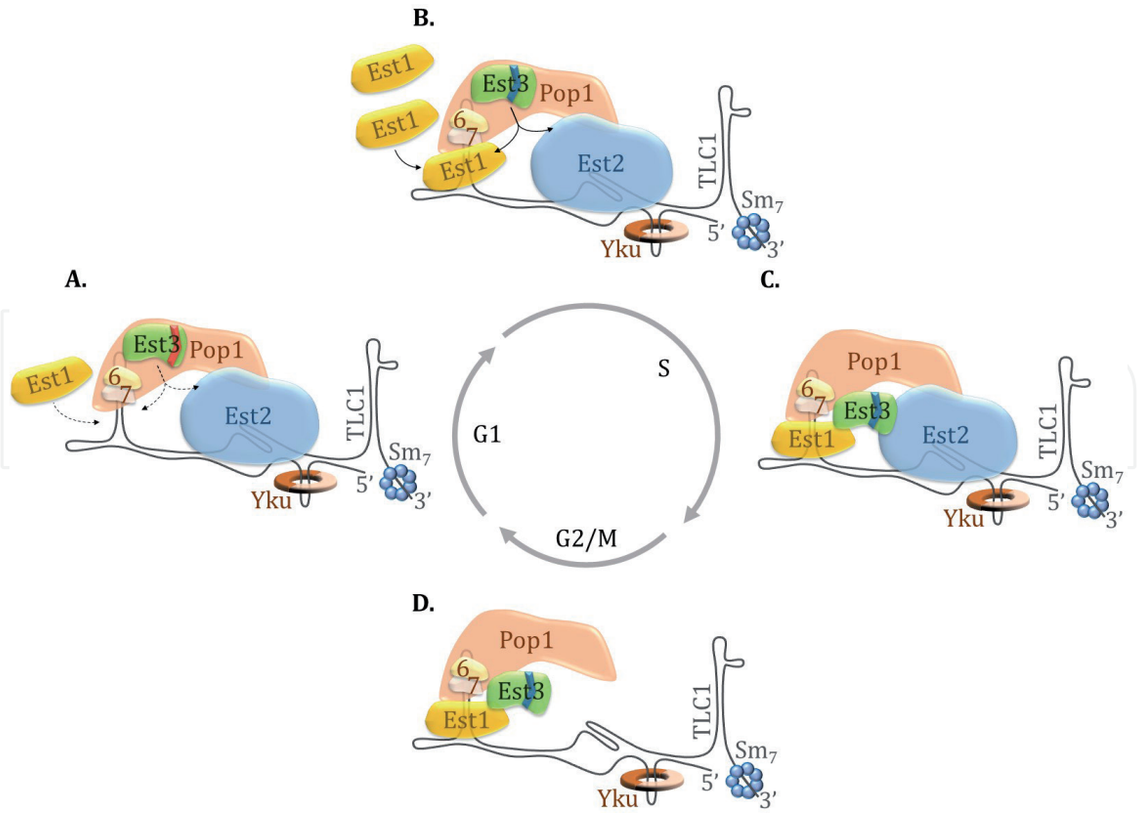

Figure 2.
Telomerase assembly and disassembly through the cell cycle. (A) The Est2 catalytic subunit associates with the TLC1 RNA during G1- and S-phase of the cell cycle. It is likely, that Pop1, 6 and 7 associate with TLC1 constitutively, thus stabilizing the RNA structure and its interactions with other proteins. The Sm_7 complex, required for TLC1 maturation and stability, and the Yku70/80 heterodimer, important for TLC1 retention in the nucleus, are also constitutive subunits of the telomerase RNP. In G1, low abundance of Est1 and activation of Est3 TEL-I patch (red shape) limit their association with the complex. Est3 cannot associate with the telomerase RNP when Est1 is not bound to TLC1. (B) Increased abundance of Est1 in S-phase promotes its association with TLC1. This complex is not active without subsequent Est3 binding promoted by its TEL patch (blue shape) activation. (C) In late S-phase, a functional telomerase complex is formed by Est3 bridging Est1 and Est2. (D) Telomerase is disassembled through the departure of Est2 in G2-phase. Est1 and Est3 remain associated with TLC1, perhaps due to stabilization by Pop1, 6 and 7, or a conformational change induced by initial Est3 association in S-phase.

However, the modes of Est2-TLC1 recruitment to telomeres as well as its consequences vary during different stages. In G1, telomere-telomerase association requires the Yku-TLC1 binding, whereas in late S-phase, it mostly depends on the Cdc13-Est1 interaction. In line with the different mechanisms of telomerase recruitment, association of Est2-TLC1 with telomeres is transient in G1- and G2-phases of the cell cycle. However, during late S-phase it gets stabilized, allowing for productive telomere elongation (see Section 4 for more details) [52].

In contrast to Est2, the total Est1 protein level fluctuates during the progression of the cell cycle (**Figure 2**). While in G1, Est1 abundance is quite low, it reaches its maximum in S-phase and is maintained at the same level for the rest of the cell cycle [48, 51, 53]. This cell cycle regulation of Est1 abundance results in its limited association with telomerase complex and telomeres in G1 [39, 48, 51, 54]. Indeed, Est2 immunoprecipitation experiments revealed that in G1, Est1 association with the telomerase RNP is 3-fold lower than that of Est2 [39, 54]. Only during S-phase does the Est1-Est2 ratio reach 1:1 and remain constant until the end of the cell cycle. Consistent with stable association of Est1 within the telomerase complex in S-phase, Est1 is robustly detected at telomeres at this point of the cell cycle and further on [48, 51]. Notably, by increasing Est1 protein level in G1, the 1:1 ratio of Est1 and Est2 can be achieved in the telomerase complex throughout the cell cycle

[48, 54]. Therefore, Est1 is absent from the telomerase RNP during G1 due to its low abundance, and as of yet there is no evidence for an active exclusion of Est1.

Cell cycle-dependent regulation of Est1 abundance occurs both at the mRNA and protein levels. During G1, the Est1 mRNA level is at its lowest, whereas at the G1-S transition, it increases, reflecting the fact that *EST1* transcription occurs late in G1 [48, 55, 56]. In G2/M-phase, the Est1 mRNA level decreases again, likely as a result of a standard mRNA decay process [55, 56].

Several studies indicate that the low abundance of Est1 in G1-phase is not solely due to cell cycle-specific control of its mRNA level, but also due to the G1-specific proteasome-dependent degradation of Est1 [48, 53, 57]. Degradation by the proteasome requires prior protein poly-ubiquitination [58]. Ferguson et al. showed that Est1 degradation is mediated by the Anaphase Promoting Complex (APC) E3 ubiquitin-ligase, that acts in G1 and ensures a smooth progression of the cell cycle via timely degradation of key regulatory proteins [53]. Disruption of the APC function and APC recognition motifs identified in Est1 was shown to abrogate the cell cycle regulation of the Est1 protein abundance, such that Est1 becomes more stable in G1.

However, this result has been challenged recently, as no change in G1-specific Est1 protein level has been observed in cells bearing mutations in the APC motifs [40]. Ferguson et al. could not detect Est1 poly-ubiquitination *in vivo*, arguing that ubiquitinated proteins would constitute a small fraction of a low abundant Est1 protein, and also would be quickly degraded by the proteasome [53]. However, single mutations of the conserved Est1 lysine residues, which act as the poly-ubiquitination substrates, did not yield a telomere defect, suggesting that Est1 might not be a subject for ubiquitination *in vivo* [40]. The poly-ubiquitination sites could be redundant though, and elimination of the whole lysine cluster might be required to abolish the ubiquitination mechanism. Furthermore, neither APC-dependent degradation, nor poly-ubiquitination of Est1 was observed *in vitro*, suggesting that the APC effect on the Est1 abundance might be indirect [53].

Yet, the idea of the G1-specific degradation of Est1 by the proteasome was supported by another study, which showed that Est1 physically interacts with the Cdc48 complex [57]. Cdc48 is a chaperone, which in complex with the E3 ubiquitin-ligases Npl4 and Ufd1, acts as a segregase to separate ubiquitinated proteins from multi-protein complexes [58]. In cells expressing the *cdc48-3* temperature-sensitive allele at the semi-permissive temperature, the G1-specific regulation of the Est1 abundance was abrogated, and the Est1 level became comparable at all stages of the cell cycle [57]. In addition, the overall Est1 protein level increased almost 40-fold, indicating that Cdc48 contributes to Est1 degradation throughout the cell cycle. Notably, in this study Est1 ubiquitination could be detected *in vivo*. In *cdc48-3* cells, the level of ubiquitinated Est1 was twice higher than in wild-type cells, consistent with the role of Cdc48 in channeling ubiquitinated proteins for degradation. However, enrichment of the ubiquitinated Est1 was observed throughout the whole cell cycle, which again suggests that the Cdc48 role in Est1 degradation is not limited to G1-stage of the cell cycle. It is plausible, that in addition to its regulatory G1 function, the Cdc48 complex promotes Est1 degradation after the disassembly of the telomerase complex in G2 (see below). However, the Est1 protein level does not decrease in G2-phase [48, 51]. This argues that accumulation of the ubiquitinated Est1 population in *cdc48-3* cells is not linked to the Est1 degradation defect, but occurs for another reason [57]. Indeed, although the proteasome-mediated degradation requires protein poly-ubiquitination, only mono-ubiquitinated Est1 was observed in *cdc48-3* cells.

Altogether, these conflicting results leave open the question about the role of the proteasome in cell-cycle regulation of Est1 abundance and require additional clarification [40, 48, 53, 57].

The Est3 protein level *per se* is not the subject of cell cycle regulation. However, in line with the Est1 behavior, Est3 association with telomerase is also cell-cycle regulated, being restricted to late S-phase [39, 48]. Similar behavior of Est1 and Est3 can at least partly be explained by the fact that Est1 is required for Est3 association with Est2 and TLC1 [23, 48] (**Figure 2**).

Notably, overexpression of Est1 in G1 leads to its association with the telomerase RNP, as well as Est3 binding, resulting in the assembly of the active telomerase [48, 54]. Despite that, no productive telomere elongation was observed during G1-stage of the cell cycle [46, 48, 52, 54]. This observation strongly suggests that the cell cycle regulated telomerase assembly is not the limiting regulatory mechanism that prevents telomere elongation during G1 (see Section 4).

As outlined above, the assembly of the telomerase complex is a tightly regulated process that occurs via regulation of abundance and inclusion of the Est1 and Est3 accessory proteins in the complex in late S-phase [39, 48, 51, 54]. However, there is evidence that telomerase disassembly may also be an actively regulated process. It may occur via a mechanism different from telomerase assembly, as it requires the dissociation of the Est2 catalytic subunit, resulting in the formation of the Est1-Est3-TLC1 disassembly complex [39] (**Figure 2D**). It is not known what serves as a signal for Est2 dissociation and how this process occurs mechanistically. Since Est3 interacts with Est2 in the TEN domain, which is also responsible for Est2 binding to the telomeric DNA, it is possible that the G2-specific disassembly of telomerase complex might be signaled via the Est3 subunit [39, 44].

One of the intriguing questions is how Est3 remains within the Est1-Est3-TLC1 complex after Est2 dissociation, given that its binding to the complex requires a simultaneous presence of Est1 and Est2 on TLC1 [39]. It is possible that once bound to the telomerase complex, Est3 can be stabilized by other proteins and consequently no longer requires Est2 to keep its position. One of the candidates for such a function is the complex of the Pop1, Pop6 and Pop7 proteins, which might stabilize Est3 binding within the telomerase complex, as they do for Est1 and Est2 [25, 37]. In general, although constitutively present in the telomerase RNP, the role of the Pop proteins in telomerase regulation remains unexplored. Therefore, it would be of a great interest to assess whether their telomerase-related function is somehow regulated during cell cycle.

4. Regulation of telomerase recruitment to telomeres during the cell cycle

As mentioned above, telomerase assembly is highly regulated during the cell cycle. However, this control is insufficient to limit telomerase function to late S-phase. In this section, we discuss how the telomerase RNP is recruited to telomeres, and how regulation of this process makes an impact on cell-cycle restriction of telomerase function.

4.1 Sir4-Yku-TLC1 as the G1-specific telomerase recruitment mechanism

Telomerase is recruited to telomeres via two different mechanisms: (1) via the Sir4-Yku70/80-TLC1 interaction, which mostly operates in G1-phase of the cell cycle, and (2) via the Cdc13-Est1 interaction, which is the predominant recruitment pathway during late S-phase, when telomere elongation takes place (**Figure 3**).

The Yku complex is not a dedicated telomeric protein, and its main function is normally associated with DSB repair via non-homologous end joining (NHEJ) [59]. Yku is a heterodimer that consists of the Yku70 and Yku80 subunits. Together the

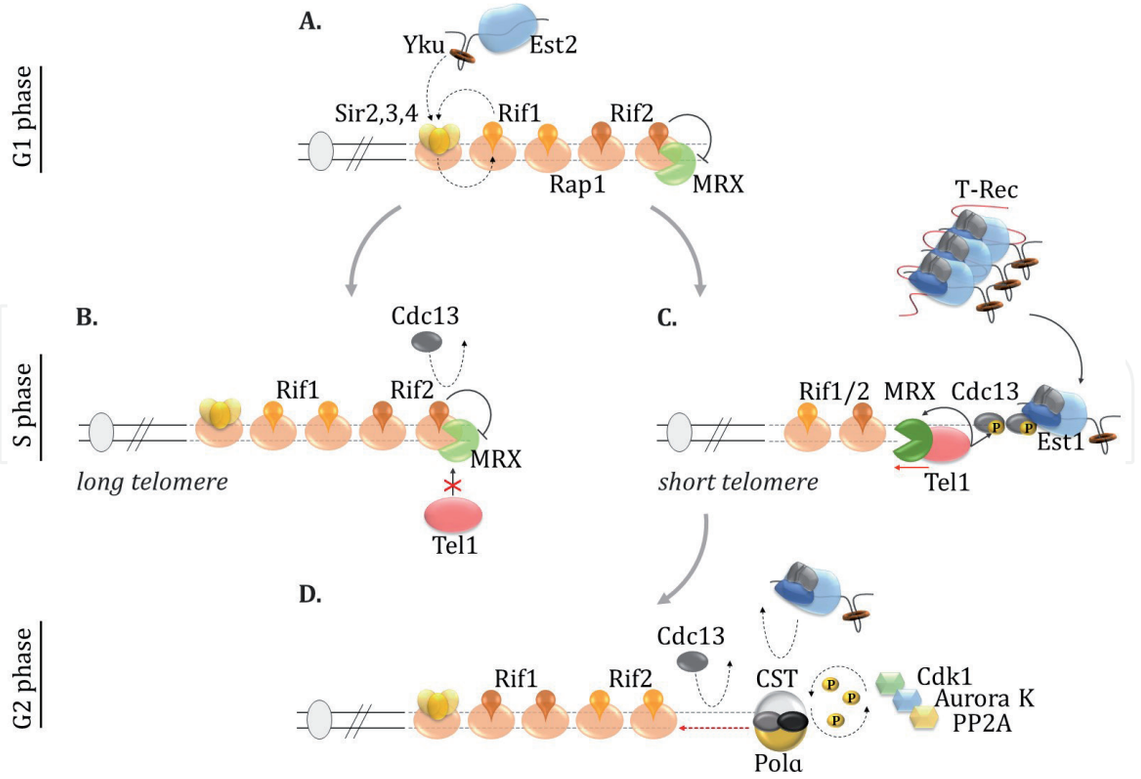

Figure 3.
Recruitment and disassociation of telomerase at short and long telomeres. (A) Inhibition of telomerase in G1: Rap1-bound Rif2 inhibits MRX-dependent telomeric C-strand resection. In addition, Rif1 and Rif2 may compete with Sir proteins for Rap1 binding, resulting in unstable telomerase recruitment via the Sir4-Yku-TLC1 pathway. (B) Inhibition of telomerase at long telomeres in S-phase: Long telomeres allow increased Rap1 binding and recruitment of Rif and Sir complexes. Rif2 competes with Tel1 to inhibit MRX activation, and subsequent 5′-end resection. This prevents the formation of a single-stranded substrate for Cdc13 and telomerase recruitment. (C) Telomerase recruitment at short telomeres in S-phase: At short telomeres, less Rap1 and Rif binding allows TERRA transcription (red line). TERRA recruits multiple telomerase molecules (T-Rec) to its telomere of origin. Tel1 can outcompete Rif2 for MRX binding, promoting 5′-end resection. Cdc13 can bind single-stranded DNA and is phosphorylated by Tel1 to recruit telomerase via Est1. (D) Elongation of telomeres by telomerase is terminated in G2-phase: Cdk1-, Aurora K- and PP2A-mediated phosphorylation and de-phosphorylation of Cdc13 and Stn1 allow formation of the CST (Cdc13, Stn1, Ten1) complex. This inhibits Est1-Cdc13 interaction and therefore decreases telomerase recruitment. Cdc13 and Stn1 recruit DNA polymerase α to fill-in the telomeric C-strand. After the C-strand has been filled-in, Cdc13 no longer has a single-stranded substrate to bind to, and hence cannot recruit telomerase.

two subunits form a ring structure, that binds a DSB end by encircling DNA strands [60]. Despite the fact that NHEJ must be avoided at all costs at telomeres, Yku is present at chromosome ends, where it plays multiple roles, such as inhibition of 5′-telomere processing [61, 62], maintenance of the telomere position effect [61, 63, 64], as well as telomere clustering and tethering to the nuclear envelope [65]. In addition, Yku is required for telomerase retention in the nucleus and its recruitment to telomeres in G1 [30, 31].

Yku70/80 associates with TLC1 via a 48 nt RNA stem-loop [30, 66] (**Figure 1**). Indeed, in *tlc1-Δ48* and *yku80-135i* mutants with abolished Yku-TLC1 interaction, the association of telomerase components with telomeres follows the same pattern as in *yku80Δ* cells. The structural element of TLC1 required for interaction with the Yku complex has been recently narrowed down to 25 nt [67]. It consists of two stems separated by a two-nucleotide (A292U293) bulge. Bending the TLC1 stem-loop in a bulge region is absolutely essential for Yku binding *in vitro* and *in vivo*. Indeed, *tlc1ΔAU* cells have slightly shortened telomeres, comparable to the effect of *tlc1Δ48* with the whole stem-loop being removed.

Structural studies indicate that Yku binds double-stranded DNA and the TLC1 stem-loop through the same aperture in the Yku70/80 heterodimer structure,

indicating that Yku cannot simultaneously interact with DNA and RNA [67, 68]. This suggests that interaction of the Yku-TLC1 complex with telomeres might be mediated by a protein-protein interaction. Indeed, Yku80 directly interacts with Sir4, a component of telomeric chromatin that is recruited to telomeres via the Rap1 double-stranded telomere binding protein [69, 70]. The Sir4-Yku80 interaction site is not in conflict with the Yku70/80-TLC1 binding surface [67]. Accordingly, the Yku70/80 heterodimer, TLC1 and Sir4 can be co-immunoprecipitated as a complex, implying that telomerase could be recruited to DNA via Sir4-Yku80 interaction. Indeed, in *sir4* mutants with abolished Sir4-Yku80 interaction, the Est2 level at telomeres is 15-25% lower than in wild-type cells, which is reminiscent of the *sir4*Δ effect. In addition, telomere length in these mutants is somewhat shorter than in wild-type cells, similar to the *yku80* mutants with an attenuated Sir4-Yku80 interaction.

Interestingly, the telomere defect in the y*ku80*Δ cells is much more pronounced than in mutants with the disrupted Sir4-Yku80 interaction. This effect can be explained by the additional role of the Yku complex in the nuclear retention of TLC1, which does not depend on Sir4 [31, 67]. Accordingly, the level of nuclear TLC1 was reduced both in the *tlc1*Δ*AU* and y*ku80*Δ cells with abolished Yku-TLC1 interaction [67]. At the same time, in the Sir4-Yku80 interaction mutants, the nuclear fraction of TLC1 was normal.

Certain evidence suggests that at telomeres, the Yku complex does not exist as a uniform population. Apart from the Sir4-bound fraction, Yku also binds to telomeres directly, as it does at DSBs [71–73]. In addition to having two different modes of telomere binding, Yku was also found at diverse telomeric loci, including the junction between telomere and subtelomere regions, as well as interstitial telomeric sequences between the subtelomeric repeats [73]. Both populations include Yku directly bound to DNA and Sir4-bound Yku. It is still unknown whether Yku binds to the very tips of telomeres, and if so, in what fashion. The position of Yku so far from the telomere end was attributed to its role in maintenance of stalled and broken replication forks. Here, Yku might load on one-ended breaks and protect them from end-resection, thus favoring telomere addition by telomerase. However, since the Sir4-bound Yku population is also present at the distal telomeric loci, it suggests that telomerase recruited to telomeres via the Sir4-Yku80 pathway might be kept far from the telomere end. By extension, since the Sir4-Yku80 pathway operates mainly in G1-phase, keeping telomerase at a larger distance from the chromosome end might be a novel regulatory step, which would ensure telomerase molecules are not engaged in productive telomere elongation in G1.

Disrupting the Sir4-Yku-TLC1 pathway of telomerase recruitment results only in mild telomere shortening [28, 66, 67]. This implies that the productive telomerase recruitment required for telomere elongation is predominantly mediated by a different mechanism. Indeed, the S-phase specific Cdc13-Est1 interaction underlies the main functional telomerase recruitment pathway, and is therefore absolutely essential for telomere maintenance [22, 26, 27].

4.2 Cdc13-Est1 as the major telomerase recruitment pathway in late S-phase

A significant feature of late S-phase telomeres that distinguishes them from G1 and G2 chromosome ends is the formation of detectable 3′-single-stranded telomeric overhangs, or G-tails [74, 75]. The formation of telomeric overhangs requires MRX-dependent 5′-end processing, but, as briefly mentioned in the introduction, this process seems to take place only on the leading-strand telomeres [4, 76–78]. This S-phase specific structure is bound by Cdc13 and facilitates telomerase recruitment to telomeres via a direct association between Cdc13 and Est1 [22] (**Figure 3C**).

The Cdc13-Est1 interaction is also favored by an increased abundance of Est1 in late S-phase (see Section 3) [51].

The direct interaction between Cdc13 and Est1 was inferred from the fact that the C-terminal Cdc13 Glu252 residue forms a salt bridge with the Lys444 of Est1 (**Figure 1B**). Abrogation of either of these partners by the *cdc13-2* or *est1-60* mutations, respectively, results in a loss of telomerase recruitment and the senescence phenotype [26, 27]. However, a combination of mutations that leads to a charge swap in those residues, suppresses the defect associated with single mutations [27]. Consistently, co-immunoprecipitation of Est1 via Cdc13 revealed that in *cdc13-2* cells, Cdc13-Est1 binding is significantly reduced, but a complementary *est1-60* mutation can restore this interaction [40, 54].

However, the importance of the $Cdc13^{Glu252}$-$Est1^{Lys444}$ salt bridge in telomerase recruitment was challenged by several later studies. For example, no interaction defect between the Cdc13-2 mutant protein and Est1 has been detected in the yeast two-hybrid and co-immunoprecipitation analyses [79]. In addition, the *cdc13-2* and *est1-60* mutations only mildly affected Cdc13-Est1 association *in vitro* [67, 80]. Furthermore, estimation of the Est1 level at telomeres yielded the opposite results, reporting either no change or a complete loss of Est1 association with telomeres in *cdc13-2* cells [51, 67, 81]. In any case, neither *cdc13-2* nor *est1-60* mutants can maintain telomeres and the cells undergo senescence, suggesting that an important functional interaction is abrogated by these mutations and can be rescued by the charge swap.

Recent studies shed some light on the *cdc13-2*/*est1-60* controversy, demonstrating that this specific Cdc13-Est1 interface might be important for activation or stabilization of telomerase after it is recruitment to telomeres [37, 67]. Cells expressing the Cdc13-Est2 fusion bypass the need for Est1 for telomerase recruitment to telomeres [22]. Due to the loss of the Est1-dependent regulatory mechanism of telomerase activity, these mutants acquire over-elongated telomeres. Notably, the long telomere phenotype of the Cdc13-Est2 cells was even further exacerbated if Est1 was ectopically expressed, indicating that Est1 indeed activates or stabilizes telomerase after its recruitment to telomeres [37, 67]. However, expression of the TLC1 variants deficient in Est1 binding in this strain background suppressed the telomere-elongation phenotype, resulting in the wild-type telomere length [37]. Furthermore, supplementing cells expressing the Cdc13-Est2 protein fusion with the *est1-60* allele resulted in telomere shortening when compared to isogenic strains expressing the wild-type Est1 protein [67]. A similar effect was observed when the Cdc13-2 mutant protein was fused with Est2. However, when the Cdc13-2-Est2 fusion was combined with the Est1-60 protein expression, telomere length was restored to that of a wild-type. This result indicates that Est1 association with the telomerase RNP and the interface between the Cdc13 and Est1 forming the $Cdc13^{Glu252}$-$Est1^{Lys444}$ salt bridge might be required for telomerase activation and/or stabilization, which comes into play downstream of telomerase recruitment [37, 67].

If the $Cdc13^{Glu252}$-$Est1^{Lys444}$ salt bridge area is important for telomerase stabilization, which Cdc13 interface supports the Est1 recruitment? Based on *in vitro* studies, the Est1-recruitment site of Cdc13 may be located within the N-terminal part of the protein, in close proximity to the salt bridge interface discussed above [67]. Disruption of this area in *cdc13-P235A* and *cdc13-F237A* mutants resulted in a large reduction of telomere length. In addition, *cdc13-F237A* cells displayed an S-phase specific decrease in Est1 and Est2 association with telomeres, whereas the level of Cdc13 was not affected. It must be noted that when compared to *cdc13-2* mutants, the *cdc13-F237A* defects both in telomere maintenance and telomerase binding to telomeres were somewhat weaker [67, 81]. However, based on the assigned functions, the recruitment-deficient *cdc13-F237A* mutant would be expected to have more pronounced telomere phenotypes. Hence, additional studies might be

required to dissect the dual role of the Cdc13-Est1 interaction in supporting telomerase recruitment to telomeres and its subsequent stabilization and/or activation.

Certain evidence suggests that Cdc13 is phosphorylated by the Tel1/Mec1 checkpoint kinases to promote the Cdc13-Est1 interaction [82, 83] (**Figure 3C**). Indeed, i*n vitro*, Tel1 phosphorylates Cdc13 at the positions 225, 249 and 255 [82]. Mec1/Tel1-dependent Cdc13 phosphorylation was also detected *in vivo* by the S249/S255 phospho-specific antibody [83]. The *cdc13-S249A/255A* mutants with abolished Tel1 phosphorylation sites display telomere shortening and the senescence phenotype, as well as a loss of telomerase-mediated telomere addition at HO sites flanked by telomeric repeats. Notably, the identified phosphorylation sites reside in the part of the Cdc13 protein, which is responsible for the formation of the $Cdc13^{Glu252}$-$Est1^{Lys444}$ salt bridge. This implies that Tel1-mediated Cdc13 phosphorylation might be required for telomerase activation/stabilization after telomerase recruitment. Consistent with this idea, lack of Cdc13 could be complemented by expression of Cdc13-Est1 as well as Cdc13-S249A/255A-Est1 fusion proteins [82]. This suggests that Cdc13 phosphorylation may promote the Est1 function downstream of telomerase recruitment.

However, there are also conflicting data on this issue. In particular, cells expressing the Cdc13 protein in which all potential consensus Tel1 phosphorylation sites were mutated, did not display any telomere defect [84]. In addition, the mass-spectrometry analysis of the Cdc13 phosphorylation sites did not detect *in vivo* phosphorylation of the S249/255 residues [85]. Therefore, the existence of Tel1-mediated Cdc13 phosphorylation *in vivo* and its role in telomerase function remains unclear.

Altogether, the data presented above demonstrates that in principle, telomerase can be recruited to telomeres via two separate mechanisms: Sir4-Yku-TLC1 which operates in G1 and Cdc13-Est1 available in late S-phase. However, live-cell imaging of TLC1 dynamics revealed that in G1 and G2 phases, telomerase-telomere interactions are very short-lived and transient [52]. Indeed, outside S-phase, TLC1 molecules move much more rapidly and diffusively as compared to telomeres. During late S-phase, however, TLC1 RNA molecules can assemble in a cluster of 6-15 molecules, named telomerase-recruitment cluster, or T-Rec, which stably associates with telomeres and follows their dynamics.

Consistent with the *in vivo* pattern of telomerase-telomere interactions, the loss of the Sir4-Yku telomerase recruitment pathway has only a mild effect on telomere length [28, 66, 67], whereas disruption of the Cdc13-Est1 interaction completely abrogates telomere maintenance and results in cellular senescence [22, 26, 27]. Therefore, it can be concluded that the Cdc13-Est1 mechanism is the only true telomerase recruitment pathway which allows efficient telomere elongation. As telomerase recruitment via Cdc13-Est1 is only possible in late S-phase, Cdc13-Est1 interaction might be the main factor that determines cell cycle restriction of telomerase function [51]. As for the Sir4-Yku80 telomerase recruitment pathway, the question about its functional significance remains open (see Section 6 for discussion).

4.3 The switch between the Cdc13-Est1 and Cdc13-Stn1-Ten1 complexes as a signal for telomerase dissociation

In addition to the Cdc13-Est1 interaction important for telomerase recruitment to telomeres, Cdc13 also forms a complex with the Stn1 and Ten1 proteins (CST) (**Figure 3D**). CST prevents degradation of the chromosome ends and their recognition by DSB repair mechanisms, collectively known as the capping function [8]. The CST complex also negatively regulates telomere maintenance, demonstrated by the fact that disrupting Cdc13-Stn1-Ten1 interactions leads to a long telomere phenotype [86]. The CST-dependent effect on telomere elongation is due to a direct inhibition of telomerase recruitment, as well as an indirect consequence of the CST

function in the re-synthesis of the telomeric C-strand, which eliminates the G-tails as a substrate for telomerase recruitment [86, 87].

The idea of CST directly inhibiting telomerase recruitment stems from two-hybrid experiments, indicating that Stn1 and Est1 compete for binding to Cdc13 [87]. Indeed, the mutant allele *cdc13-2*, that disrupts the Cdc13-Est1 interaction, also abolishes the Cdc13-Stn1 binding, suggesting that Est1 and Stn1 might share the same Cdc13-binding interface. Consistently, the senescence phenotype of the *cdc13-2* mutants is partially suppressed by Est1 overexpression, while overexpression of Stn1 enhances it. To support the idea of the Est1-Stn1 competition, an interesting experiment was performed with mutants expressing only the N-terminus of Stn1, which does not bind Cdc13 [86]. As in other strains where the Cdc13-Stn1 interaction is disrupted, these cells harbored long telomeres. However, an additional expression of the C-terminal portion of Stn1 suppressed this phenotype. Evidently, the C-terminus, expressed as a separate protein unit, cannot simply attach itself to the rest of Stn1, and thus, mediate the Cdc13-Stn1 interaction. The more likely explanation would be that the Stn1 C-terminus interacts with Cdc13 independently of the rest of Stn1 to outcompete Est1 for Cdc13 binding. Hence, Est1 and Stn1 compete for the same Cdc13 interface, implying that the formation of the CST complex can directly inhibit telomerase recruitment.

Telomerase-mediated elongation of the G-strand is followed by C-strand fill-in synthesis, which restores the double-stranded portion of the telomere (**Figure 3D**). CST plays an essential role in this process, as it is responsible for the recruitment of the DNA polymerase α/primase complex to telomeres. For example, it has been shown that Cdc13 interacts with Pol1, the catalytic subunit of this complex [79], while Pol12, the B subunit of the complex, associates with Stn1 [86, 88]. As a consequence of C-strand synthesis, the single-stranded telomeric overhangs that serve as substrates for Cdc13 binding are eliminated. Hence, this effect contributes to the CST role as a negative regulator of telomerase recruitment. Consistently, cells deficient in the Cdc13-Pol1 interaction have elongated telomeres, and this phenotype is dependent on telomerase interaction with Cdc13 [87]. Surprisingly, cells expressing Cdc13 with an N-terminal truncation are proficient in Pol1 interaction, but still have a telomere elongation phenotype as well as longer telomeric overhangs. The latter phenotype was attributed to the defect in the C-strand fill-in, as it was telomerase-dependent, the overhangs were only visible during late S-phase, and the C-strand was also slightly elongated when compared to wild-type cells. Overexpression of Stn1 almost completely suppressed the long telomere and G-tail phenotype in these mutants. This suppression mechanism might be mediated by the Stn1-Pol12 interaction, which could stabilize DNA polymerase α at telomeres. Consistently, certain *pol12* alleles also confer telomere elongation and longer single-stranded telomeric overhangs [88].

Altogether, the above results suggest that the CST complex can affect telomerase recruitment via two mechanisms: (1) directly, via competition between Stn1 and Est1 for Cdc13 binding; and (2) indirectly, via its role in the restoration of the double-stranded telomere structure and elimination of the G-tails. If the Cdc13-Est1 complex permits telomerase recruitment, and the CST complex prohibits it, the switch between these two complexes might serve as an additional regulatory mechanism, which determines the window of telomerase function at telomeres.

Indeed, the transition from the Cdc13-Est1 interaction to the CST complex formation seems to be regulated in part by sequential phosphorylation of Cdc13 and Stn1 by Cdk1 [89, 90] (**Figure 3D**). Cdk1-dependent phosphorylation of Cdc13 and Stn1 is mediated by S- and M-phase cyclins, respectively [90]. During S-phase, Cdc13 phosphorylation by Cdk1 leads to its association to telomeres, where Cdc13 can form a complex with Est1 and recruit telomerase. Moreover, S-phase

cyclin-dependent Cdc13 phosphorylation also delays the phosphorylation of Stn1 by M-phase cyclins. In turn, Cdk1-dependent Stn1 phosphorylation promotes the formation of the CST complex.

In addition, Tel1/Mec1 checkpoint kinases also contribute to the balance between the Cdc13-containing telomeric complexes. As was mentioned above, Tel1/Mec1-mediated Cdc13 phosphorylation promotes the Cdc13-Est1 interaction [82, 83]. In turn, this effect is counteracted by the PP2A phosphatase and the Aurora kinase [83]. Pph22, the catalytic subunit of the PP2A phosphatase, dephosphorylates Cdc13 sites phosphorylated by Tel1 or Cdk1. This facilitates telomerase dissociation from telomeres in G2/M-phase, which is demonstrated by prolonged Est1 and Est2 telomere association in *pph22*Δ strains. Aurora kinase on the other hand, phosphorylates Cdc13 in G2/M, thus promoting TLC1 departure from telomeres, possibly by allosterically influencing its interactions with Est1.

Therefore, a tightly regulated sequence of Cdc13 and Stn1 phosphorylation events, first, promotes the formation of the Cdc13-Est1 complex, and next, mediates the shift to the Cdc13-Stn1-Ten1 complex. Such a precise order of events defines a narrow time frame, which permits telomerase function at telomeres, explaining how the cell-cycle regulated restriction of telomerase function is orchestrated.

5. Regulation of telomerase preference for short telomeres

5.1 Rif2-MRX-Tel1 mechanism

Telomerase function is restricted not only by the cell cycle, but also by telomere length, being preferentially targeted to short telomeres. Indeed, within a particular cell cycle, only 6–8% of wild-type length telomeres (~300 bp) get extended, whereas short telomeres of about 100 bp are elongated in almost 45% cases [91]. Such preference for short telomeres is mediated by the Rap1-interacting partners Rif1 and Rif2. Altogether, Rap1, Rif1, and Rif2 form a negative feedback loop, which regulates telomere elongation in a length-dependent manner [92, 93] (**Figure 3**). Therefore, telomeres become over-elongated in the absence of Rif1 or Rif2, due to the increased frequency of telomerase function at all telomeres [91, 94–96]. The Rap1-Rif1-Rif2 regulatory mechanism relies on the number of the telomere-associated Rif proteins as a readout of an individual telomere length [93]. As a result of such "protein counting," only those telomeres that have the low number of Rif1 and Rif2, i.e., short ones, will be elongated. Once telomeres get extended and the sufficient amount of the Rif proteins is restored at telomeres, the negative feedback loop inhibits telomere extension.

The mechanism of the Rif protein counting, and hence, targeting telomerase to short telomeres, depends on the intricate network of physical and functional interactions between the Rif1 and Rif2 proteins, Tel1 and the MRX (Mre11-Rad50-Xrs2) complex.

The checkpoint kinase Tel1 preferentially localizes to short telomeres, and as a result, also mediates Est1 and Est2 preference to short telomeres [97–100]. Tethering Rif1 and Rif2 to DSB ends leads to the reduction in Tel1 binding, suggesting that Rif proteins might out-compete or displace Tel1 from chromosome ends. Indeed, both Rif2 and Tel1 are recruited to telomeres via the Xrs2 subunit of the MRX complex, whereas Rif1 recruitment is partially mediated by Rif2 [96, 101]. *In vitro,* the Tel1-Xrs2 interaction is inhibited by Rif2, suggesting that Rif2 and Tel1 compete for Xrs2 binding. However, it is unclear whether Rif2 and Tel1 binding to Xrs2 is mutually exclusive. Alternatively, binding of Rif2 may stimulate Tel1 dissociation [96].

As was mentioned above, timely generation of the single-stranded 3′-telomeric overhangs is crucial for telomerase function. This process largely relies on the MRX/Sae2 complex, which resects the telomeric 5′-strand and generates a short G-tail in late S-phase after the passage of the replication fork [50, 76, 77]. In addition, the Mre11-Sae2 complex is essential for opening the hairpin structures that might be formed at telomeres by G-strand foldbacks, followed by DNA synthesis and hairpin closure [102]. If not resolved, replication of such hairpins will result in the formation of dicentric chromosomes and initiation of a breakage-fusion-bridge cycle that is detrimental for genome stability.

It was shown, that a balance of the Tel1 and Rif2 activities defines the extent of telomere processing by MRX [103–105]. On one hand, Tel1 enhances MRX-dependent 5′-telomere processing [103]. Rif2, on the other hand, inhibits MRX activity [104, 105]. Tel1 also increases association of MRX at the breaks flanked with telomeric repeats, although it is unclear, if this effect also exists at native telomeres [96].

Based on these observations, the following model for the regulation of telomerase preference for short telomeres by the Rap1-Rif1-Rif2 counting mechanism can be proposed. At short telomeres, Rif2 levels are reduced [99], which relieves inhibition of MRX-mediated telomere processing and also allows unrestricted Tel1 interaction with MRX. In turn, Tel1 may stabilize MRX association with telomeres and enhance its resection activity. As a result, short telomeres acquire single-stranded G-overhangs that serve as a substrate for Cdc13 binding and subsequent Est1-mediated recruitment of telomerase [84, 103]. Once telomeres are extended, the double-stranded Rap1-binding sites are restored. As a result, more abundant Rif2 binding to telomeres decreases Tel1 association with MRX and inhibits MRX-dependent resection, thus blocking Cdc13 and telomerase access to telomeres. Hence, the availability of the G-tail as a substrate for telomerase binding is the main feature that distinguishes short telomeres from the long ones and allows their preferential elongation.

As an alternative to the Rap1-Rif1-Rif2 protein-counting mechanism described earlier, the telomerase preference for short telomeres could also be explained by a replication fork model, proposed recently by Carol Greider [106]. This model predicts that telomerase associates and progresses with the conventional replication fork, and telomere elongation can happen only if telomerase successfully reaches the chromosome end. The telomeric proteins (such as Rap1-Rif1-Rif2) may pose an obstacle for telomerase movement with the replication fork. Hence, the longer the telomere, the longer the distance telomerase must cover to reach the telomere, and the higher the chance for its premature dissociation. At short telomeres, the probability that telomerase will reach the chromosome end increases, explaining why short telomeres are preferentially extended in a given cell cycle. The late timing of telomere replication also justifies the cell cycle-restricted mode of the telomerase function, which also occurs in late S-phase.

However, in direct conflict to the replication fork model, Gallardo et al. showed that in the absence of Rif1 and Rif1 telomeric proteins, telomerase can elongate telomeres in G1-phase [52]. Since, replication does not take place in G1, these data show that a functional telomere-telomerase interaction as well as telomere elongation by telomerase do not depend on the replication fork. However, it is possible, that the replication fork might operate as an alternative pathway, which functions under specific circumstances.

5.2 TERRA-mediated pathway

Another mechanism promoting telomerase recruitment to short telomeres depends on the long noncoding RNA, TERRA (telomeric repeat-containing RNA), transcribed from telomeres [107, 108]. In yeast, TERRA is preferentially expressed

from short telomeres and reaches its maximum level in S-phase [109, 110]. During late S-phase, TERRA may act as a scaffold to allow the formation of a cluster of telomerase molecules (T-Rec), and promote telomerase recruitment to the short telomere of the TERRA origin [52, 109] (**Figure 3C**). Indeed, FISH experiments revealed that TERRA interacts with TLC1 RNA, and the complex of TERRA and T-Rec co-localize with a short telomere, from which TERRA is expressed [109].

Interestingly, TERRA expression is inhibited by Rif1 and Rif2 [111]. Therefore, it is possible that at short telomeres, containing fewer Rap1-binding sites and less Rif proteins, TERRA expression might be de-repressed, in order to favor recruitment of telomerase to short telomeres [109, 111]. Hence, the Rif counting mechanism, which allows preferential extension of short telomeres, may operate not only via Tel1-MRX-dependent formation of G-tails, but also via TERRA expression from short telomeres [91, 96, 103, 109].

6. Why telomerase is not welcome in G1-phase

As was discussed in Section 3, Est1 and Est3 do not associate stably with the telomerase RNP in G1-phase [39, 48, 51, 54]. However, restoration of telomerase composition in G1, still did not result in telomere extension [48, 54]. This implies that lack of telomerase components is not the main factor that restricts telomerase function in G1. Instead, the Rif1 and Rif2 telomeric proteins might govern such a regulatory mechanism.

Indeed, in the absence of Rif1 or Rif2, telomerase clusters are no longer restricted to late S-phase of the cell cycle and also appear in G1 [52]. Moreover, these G1-born T-Recs are functional in *rif1*Δ or *rif2*Δ mutants, as they can efficiently elongate short telomeres.

The most plausible explanation for this phenomenon lies in the role of Rif proteins in inhibition of telomere processing and formation of 3′-single-stranded overhangs [103, 104] (**Figure 3A**). Indeed, Rif2 was shown to inhibit processing in both G1 and G2 [104]. This means that in *rif2*Δ mutants, G-tails might be generated outside S-phase, followed by Cdc13-dependent telomerase recruitment. In addition, unrestricted expression and accumulation of TERRA in *rif1*Δ or *rif2*Δ mutants may contribute to unconstrained telomerase function, by promoting formation of telomerase clusters at any stage of the cell cycle [109].

Alternatively, telomere elongation may not take place in G1 due to unstable association of telomerase with telomeres outside S-phase [52]. Rif1 and Rif2 are recruited to telomeres via interaction with Rap1, and hence, compete with Sir3 and Sir4 which also bind to Rap1 [95, 112]. As a result of this competition, Sir4-Yku binding to telomeres might be unstable, providing an explanation for transient telomerase recruitment to telomeres in G1 (**Figure 3A**). Indeed, the role of Rif proteins as negative regulators of telomere length affects the Sir4-Yku pathway of telomere maintenance [70]. *rif1Δ* and *rif2Δ* mutants have hyper-elongated telomeres [94, 95]. The *tlc1Δ48* mutation, which abrogates Yku-TLC1 interaction, resulted in ~500 bp shortening of over-elongated *rif1Δ* and *rif2Δ* telomeres, which is a far bigger effect than in the wild-type background (only 70 bp) [70]. This observation suggests that Rif proteins inhibit Yku-mediated telomere lengthening. Altogether, these data support the idea for the role of Rif proteins in destabilizing Yku-Sir4 binding to telomeres, and hence, transient telomerase recruitment in G1-phase. It must be noted that the *tlc1Δ48* mutation does not completely suppress the telomere over-elongation phenotype of *rif1Δ* and *rif2Δ* mutants, implying that the negative role of Rif proteins in telomere length maintenance is also linked to the Cdc13-Est1 mechanism of telomerase recruitment.

Therefore, it is possible that G1-specific inhibition of telomerase function is controlled by Rif1 and Rif2 proteins at two different levels: (1) by ensuring that G-tails are not produced in G1, and hence, the Cdc13-Est1 telomerase recruitment pathway is not available; and (2) by out-competing Sir4-Yku from telomeres, therefore destabilizing telomerase association with telomeres.

As we can see, Sir4-Yku interaction does not provide stable telomerase recruitment in G1 and is mostly dispensable for telomere maintenance. Then why having a specialized but unproductive telomerase recruitment pathway in G1? We suggest that via short-lived interactions, cells could ensure that telomerase does not localize to sites of DSB repair and engage in unsanctioned repair mechanisms. Indeed, sites of telomere elongation and DSB repair were shown to be spatially separated between different nuclear compartments [113, 114]. Although in this scenario, telomerase is kept at telomeres in G1, it does not engage in productive interactions with telomeres, possibly due to the competition between Rif and Sir proteins for Rap1 binding. Interestingly, the fact that Sir4-Yku80 complexes locate distally from telomere ends [73], might also ensure that telomerase recruitment to telomeres in G1 remains unproductive.

7. Inhibition of telomerase function at DSBs

Due to a similar structure of telomeres and DSBs, telomerase has a potential to add telomeres to broken DNA, a phenomenon called *de novo* telomere addition. The consequences of telomere addition to DSBs could be fatal, because the chromosome fragment distal to the DSB is either lost or can initiate further genomic rearrangements [115, 116]. On the other hand, unsanctioned access of DNA repair mechanisms to telomeres will lead to chromosome fusions and massive genome rearrangements with devastating consequences for cell function [117–120].

To ensure that these accidents do not happen, telomeres and DNA DSBs are spatially separated in the nucleus. DSBs are mainly localized to the nuclear pores, whereas telomeres anchor at the nuclear envelope, although exceptions from this general rule can be observed [113, 114]. In addition, local mechanisms operate both at DSBs and telomeres to ensure that telomerase and DSB repair machinery do not mix up their substrates.

7.1 Telomere anchoring at the nuclear envelope

During G1- and S-phase, telomeres are clustered in approximately 8 foci, which are tethered to the nuclear envelope [113, 121]. Telomere tethering requires interaction between telomeric and nuclear envelope proteins. One telomere tethering pathway involves Sir4 interaction with Esc1 and Mps3 nuclear envelope proteins, whereas the other one depends on the Yku70/80 and Mps3 [122–124]. The two telomere tethering pathways are redundant to a certain extent, although the details of their exact functional interaction remain elusive [121].

Unlike Sir4, Yku70 cannot directly bind Mps3 *in vitro* [123]. This indicates that the Yku-mediated telomere anchoring pathway requires a "bridge" between Yku on the telomere side and Mps3 on the nuclear envelope side. It seems that telomerase might function as such a "bridge," at least during S-phase. It was shown, that on the nuclear envelope side, Est1 interacts with Mps3 [125]. In turn, on the telomere side of this "sandwich," TLC1 and Est1 interact with Yku [126]. In G1-phase, these interactions do not contribute to telomere anchoring, suggesting that another factor may mediate Yku-Mps3 bridging and telomere tethering.

An elegant single-telomere extension experiment revealed that telomeres detach from the nuclear envelope during extension [127]. In this assay, a telomerase-deficient recipient mutant with short telomeres was mated with a wild-type donor cell. In the zygote, short recipient telomeres can be efficiently elongated by the donor telomerase. To track short recipient telomeres, Tel5R was tagged with *lacO*/LacI-GFP, and the nuclear envelope was marked with Nup49-GFP. It was observed that the short Tel5R telomere was released from the nuclear periphery in the first S-phase after mating, when telomere elongation was expected to happen. However, in the second cell cycle, when the short telomere should already have been extended, Tel5R stayed anchored to the nuclear envelope. Notably, in the telomerase-negative zygote, the short telomere did not detach from the nuclear envelope in the first cell cycle. This indicates that telomerase function or the process of telomere elongation in general somehow triggers telomere dissociation from the nuclear envelope.

Deletion of *SIZ2*, encoding for the SUMO E3 ligase, causes long untethered telomeres. This indicates that telomere anchoring to the nuclear envelope is achieved via Siz2-dependent sumoylation of certain tethering components. Accordingly, Siz2 sumoylates Yku70/80 and Sir4 *in vivo.* Moreover, fusing Yku70 with SUMO suppresses the *siz2*Δ telomere tethering defect.

Altogether, these observations suggest that telomere anchoring may not only separate telomere-extension and DSB repair activities in space, but also act as an additional regulatory mechanism, which ensures cell-cycle restriction of telomere elongation.

Although normally clustered at the nuclear envelope, some telomeres can occasionally localize to nuclear pores, the sites of DSB repair. For instance, when a DSB occurs in a subtelomeric region, it must re-localize to the nuclear pore for efficient DSB repair [128]. In addition, eroded telomeres in telomerase-negative cells were also shown to re-localize to nuclear pores [129]. As a result, such critically short telomeres can undergo recombination-dependent elongation that allows cell survival in the absence of telomerase [130].

7.2 Local mechanisms restricting telomerase access to DSBs

De novo telomere addition at DSBs occurs mostly at telomere-like TG-rich regions [131–134]. However, the molecular mechanism of this process is not very well understood. In particular, it is still unknown how telomerase is recruited to the break. Cdc13 is one of the most likely candidates for this role. Consistently, Cdc13 and telomerase can be detected at both TG-flanked and non-TG DSB ends [135]. The single-stranded DNA binding protein complex RPA is another candidate for recruiting telomerase to DSBs. RPA is abundantly present at processed DSB ends and was also shown to mediate telomerase association with telomeres [136, 137].

Chromosome healing via *de novo* telomere addition is an extremely rare event, suggesting that telomerase function is efficiently inhibited at DSBs [115, 134]. In yeast, two mechanisms are implicated in the inhibition of *de novo* telomere addition. The first one involves Mec1-mediated Cdc13 phosphorylation at the S306 residue [138]. In cells expressing the Cdc13-S306A unphosphorylatable protein, the frequency of *de novo* telomere addition to DSBs is increased. This Cdc13 modification does not prevent Cdc13-Est1 interaction, but rather affects Cdc13 association with the breaks. Accordingly, *cdc13-S306A* mutants have increased accumulation of Cdc13 at DSBs. The Mec1-dependent phosphorylation of Cdc13-S306 can be reversed by Pph3, the yeast protein phosphatase 4, and Rrd1, the yeast ortholog of human phosphotyrosyl phosphatase activator. Reversible Cdc13 phosphorylation kept under the control of the DNA damage checkpoint kinase is reminiscent of Cdc13 regulation at telomeres (discussed in Section 4). Therefore, by targeting different phosphorylation sites, cells modulate Cdc13 functions both at telomeres and DSBs.

Another mechanism inhibiting telomerase at DSBs relies on Pif1, a multifunctional 5′-3′ helicase, expressed both in the nucleus and mitochondria [139]. The nuclear Pif1 isoform contributes to DNA replication and repair by promoting Okazaki fragment processing, resolution of G-quadruplex DNA structures, ribosomal DNA replication and DSB repair via Rad51-dependent break-induced replication. In addition, Pif1 inhibits telomere addition both at telomeres and DSBs. Accordingly, cells expressing the *pif1-m2* allele that lack the nuclear Pif1 isoform, have long telomeres, whereas overexpression of *PIF1* leads to telomere shortening [134]. The frequency of *de novo* telomere addition to spontaneous and HO-induced DSBs is increased 200-1000-fold in the absence of Pif1 [131, 132, 134]. The inhibitory effect of Pif1 on *de novo* telomere addition is telomerase-dependent, as inactivation of telomerase function suppresses the high rate of gross chromosomal rearrangements and long telomere phenotype observed in *pif1-m2* cells [132, 140].

Similar to Cdc13, Pif1 function can be modulated by specific phosphorylation events. It was shown that Mec1-dependent phosphorylation specifically regulates Pif1 activity at DSBs [141]. Cells expressing Pif1-4A, which cannot be phosphorylated by Mec1, have the same level of *de novo* telomere addition as *pif1-m2* cells. However, additional telomere lengthening associated with *pif1-m2* does not occur in *pif1-4A* cells. Hence, Mec1-dependent Pif1 phosphorylation is required for telomerase inhibition specifically at DSBs.

In vitro, Pif1 preferentially unwinds RNA-DNA structures [142]. In addition, the helicase activity of Pif1 is required for its telomeric function [140]. Therefore, Pif1 role in inhibition of telomere elongation could be explained by its ability to disrupt the base-pairing between the TLC1 RNA and telomeric DNA, leading to displacement of telomerase from telomeres. Consistent with this model, Pif1 expression is cell-cycle regulated and reaches its maximum during late S-phase, coinciding with the time of telomerase action at telomeres [51, 143]. In addition, Pif1 overexpression reduces telomerase association with telomeres [144]. However, the novel function of the TERRA RNA in recruitment of telomerase clusters to telomeres raises the possibility that TERRA might also be a target for the Pif1 helicase activity, which would explain its negative effect on telomere lengthening [109].

Replication forks can frequently stall at TG-rich sequences, resulting in formation of DSBs flanked with short TG-sequences. At the same time, native telomeres can also reach a critically short length. Despite this structural resemblance, the first type of substrate must be channeled into proper DSB repair mechanisms, whereas telomerase must be inhibited. In turn, short telomeres must not be recognized by DNA repair mechanisms, and instead, must be elongated by telomerase.

How does a cell distinguish between these very similar structures? Recent evidence suggests that cooperation between Cdc13 and Pif1 might channel particular substrates into proper repair pathways [145]. It was shown, that if either a natural telomere or a TG-seed flanking a DSB is shorter than 35-40 bp, it is recognized as a break and is protected from telomerase activity by Pif1. As a result, such a substrate will be processed by DSB repair mechanisms. In turn, when the length of the TG-tract exceeds the 35-40 bp threshold, it is considered as a short telomere, irrespective of whether it is at a break or at the natural chromosome end. Pif1 will not protect such a substrate from telomerase function, thus making it available for telomerase-mediated elongation.

Mec1-dependent Pif1 phosphorylation, which specifically modulates its function at the breaks, does not make Pif1 selectively sensitive to shorter TG-sequences and insensitive to the longer ones [141, 145]. Instead, it was shown that Pif1 selectivity for different TG-substrates is mediated by Cdc13 [145]. If Cdc13 binding to DSBs is attenuated, longer telomeres become sensitive to Pif1. This implies that Cdc13 protects longer TG-sequences from the inhibitory Pif1 activity, thus allowing telomere

addition. Interestingly, this effect of Cdc13 is not linked to its role in telomerase recruitment. The Cdc13-Est1 or Cdc13-Est2 fusions did not result in more frequent telomere addition at short TG-sequences when Pif1 was present. It suggests that the role of Cdc13 at the threshold DSB TG-sequences is not due to telomerase recruitment, but could possibly be explained by other Cdc13 functions, such as telomere capping or promoting the C-strand synthesis.

Pif1 was previously implicated in promoting resection in *cdc13-1* cells, where Cdc13 is inactive [146, 147]. Hence, Strecker et al. suggested that at short TG-stretches, less Cdc13 may allow more extensive processing by Pif1 and telomerase inhibition [145]. Once telomere length is over the threshold, it can be protected from Pif1 resection and thus Pif1 no longer inhibits telomerase.

We suggest an alternative explanation that may explain the Pif1 and Cdc13 effect on telomere addition to threshold substrates by their role in Okazaki fragment synthesis. When the replicative helicase runs off telomeric DNA ends ahead of the replisome, single-stranded DNA stretches may become exposed. The CA-rich strand will be rapidly converted into double-stranded DNA by leading-strand synthesis, whereas the TG-rich strand must be filled-in by the lagging-strand replication. It is possible, that if the TG-tract is shorter than 35–40 bp, lagging-strand initiation will be inefficient. In this scenario, the TG-tract, as well as the upstream non-TG sequence, will remain single-stranded. RPA bound to the non-TG sequence will signal for the DNA damage response activation, resulting in the engagement of the 3′-overhang in homology search and DSB repair. However, when the TG-sequence is longer than the threshold, priming for lagging-strand synthesis may be efficient. As a result, single-stranded DNA will get converted into a double-stranded DNA tract, and RPA displaced from the non-TG sequence will no longer signal for the DNA damage response activation. Finally, Cdc13 bound to the leftover single-stranded TG-sequence will recruit telomerase.

Why would the initiation of the lagging-strand synthesis be inefficient at short TG-tracts? In mammals, the CST complex cannot bind single-stranded DNA tracts that are shorter than 32 bp [148]. This suggests that at shorter TG-tracts, the CST complex might not be stable, and hence, cannot efficiently recruit/stabilize DNA polymerase α/primase complex. Consequently, Okazaki fragment synthesis will be inefficient, and the RNA primer could be displaced from DNA via the Pif1 helicase activity. Therefore, the RPA-bound 3′-overhang containing non-TG and TG-sequences will persist and signal for DNA damage response activation. Long TG-tracts, on the other hand, will efficiently accommodate the CST complex, resulting in stable recruitment of the lagging-strand synthesis machinery. The RNA primer would be quickly extended into a full Okazaki fragment, making it inaccessible for Pif1. This can explain, why at longer TG-tracts, telomere addition is not under Pif1 control. According to this scenario, abolishing lagging-strand DNA synthesis, but not RNA priming, should render long TG-tracts sensitive to Pif1, leading to inhibition of telomere addition.

Therefore, at telomeres and DSBs, Pif1 might act at two consecutive steps. First, Pif1 may decide the fate of the telomeric end, and either channel it to the DSB repair pathway or leave it accessible for telomere elongation. As discussed above, this could be due to the Pif1 role in Okazaki fragment synthesis, rather than direct telomerase displacement. At the next step, Pif1 might regulate the extent of telomere elongation by telomerase. Accordingly, Pif1 was shown to inhibit telomerase preferentially at long telomeres [149]. Unlike its function at the threshold TG-tracts, this effect could be due to Pif1 stripping telomerase from telomeric 3′-overhangs.

Although most DSBs are localized to nuclear pores to be repaired, some persistent breaks are brought to the same regions as telomeres [114]. If such a break is deemed "nontelomeric," it will subsequently re-localize to the nuclear pore. Why are these "questionable" DSBs brought so dangerously close to the telomeric sites?

Such behavior can be nicely explained by the role of Pif1 in sorting DSBs from telomeres based on their threshold TG-sequence [145]. By extension, this Pif1-mediated process should co-localize with telomeric clusters tethered to the nuclear envelope.

8. Conclusions and future perspectives

In this review, we have outlined the main regulatory mechanisms that tightly cooperate in order to control telomerase function at telomere and at the breaks. Although our knowledge on this subject is quite extensive, many questions remain. Do the Pop1, Pop6 and Pop7 proteins functionally interact with any other telomerase subunits or telomeric proteins, in addition to Est1 and Est2 (e.g., Est3, Cdc13, Yku). How do the Pop proteins affect telomerase composition and function during cell cycle? Are there any unknown telomerase subunits that are yet to be discovered, and if so, what are their roles? What is the functional significance of the Sir4-Yku-TLC1 telomerase recruitment pathway? Is it related to the sequestration of telomerase from the DSB repair sites, as we proposed above, or does it play another role? Telomere anchoring at the nuclear periphery seems to affect their elongation. How is this process mediated, and what is the significance of telomerase in telomere anchoring at the nuclear envelope? What is the mechanism that allows Pif1 to distinguish DSBs from short telomeres and inhibit telomerase only at DSBs? All these questions and many more wait for answers, which will help to understand better the intricate network of telomerase regulatory pathways.

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Conflict of interest

The authors declare no conflict of interest.

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