Chromosomes and Gene Expression
Genetics and Genomics

Control of telomere length in yeast by SUMOylated PCNA and the Elg1 PCNA unloader

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Abstract

Telomeres cap and protect the linear eukaryotic chromosomes. Telomere length is determined by an equilibrium between positive and negative regulators of telomerase activity. A systematic screen for yeast mutants that affect telomere length maintenance in the yeast *Saccharomyces cerevisiae* revealed that mutations in any of ~500 genes affects telomere length. One of the genes that, when mutated, causes telomere elongation is *ELG1*, which encodes an unloader of PCNA, the processivity factor for replicative DNA polymerases. PCNA can undergo SUMOylation on two conserved residues, K164 and K127, or ubiquitination at lysine 164. These modifications have already been implicated in genome stability processes. We report that SUMOylated PCNA acts as a signal that positively regulates telomerase activity. We also uncovered physical interactions between Elg1 and the CST (Cdc13-Stn1-Ten) complex, and dissected the mechanism by which Elg1 and Stn1 negatively regulates telomere elongation, coordinated by SUMO. We present a model that provides mechanistic insights on how chromosomal replication and telomere elongation are coordinated.

eLife assessment

This **important** study aims to discover the mechanisms governing the switch between conventional DNA replication and the specialized mechanism of telomere end replication. **Solid** genetic and biochemical assays suggest an interplay between sumoylated PCNA and chromosome terminal capping proteins. The questions addressed have implications for several fields, such as genome stability.

Introduction

Telomeres cap the ends of linear eukaryotic chromosomes and safeguard them against genome instability. Telomeric DNA consists of G/C-rich DNA repeats, with the G-rich strand extending to form a 3' single-stranded overhang. Telomeres are primarily involved in counteracting the gradual shortening of the telomeric DNA caused by conventional DNA replication, thereby solving the

"end-replication problem" (Bonnell, Pasquier, & Wellinger, 2021; Soudet, Jolivet, & Teixeira, 2014). Another important function of telomeres is to protect chromosome ends from improper recognition as a DNA double-strand break (DSB) [reviewed in (de Lange, 2018)].

An important, conserved protein complex involved in regulation of telomere length is the CST, which in *Saccharomyces cerevisiae* is composed of Cdc13, Stn1, and Ten1 (L. Y. Chen & Lingner, 2013; Ge et al., 2020; Lim et al., 2020; Puglisi, Bianchi, Lemmens, Damay, & Shore, 2008; Rice & Skordalakes, 2016). The CST has similarity to the ssDNA binding complex RPA and localizes specifically to the single-stranded telomeric DNA, where it is involved in chromosome end capping and telomere length regulation (Hughes, Weilbaecher, Walterscheid, & Lundblad, 2000; Lin & Zakian, 1996; Mersaoui & Wellinger, 2019). The CST facilitates telomerase-mediated telomere elongation (Evans & Lundblad, 1999, 2000; Nugent, Hughes, Lue, & Lundblad, 1996), participates in telomere processing during DNA replication (Soudet et al., 2014), and helps "capping" the telomere (Mersaoui & Wellinger, 2019). In late S-phase Cdc13 interacts with the Est1 subunit of telomerase and promotes telomerase activity (Chandra, Hughes, Nugent, & Lundblad, 2001; H. Chen et al., 2018). Stn1 binds Cdc13 in a region that overlaps with that bound by Est1. Recruitment of Stn1 evicts Est1 and prevents further elongation of the G-strand (Chandra et al., 2001; Wang et al., 2000). The amino terminus of Stn1 binds Ten1, whereas its C-terminus interacts with both Cdc13 and Pol12, a subunit of Polymerase Alpha (Grossi, Puglisi, Dmitriev, Lopes, & Shore, 2004; Petreaca et al., 2006; Puglisi et al., 2008). Thus, the CST coordinates leading strand elongation by telomerase with lagging strand DNA synthesis by the cell's replisome (Grossi et al., 2004).

Very often the integrity of the genome is compromised by internal and external sources of DNA damage. This vulnerability increases during S-phase, when the DNA is unpacked and copied. Elg1, the major subunit of a replication factor C-like complex (RLC), plays a central role in DNA replication and is critical for genome maintenance. In *Saccharomyces cerevisiae*, loss of the *ELG1* gene causes gross chromosomal rearrangements, chromosome losses, defective sister chromatid cohesion and recombination, increased sensitivity to DNA damaging agents and abnormal telomere length maintenance [reviewed in (Arbel, Choudhary, Tfilin, & Kupiec, 2021)]. The function of the Elg1 RLC is to unload the processivity factor PCNA from chromatin, in particular after Okazaki fragment processing and ligation in the lagging strand, and during DNA repair (Kubota, Katou, Nakato, Shirahige, & Donaldson, 2015; Kubota, Nishimura, Kanemaki, & Donaldson, 2013; Parnas et al., 2010; Shemesh et al., 2017). The mammalian ortholog of *ELG1* (ATAD5) participates in the Fanconi Anemia pathway (Kim et al., 2020; Yang et al., 2011) and, when mutated, leads to genome instability and cancer in mice and humans (Bell et al., 2011; Kuchenbaecker et al., 2015; Maleva Kostovska et al., 2016).

Post-translational modifications of PCNA (in particular ubiquitination and SUMOylation) orchestrate the activity of a large number of interacting proteins during DNA replication and repair. Many of these proteins interact through a PIP (PCNA Interacting Peptide) motif. PCNA can undergo SUMOylation on two conserved residues, K164 and K127, or ubiquitination at lysine 164. Although the Elg1 RLC shows affinity to SUMOylated PCNA, the complex is able to unload modified and unmodified versions of the clamp (Kang et al., 2019; Kubota et al., 2015; Kubota et al., 2013; Parnas et al., 2010; Shemesh et al., 2017; Shiomi & Nishitani, 2013). Thus SUMOylation may assist but is not essential for PCNA unloading. Retention and accumulation of PCNA on DNA is the major cause of genome instability in *elg1Δ* (Itzkovich, Choudhary, Arbel, & Kupiec, 2023; Johnson, Gali, Takahashi, & Kubota, 2016; Shemesh et al., 2017).

Previous studies found that telomeric proteins, and in particular the CST, can be regulated by SUMO modification (Hang, Liu, Cheung, Yang, & Zhao, 2011). Here we show that SUMOylation of PCNA plays a role in the regulation of telomere length, and uncover an interaction between the Elg1 RLC and the CST. We report that PCNA SUMOylation positively regulates telomerase activity,

and its unloading by the Elg1-RLC is essential for normal telomere size regulation. The N-terminus of Elg1 interacts with Stn1 during late S phase, and mediates the interaction between Stn1 and Cdc13 after PCNA has been unloaded from the telomeres.

Results

The long telomeres phenotype of *elg1*Δ strains requires PCNA SUMOylation

Previous results suggested that the Elg1 protein preferentially interacts with the SUMO machinery, and with SUMOylated PCNA (Parnas, Amishay, Liefshitz, Zipin-Roitman, & Kupiec, 2011; Parnas et al., 2010; Shemesh et al., 2017). To test whether PCNA modifications play any role in the elongated telomere phenotype of *elg1Δ*, we combined the *ELG1* deletion with mutations in the *POL30* gene, encoding PCNA. We mutated either lysine 127, lysine 164, or both (hereafter referred as *pol30-K127R, pol30-K164R,* and *pol30-RR*, respectively) in the *elg1*Δ background. **Figure 1** shows that the mutations in *POL30* have little effect by themselves. Importantly, whereas the single mutants did not affect the long telomeres of *elg1*Δ, mutating both lysine residues of each PCNA subunit completely abrogated the long telomere phenotype. Lysine 164 can be modified by both SUMO and ubiquitin; mutating only this residue prevents ubiquitination, but not SUMOylation; figure 1demonstrates that this mutant shows no effect. To ensure that SUMO, and not ubiquitin, is the modification responsible for the long telomeres of *elg1*Δ, we deleted *RAD18*, encoding the E3 ubiquitin ligase required to ubiquitinate PCNA (Hoege, Pfander, Moldovan, Pyrowolakis, & Jentsch, 2002; Stelter & Ulrich, 2003). **Figure 1** shows that lack of Rad18 has no effect on the elongated telomeres of *elg1*Δ. [Deletion of the SUMO E3 ligases *SIZ1* and *SIZ2* (to prevent SUMOylation) by itself alters telomere length (Hang et al., 2011) and thus they could not be used here]. We conclude that SUMOylation of PCNA is required for the long telomeres phenotype of *elg1*Δ mutants.

These results suggest that SUMOylated PCNA may play a central role in determining telomere length also in cells with wild type Elg1 activity. We thus next asked whether artificially overexpressing modified and unmodified PCNA may mimic the long telomere phenotype of *elg1*Δ. Since it is hard to force ubiquitination or SUMOylation of PCNA *in vivo*, we used covalent fusions between PCNA and either ubiquitin or SUMO. These have been shown in the past to mimic naturally modified PCNA molecules (Parker, Bielen, Dikic, & Ulrich, 2007; Takahashi, Wollscheid, Lowther, & Ulrich, 2020). We overexpressed either wild type Pol30, Pol30-RR (which cannot be modified) or Pol30-RR fused either to ubiquitin or to SUMO, in *pol30-RR* cells, unable to carry out modifications of the PCNA encoded by the genome. **Figure 1B** shows that telomeres become elongated upon overexpression of the wild type protein (which can be modified), or the Pol30-RR version fused to SUMO, but do not elongate upon overexpression of the unmodifiable Pol30-RR version, or the one fused to ubiquitin. These results, together with those of **Figure 1A**, show that SUMOylated PCNA, and not unmodified or ubiquitinated PCNA, is both necessary and sufficient for telomere elongation in the presence or in the absence of Elg1.

Genetic and Physical interactions between Elg1 and Stn1

The elongated telomere phenotype of Elg1 was identified in systematic screens of the yeast knockout collection (Askree et al., 2004; Gatbonton et al., 2006), together with ~200 additional mutants of similar phenotypes. To identify the pathway of telomere length maintenance in which Elg1 participates, we systematically combined the *elg1*Δ mutant with all the other mutants with long telomeres. Most double mutants exhibited telomeres longer than each of the single mutants, indicating that they affect different pathways. One of the mutants showing clear epistasis (the double mutant was not longer than the single mutants) carried a deletion of *RRP8*, a gene involved in modification of the ribosomal RNA. Further analysis demonstrated that the knockout of *RRP8*

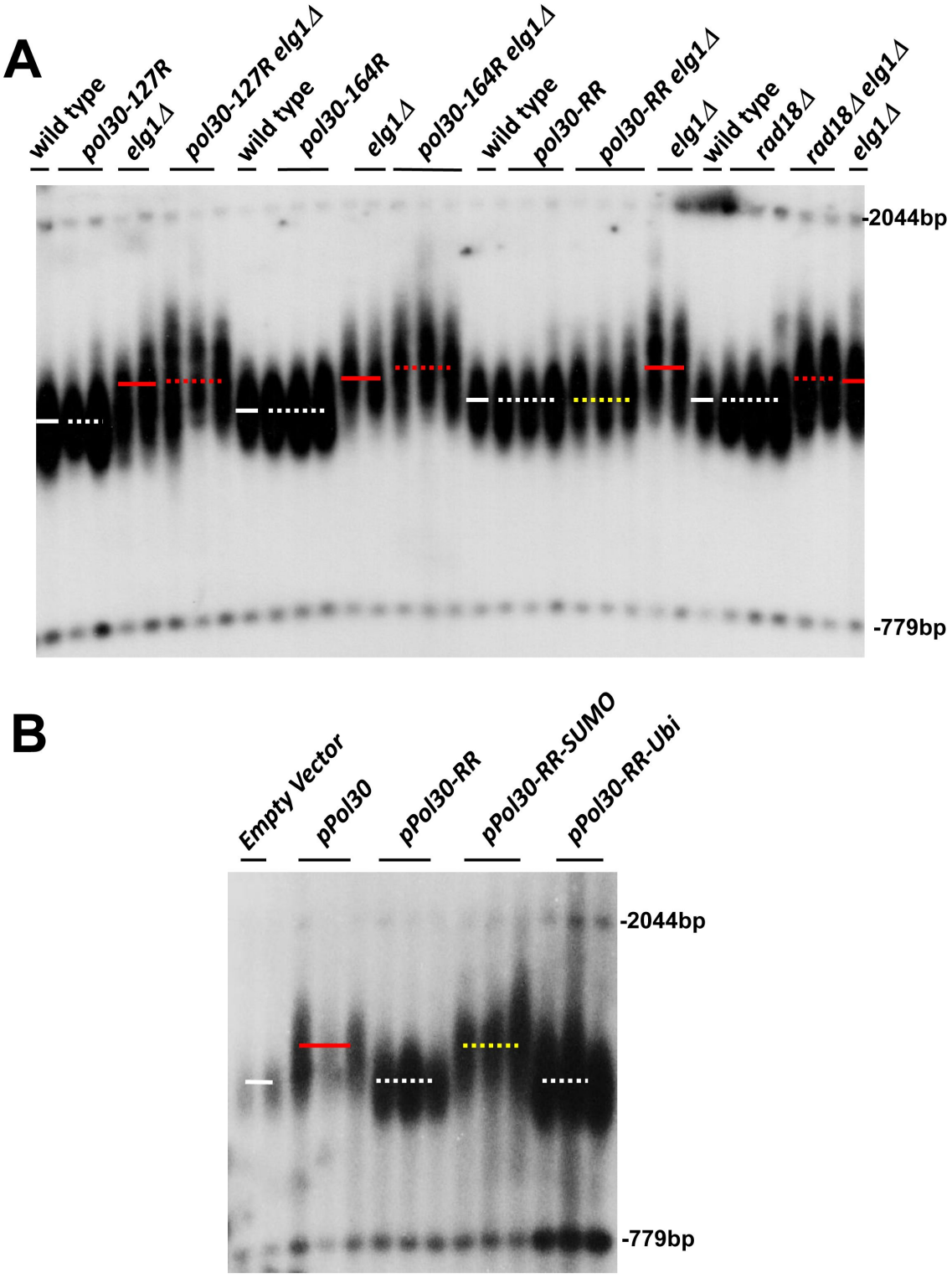

Figure 1

SUMOylated PCNA regulates telomere length.

A. Southern blot (Teloblot) showing that lack of SUMOylation of PCNA prevents telomere elongation. Independently-created colonies were passaged ten times, its DNA extracted, digested with *Xho*I and run in an agarose gel. The DNA was then transferred to a nitrocellulose membrane, which was incubated with a radioactive probe that detects telomeres, and a size marker. **B.** Teloblot showing that overexpression of wild type PCNA or Pol30-RR-SUMO fusion, but not Pol30-ubiquitin fusion or Pol30-RR causes telomere elongation.

exhibited the “neighboring gene effect”, and in fact the deletion of *RRP8* caused a decrease in the expression of its neighboring gene, *STN1* (Ben-Shitrit et al., 2012). We confirmed the genetic interactions between *ELG1* and *STN1* alleles by combining *elg1*Δ with the *stn1-13* and *stn1-164* alleles (Grandin, Reed, & Charbonneau, 1997), which exhibit long telomeres. The telomeres of the double mutants are not longer than those of the single mutants (**Figure 2A**), indicating that Elg1 works in the same TLM pathway as Stn1.

Given the epistatic relations between the *ELG1* and *STN1* genes, we addressed, by co-Immunoprecipitation (IP) and Yeast-Two-Hybrid (YTH) assay, whether the proteins in the CST complex interact with Elg1. IP of epitope-tagged Elg1 pulled down Stn1 but not the other two members of the CST complex, Cdc13 or Ten1 (**Figure 2B**). For our YTH experiments, the Elg1 protein was divided into three main functional domains: an N-terminal domain (amino acids 1-234), a central AAA domain (amino acids 235-514) and a C-terminal domain (amino acids 515-791) (Itzkovich et al., 2023), **Figure 3A**. The AAA and C-terminal domains contain the conserved RFC boxes and are required for the interactions with the small RFC subunits, shared by all RFC-like domains. The N-terminal domain (hereafter referred to as NTD) is unique to Elg1 (Arbel et al., 2021). We thus concentrated on this domain, and used the C-terminus as a negative control, as it does not interact with any of the proteins tested here.

The NTD contains SUMO-interacting motifs (SIMs), which were previously shown to interact with SUMO and the SUMO machinery (Parnas et al., 2011; Parnas et al., 2010) (**Figure 3A**). We used the YTH technique to test potential interactions between Elg1 and the CST components, and their dependency on SUMO. The N-terminus of Elg1 interacted strongly with Cdc13 and Stn1, and weakly with Ten1 (**Figure 3B,C**, **Figure S1**). In contrast, no interaction was detected with Elg1’s CTD. We could also detect interactions of Cdc13, Stn1 and Elg1’s NTD with SUMO (**Figure 3**). These were used as positive controls in all our experiments. Since the results with Ten1 were much weaker than those with Cdc13 and Stn1, we concentrated on these two last proteins. Below, we dissect, using YTH in various genetic backgrounds, the interactions between Elg1, Stn1, Cdc13 and PCNA. The results are also summarized in **Table 1**.

elg1-sim: mutation in the SUMO-interacting motif of Elg1; *elg1-DD: TT386/7DD*; *elg1-sim+DD*: combination of mutations in the SIM and in TT386/7; *elg1-WalkerA*: mutation that eliminates ATPase and unloading activity of Elg1. *cdc13-snm:* allele of Cdc13 that cannot be SUMOylated.

Interestingly, the interaction of Elg1’s NTD with Stn1, but not with Cdc13, was abolished when the SIM motifs (amino acids I27A, I93K, I121A, and I122A) were mutated (**Figure 3B,C**). We confirmed these results by co-Immunoprecipitation (co-IP) (**Figure 2B**). These results suggest that the interaction with Stn1 is mediated by SUMOylation of a protein, whereas the interaction of Cdc13 with Elg1-NTD is independent of the SIM in the plasmid-borne copy of Elg1. We confirmed this result by deleting the genes encoding the SUMO-specific E3 enzymes, *SIZ1* and *SIZ2* in the genome (a double deletion is necessary, as many times each protein can compensate for the lack of the other). **Figure 3D** shows that indeed, deletion of the genes encoding these enzymes abolishes the interaction of Stn1 and Cdc13 with Elg1’s NTD. We conclude that SUMO plays a role in mediating the Elg1-CST interactions. Since Elg1 preferentially interacts with SUMOylated PCNA (Parnas et al., 2010), and Stn1 can bind SUMO non-covalently in YTH (**Figure 3C**), we reckoned that SUMOylated PCNA may mediate the interaction between Stn1 and Elg1. We therefore tested whether abolishing PCNA’s SUMOylation sites in the genome (*pol30-RR*) had an effect on the Stn1-Elg1 interaction. We found, however, that Elg1 and Stn1 could still interact in the *pol30-RR* strain (**Figure 3D**), despite the fact that PCNA cannot become SUMOylated. Consequently, a different SUMOylated target seems to mediate the interaction between Elg1 and Stn1, or else either one of the proteins is SUMOylated. Despite numerous attempts, we were unsuccessful in observing SUMOylation of either Stn1 or Elg1.

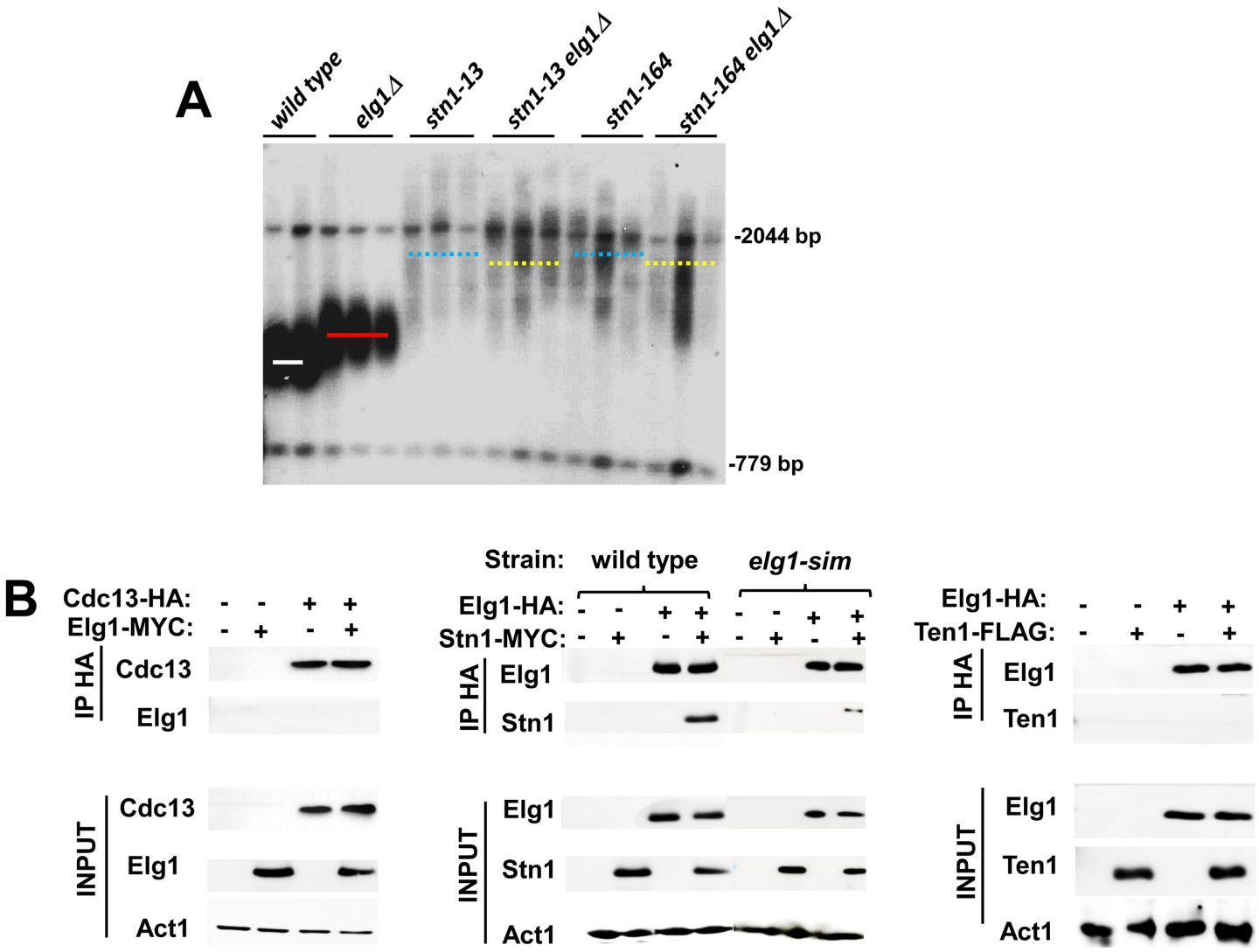

Figure 2

Genetic and physical interaction between *ELG1* and *STN1.*

A. Teloblot showing epistasis between *elg1*Δ and *stn1* mutants. **B.** Co-Immunoprecipitation experiment showing physical interaction between Elg1 and Stn1 and reduced physical interaction between Elg1-sim and Stn1. No interaction could be detected between Elg1 and Cdc13 or Ten1.

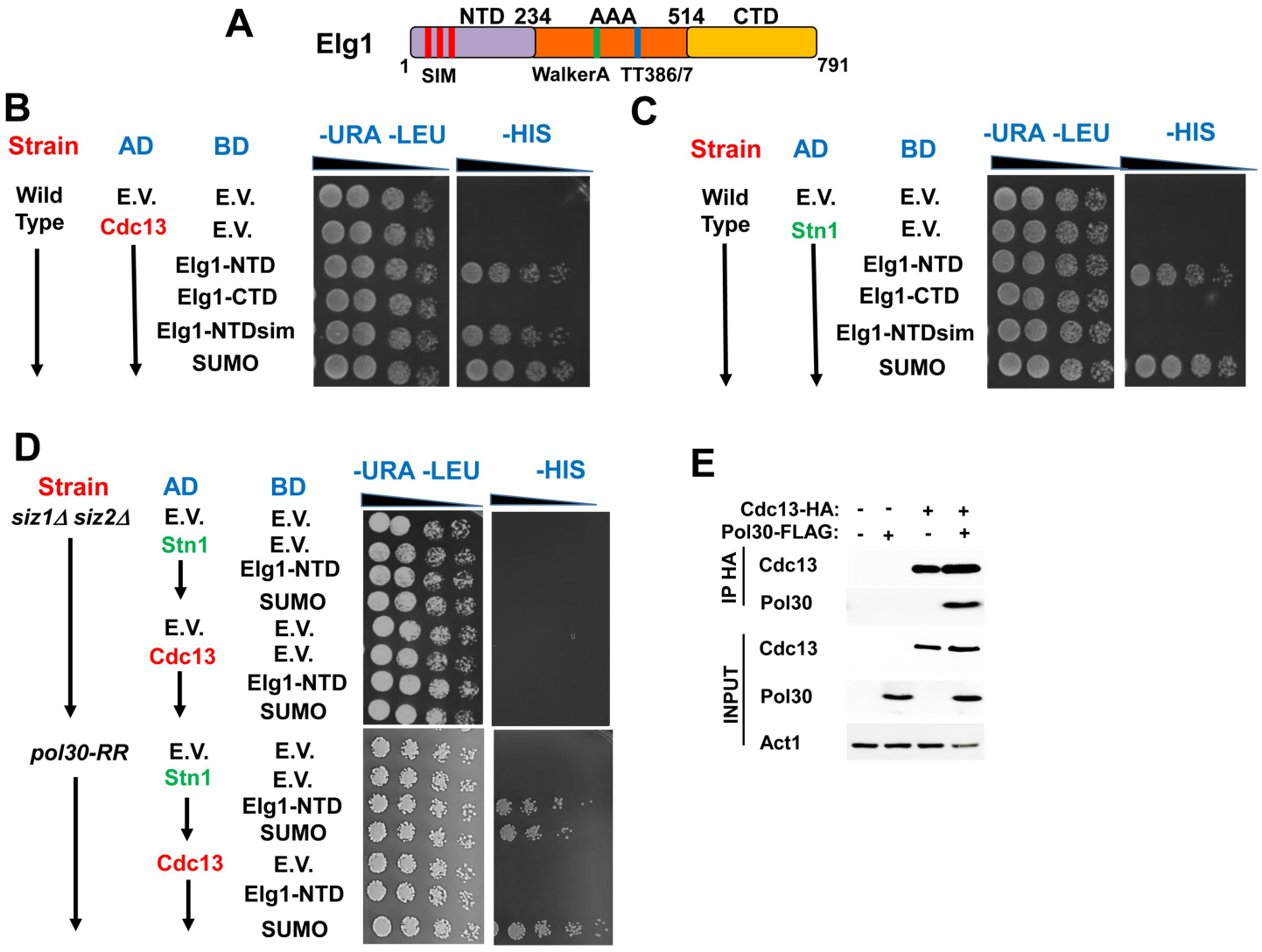

Figure 3

Determinants of the Elg1-Stn1 interaction.

A. Schematic representation of the Elg1 protein. The 3 SIM motifs, the WalkerA motif and the 2 threonines at the interface with PCNA are shown. **B.** Yeast-Two-Hybrid (YTH) interaction of Cdc13 and Elg1 in a wild type strain. AD: protein fused to the activating domain of Gal4; BD: protein fused to the DNA binding domain of Gal4. e.v.: empty vector.**C.** YTH interaction of Stn1 and Elg1 in a wild type strain. **D.** YTH experiments in the *siz1*Δ *siz2*Δ and *pol30-RR* background. **E.** Co-Immunoprecipitation experiment showing physical interaction between Cdc13 and PCNA.

Strain	Plasmid	Interaction w/Stn1	Interaction w/Cdc13
wild type	Elg1-NTD	**YES**	**YES**
wild type	Elg1-sim	**NO**	**YES**
*siz1*Δ *siz2*Δ	Elg1-NTD	**NO**	**NO**

Table 1

Summary of all YTH interactions presented.

pol30-RR	Elg1-NTD	**YES**	**NO**
elg1Δ	Elg1-NTD	**YES**	**NO**
elg1-sim	Elg1-NTD	**YES**	**YES**
elg1-DD	Elg1-NTD	**YES**	**NO**
elg1-sim+DD	Elg1-NTD	**YES**	**NO**
elg1-WalkerA	Elg1-NTD	**YES**	**NO**
cdc13-snm	Elg1-NTD	**YES**	**YES**
wild type	Elg1-NTD	**-**	***snm:* NO**

Table 1 (continued)

We also noted that, despite the fact that mutation in the plasmid-borne NTD's SIM had no effect on the interaction with Cdc13 (**Figure 3B**), deletion of *SIZ1* and *SIZ2*, and mutation in the SUMOylation sites in PCNA completely abolished the interactions between Elg1's NTD and Cdc13 (**Figure 3D**). This implies that PCNA SUMOylation is necessary for the interaction between Elg1 and Cdc13. We confirmed by co-IP that Cdc13 indeed physically interacts with PCNA (**Figure 3E**). The interaction between Cdc13 and PCNA was almost completely abolished when PCNA could not be SUMOylated (**Figure S2**), In summary, the interaction between Cdc13 and both PCNA and Elg1, but not that of Elg1 with Stn1, is dependent on SUMOylation of PCNA.

Elg1's functional activity is essential for its interaction with Cdc13

When we repeated the YTH assays in a strain in which the *ELG1* gene is deleted from the genome, we were surprised to see that now Cdc13 failed to interact with the plasmid-borne Elg1-NTD (**Figure 4A**, compare to **Figure 3B**). In contrast, the interaction between Stn1 and Elg1-NTD was not affected by the deletion of the genomic *ELG1*.

Taken together, these results imply that whereas Stn1 interacts directly with the plasmid-borne Elg1-NTD, via its SIM-mediated SUMO-binding, the interaction with Cdc13 require both PCNA SUMOylation and the genomic *ELG1* gene. We can envision two formal possibilities for the latter requirement: either 1) the interaction with Cdc13 requires parts of the Elg1 protein not present in the plasmid-borne NTD (e.g., the AAA or CTD domain), or 2) Elg1's activity (e.g., PCNA unloading) may be required to facilitate the physical interaction of the NTD with Cdc13.

To distinguish between these two possibilities, we carried out YTH experiments with strains carrying different alleles of *ELG1* in the genome. Three mutations were tested first: *elg1-sim* (mutated in amino acids I27A, I93K, I121A I122A), which does not interact with SUMO; *elg1-TT386/387DD*, hereafter referred to as *elg1-DD*, which affects the interface Elg1-PCNA (Shemesh et al., 2017); and *elg1-sim+DD*, an allele that combines both mutations. Whereas the *SIM* mutation has a very mild effect on the ability of cells to unload PCNA, and displays no phenotypes, the *elg1-DD* is more impaired and has a stronger phenotype, and the *elg1-sim+DD* allele has essentially a null phenotype, similar to that of *elg1*Δ, but expressing the protein at normal levels (Itzkovich et al., 2023; Shemesh et al., 2017).

We found that in strains carrying genomic *elg1-DD* and *elg1-sim+DD* alleles, Cdc13 failed to interact with plasmid borne Elg1-NTD, whereas in the *elg1-sim* strain the interaction was weak but clearly seen (**Figure 4B-D**). This observation indicates that the PCNA unloading activity of Elg1 is essential to allow the interaction between Cdc13 and the plasmid borne Elg1-NTD: this activity is high in *elg1-sim* mutants, intermediate in the *elg1-DD* strain and is abolished by the *elg1-sim+DD* mutations. Note that the presence of a functional SIM at the plasmid-borne NTD is sufficient to ensure a mild interaction with Cdc13, but only if the genomic copy retains PCNA unloading activity (as in the *elg1-sim* mutant). On the other hand, the presence of a functional SIM in the inactive *elg1-DD* allele does not warrant an interaction of Elg1's NTD (which has SIM motifs) with Cdc13. We confirmed this idea by analyzing the interaction between Cdc13 and Elg1's NTD in another SIM-containing mutant strain devoid of PCNA unloading activity, *elg1-WalkerA* (KK343/4DD), which lacks ATPase and PCNA unloading activity (Itzkovich et al., 2023). This mutant expresses wild type levels of protein, and the mutant has essentially the same phenotype of an *elg1*Δ allele. **Figure 4E** shows that no interaction between the plasmid-borne Elg1 NTD and Cdc13 could be detected, despite the fact that functional SIMs are present both at the NTD and in the genome-encoded Elg1-WalkerA protein. All the mutants expressed the Elg1 protein at wild type levels (**Figure S3**).

We conclude that whereas the interaction with Stn1 is direct and mediated by the SUMOylation of a protein (different from PCNA), the interaction between Elg1's NTD and Cdc13 is indirect, and can only take place after PCNA has been SUMOylated and unloaded. This suggests a model for the coordination of telomerase activity and DNA replication: the presence of SUMOylated PCNA at

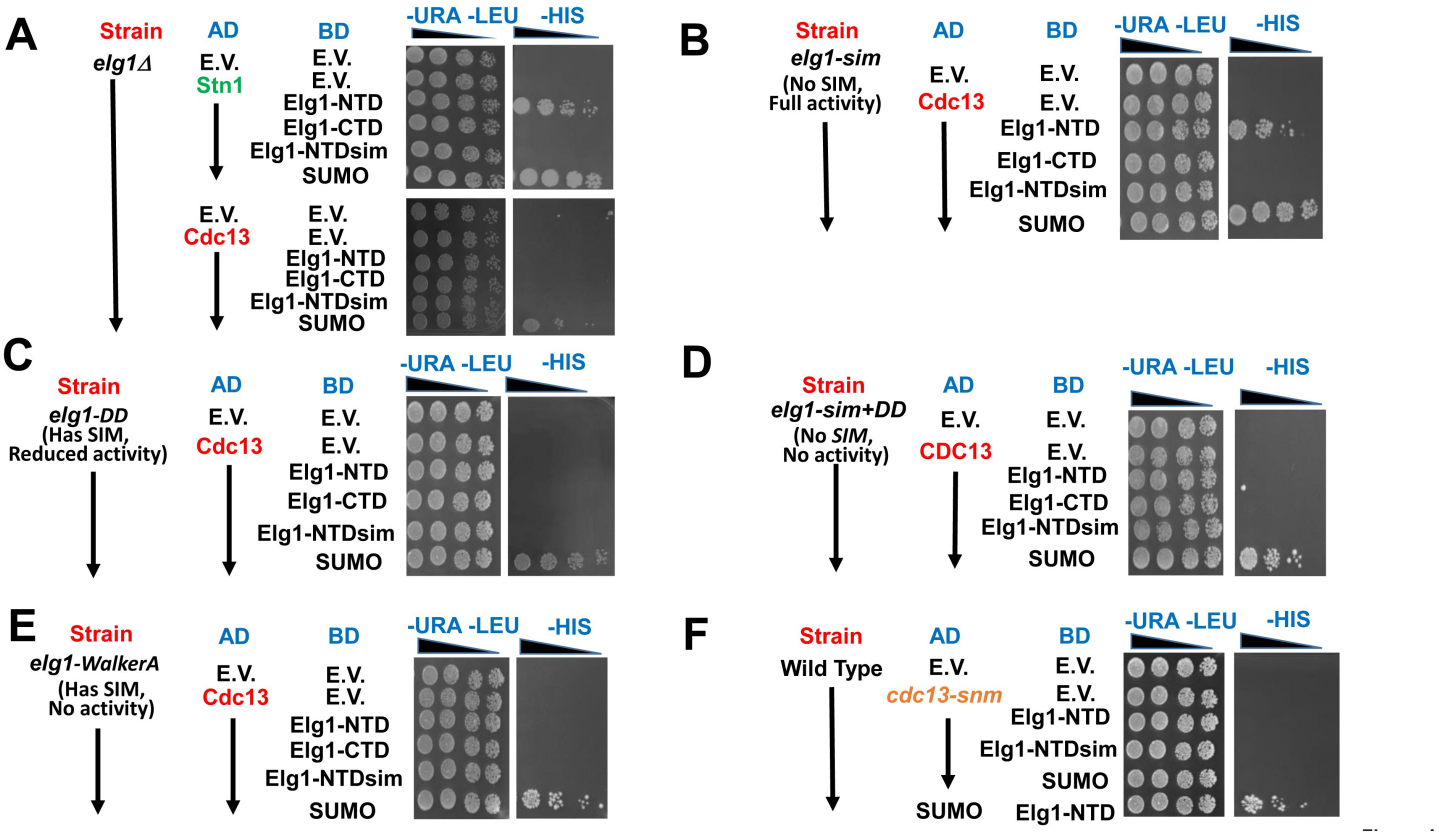

Figure 4

The unloading activity of Elg1 and Cdc13 SUMOylation are necessary for the Elg1-Cdc13 interaction.

A. YTH experiment in a *elg1*Δ strain. **B.** YTH experiment in a *elg1-sim* strain. **C.** YTH experiment in a *elg1-TT386/7DD* strain. **D.** YTH experiment in a *elg1-sim+DD* strain. **E.** YTH experiment in a *elg1-Walker AB* strain. **F.** Lack of interaction between *cdc13-snm* and Elg1 (in a wild type strain).

telomeres may serve as a positive signal for telomerase activity. Once Elg1 unloads PCNA, however, Stn1-Elg1 can bind Cdc13, ending telomerase activity. In this context, it is possible to understand the results seen in **Figure 3D**, showing that mutations in the SUMO E3 enzymes or in the sites of SUMOylation of PCNA, both of which prevent PCNA SUMOylation, show the same effect as the *sim+DD* or *walkerA* mutations in the genomic *ELG1* copy, which prevent PCNA unloading activity.

SUMOylation of Cdc13 is needed for interaction with Elg1

Cdc13 undergoes SUMOylation, and this modification plays an essential role in the negative regulation of telomere length (Hang et al., 2011). Thus, Cdc13 is a good candidate for the target of SUMOylation required for the interaction between Elg1 and Stn1. Accordingly, mutations of the Cdc13 SUMOylation sites (*cdc13-snm*, "SUMO no more") weaken its interaction with Stn1 and lead to elongated telomere phenotype (Hang et al., 2011). We therefore checked whether the *cdc13-snm* mutation affects also the interaction with Elg1. **Figure 4F** shows that indeed preventing SUMOylation of Cdc13 abolishes the interaction between this protein and Elg1. Note that the strain used expresses a wild type genomic copy of *CDC13*. Thus, SUMOylation **of the plasmid-borne version of Cdc13** (i.e., in *cis*) is required for these interactions in the YTH assay. No effect was observed when the YTH plasmid carried a wild type copy of *CDC13*, and the genomic copy was the *cdc13-snm* allele (**Figure S4A**). In summary, the N-terminus of Elg1 interacts with Cdc13 only if 1) Cdc13 can be SUMOylated (**Figure 4F**), 2) PCNA can be SUMOylated (**Figure 3D**), and 3) PCNA can be unloaded (**Figure 4A, C, D,E**).

The interaction of Elg1 with Stn1 takes place only at late S-phase

The fact that the interactions of the Elg1 NTD with Cdc13 are dependent on PCNA modification and unloading, whereas those with Stn1 are not, suggests that the interaction with Cdc13 may be mediated by, or dependent on, the interaction of Elg1 with Stn1. To further dissect this point, we divided the Stn1 protein into an NTD (first 281 amino acids) and a CTD (amino acids 282-495). The later has been shown to be the region that interacts with Cdc13 (Petreaca, Chiu, & Nugent, 2007). **Figure S4B** shows that the NTD of Elg1 also interacts with Stn1 via its C-terminal domain, both in *WT* and *elg1*Δ strains.

Having established Stn1 as the main interactor of Elg1 in the CST complex, we next examined the interaction between these proteins throughout the cell cycle phases. We arrested a strain with tagged Elg1, Stn1 and DNA Polymerase Delta in G1 with alpha factor. Cells were released into the cell cycle and samples were taken at intervals during two cell cycles. This allowed us to map the timing of interaction between Elg1 and Stn1 proteins, and whether this interaction is codependent on the movement of replication fork. **Figure S5** shows that the total level of these proteins does not change throughout the cell cycle. We immunoprecipitated Elg1, and measured the level of the other two proteins by Western blot (**Figure 5A-C**). Pol3 was co-IPed with Elg1 strongly only during the S phase (40-70 minutes, 120-140 minutes) whereas Stn1 was only detected in late S phase (60-70 minutes, 140-160 minutes) for both cell cycles and was barely detected in G1 (**Figure 5A-C**). This pattern coincides with telomerase activity at telomeres, which is low in early to mid S phase and peaks in late S-phase (Bianchi et al., 2008, Giraud et al., 2008, Taggart et al., 2002).

To monitor the arrival of the replication fork to telomeres, we performed chromatin immunoprecipitation at telomeres (Telo-ChIP) in synchronized cells. **Figure 5D** shows that the timing of arrival of PCNA to the telomeres (60-70 minutes into the cell cycle) coincides with the timing of the co-IP between Elg1 and Stn1 (**Figure 5A-C**). Thus, our results are consistent with the idea that Elg1 moves with the replication fork, and interacts with Stn1 at the end of S-phase, when telomeres are replicated and telomerase is activated.

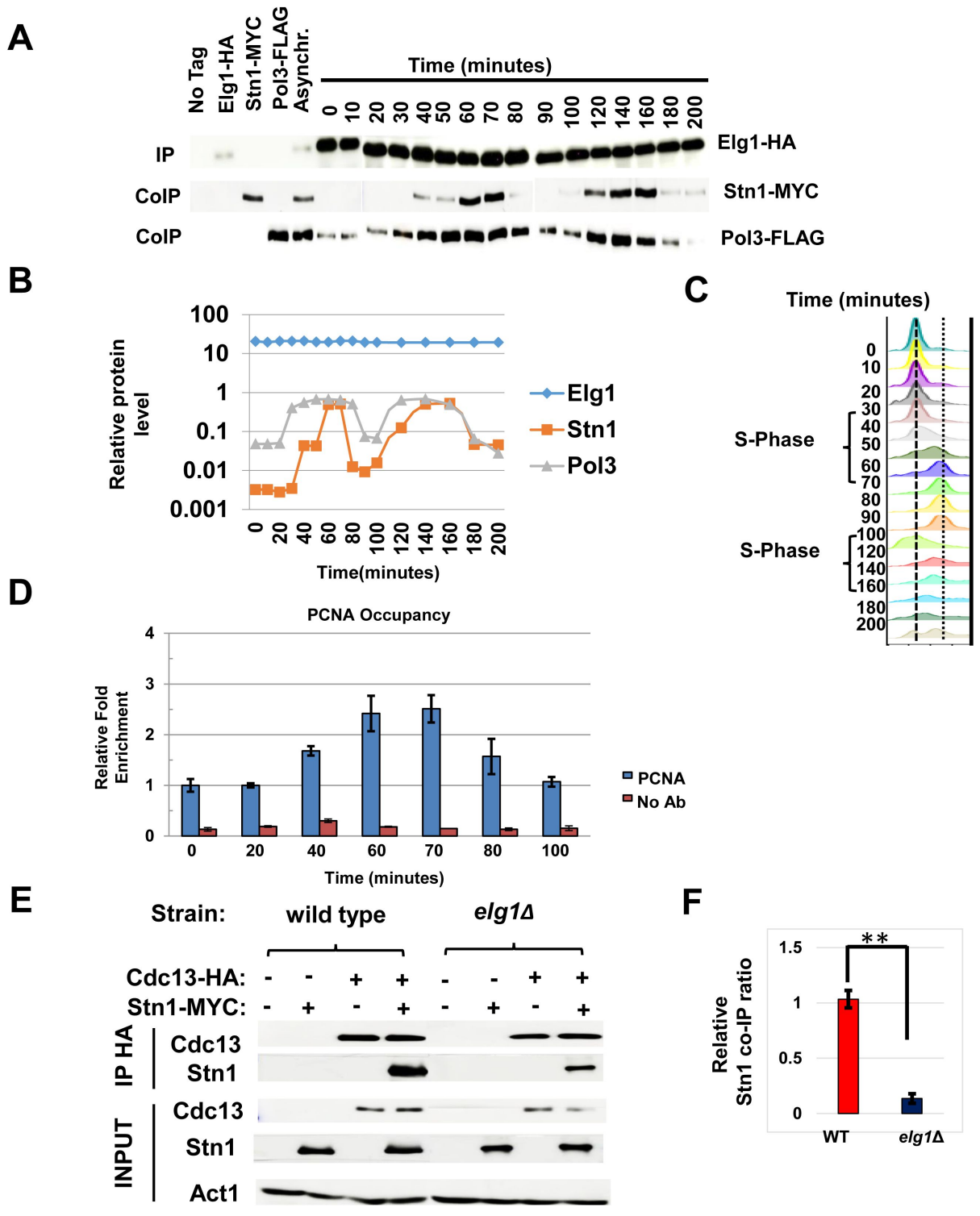

Figure 5

Timing of Elg1-Stn1 interaction.

A. Co-IP experiment with synchronized cells. Aliquots were taken at time intervals, Elg1 was immunoprecipitated, and the level of Stn1 and Pol3 (the large subunit of DNA polymerase Delta) were monitored by Western blot. Strains with single tags are shown as controls. Whole Cell Extract results are shown in **Figure S5**. **B.** Quantitation of the Western shown in A. **C.** DNA content of the cells used in A, by cell cytometry. **D.** Chromatin immunoprecipitation at telomeres (Telo-ChIP) in synchronized cells showing PCNA occupancy. **E.** Interaction between Stn1 and Cdc13 in a wild type and a *elg1*Δ strain. **F.** Quantitation of three independent biological repeats of the experiment shown in E. **:p<0.001.

The interaction between Cdc13 and Stn1 is dependent on Elg1

Our YTH and IP data suggest that unlike the relationship between Cdc13 and Elg1, the interaction between Stn1 and Elg1 is direct (**Figures 2 and 3**). Since both Elg1 and Cdc13 bind Stn1 at its C-terminus [**Figure S3** and (Petreaca et al., 2007)], it is possible that the absence of Elg1 may affect the interaction between the two CST members. We thus measured the co-IP of Cdc13 and Stn1 in a wild type or an *elg1*Δ strain. Cells were cell-cycle synchronized in S-phase, as the interaction between these two proteins was barely seen when immunoprecipitated in an asynchronous culture. When Elg1 was absent, Stn1 exhibited a strong reduction in its co-precipitation with Cdc13 (**Figure 5E,F**). This suggests that the interaction between Stn1 and Cdc13 is, at least partly, dependent on Elg1.

Model: Elg1 negatively regulates the telomere length by forming an interaction with the CST complex

Taken together, our results suggest the following model (**Figure 6**):

Following firing of the origin proximal to the telomeres at late S-phase, Elg1 moves with the fork, unloading PCNA from the lagging strand as the fork progresses (Kubota et al., 2015; Kubota et al., 2013; Shemesh et al., 2017) (**Figure 6A**). SUMOylation of PCNA acts as a signal for the activation of telomerase (**Figures 1A,B and 6B**). Once Elg1 reaches the telomeres, it interacts with Stn1 (**Figure 5**). This interaction requires SUMOylation of Stn1 or of some other protein, which is not PCNA (**Figure 3D**) nor Cdc13 (**Figure S3A**) and could be Stn1 itself or another telomeric protein (Hang et al., 2011). The Elg1-Stn1 complex then unloads the SUMOylated PCNA at the telomeres, and Cdc13 becomes SUMOylated (**Figure 6C**), now interacting with Elg1-Stn1. SUMOylation of Cdc13 allows its interaction with Stn1, and presumably with Ten1 (Hang et al., 2011), creating the complete CST complex, and preventing further telomerase activity (**Figure 6D**). The fact that co-IP experiments detect a strong interaction with Stn1 but fail to detect an interaction with Cdc13 (**Figure 2B**) suggests that indeed the interaction Elg1-Cdc13 is very transient, and Elg1 leaves chromatin immediately, leaving the C-terminus of Stn1 free to interact with Cdc13. It is possible that a single “wave” of SUMOylation at the telomeres coordinates PCNA unloading with the interaction between Elg1 and Cdc13. Alternatively, unloading of PCNA by Elg1 may allow the recruitment of SUMO ligases to modify Cdc13. In support for this idea, YTH interactions between Stn1 or Cdc13 with SUMO are abolished in *siz1*Δ *siz2*Δ strains (**Figure 3D**).

Discussion

We have presented evidence for the fact that two negative regulators of telomerase activity, Elg1 and Stn1, work in the same pathway to coordinate chromosomal DNA replication and telomere elongation. We present a model (**Figure 6**) in which the unloading of SUMOylated PCNA by Elg1 at telomeres facilitates the interaction between Stn1 and Cdc13 that negatively regulate telomerase activity.

This model is supported by several observations. First, the double mutants of Elg1 and Stn1 do not show increased telomere length phenotype in comparison to the single mutants, indicating that both proteins work in the same pathway that negatively regulates telomere length (**Figure 2A**). In addition, the interaction between Elg1 and Stn1 seems direct and occurs exclusively during the late S-phase (**Figures 2B** and **5A-C**). This interaction is neither dependent on SUMOylation of PCNA (**Figure 3D**) nor on Cdc13 (**Figure S3A**). In contrast, the interaction between Cdc13 and Elg1 is dependent on both the SUMOylation of PCNA (**Figure 3D**) and of Cdc13 itself (**Figure 4F**). We suggest that Elg1 participates in the negative regulation of telomere length by unloading SUMOylated PCNA from the telomeres. This eliminates a positive signal for telomerase activity, and causes the exchange of Est1 by Stn1 as interactor of Cdc13, effectively terminating telomerase

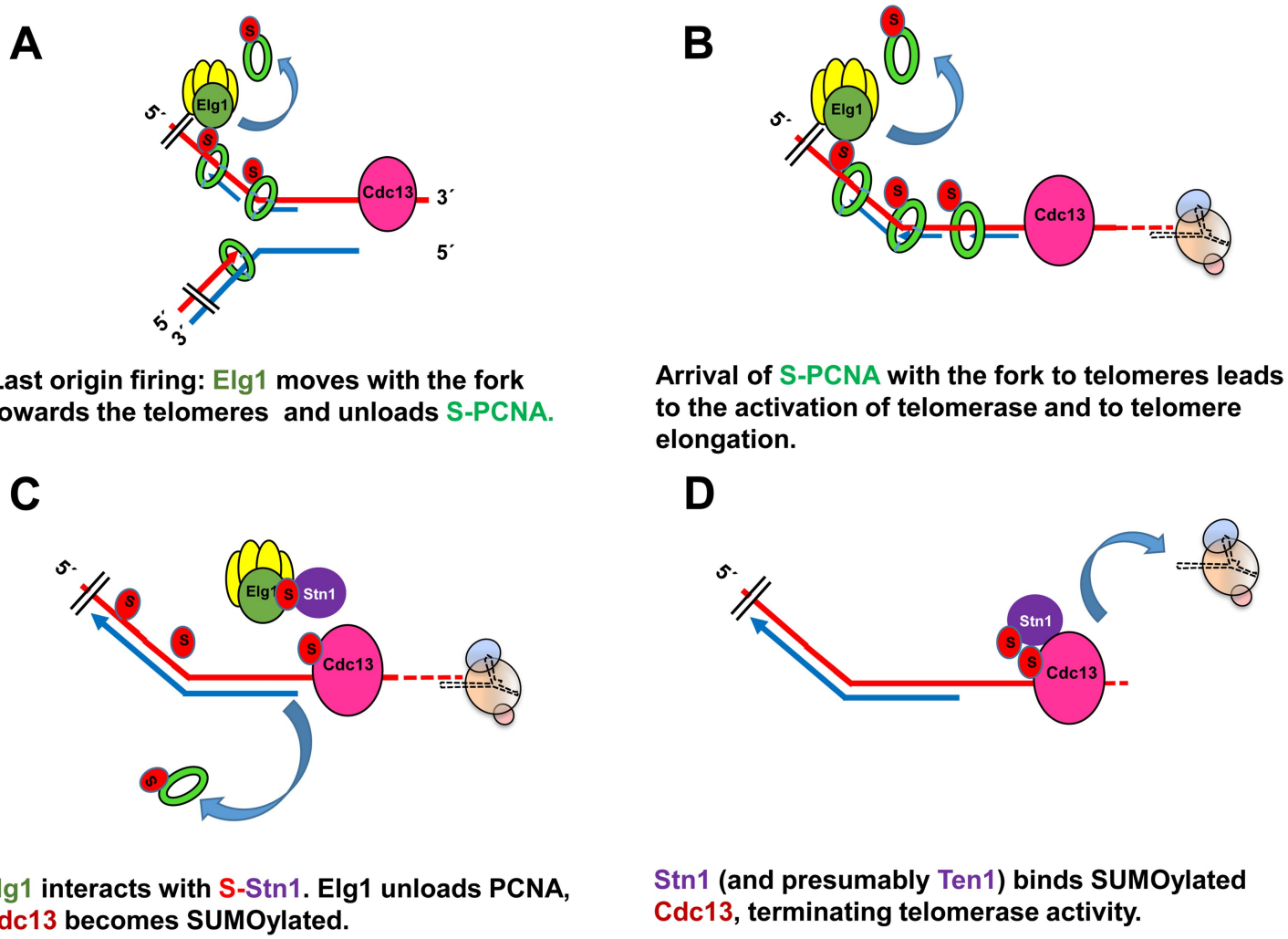

Figure 6

Model for the role of SUMOylated PCNA and Elg1 in telomere length regulation.

A. Cdc13 binds ssDNA at the telomeres, Elg1 moves with the replisome at the lagging strand, unloading PCNA in each Okazaki fragment. **B.** Arrival of the SUMOylated PCNA at the fork to Cdc13 promotes telomerase activity. **C.** Elg1 interacts with Stn1, which could be SUMOylated. Cdc13 becomes SUMOylated. Elg1 unloads PCNA and leaves telomeres. **D.** Stn1 is now able to interact with Cdc13, evicting Est1 and terminating telomerase activity.

activity (Chandra et al., 2001; Gao, Cervantes, Mandell, Otero, & Lundblad, 2007; Ge et al., 2020; Hang et al., 2011; Puglisi et al., 2008). As expected from the model, the interaction Elg1-Cdc13 is transient, and cannot be detected by co-IP.

We present evidence for the fact that SUMOylated PCNA serves as a positive signal for telomere elongation (**Figure 1**). Preventing its unloading (by mutations in the *ELG1* gene) leads to elongated telomeres, and so does PCNA overexpression, provided the protein is able to be SUMOylated, or is fused to SUMO (thus mimicking SUMOylated PCNA) (**Figure 1**). *ELG1* is the only PCNA unloader in eukaryotes; however, it is not an essential gene, implying that there are alternative ways in which PCNA can get unloaded (either spontaneously or with the help of RFC) (Arbel et al., 2021). Once this step does take place, Stn1 can replace Est1 as Cdc13's partner, and stop the activity of telomerase, but at a longer telomere steady state equilibrium point. This explains both the long telomere phenotype of *elg1* mutants, and the fact that it is less severe than that of *stn1* alleles (**Figure 2**). The fact that *pol30-RR* mutants have stable, normal-sized telomeres (**Figure 1**), imply that despite lacking PCNA SUMOylation, they are able to solve the "end replication problem". Our results support the notion (Maicher et al., 2017) that there are two alternative modes of telomerase: one carrying "basal activity", with minimal genetic requirements, able to maintain most telomeres at normal size, and another, "sustained activity", required for increased elongating activity (Maicher et al., 2017). Previous results have shown that the first mode does not require Rnr1, the large subunit of the ribonucleotide reductase enzyme, whereas the second depends on it (Maicher et al., 2017). Similarly, SUMOylated PCNA is only needed for the second mode of telomerase. In our experiments, the phenotype that we monitored was elongated telomeres (e.g., by deletion of *ELG1* or by overexpression of PCNA) which required sustained telomerase activity. To solve the "end replication problem" and maintain normal telomere length, very little telomerase activity might be needed, and even unmodified PCNA or telomeric proteins might be able to provide that basal level.

Many proteins in the cell become SUMOylated at one point or another. This modification is usually transient, due to the activity of de-SUMOylases (which eliminate the modification) and STUbLs (SUMO-dependent ubiquitin ligases, which usually send the protein to degradation). It has been proposed that SUMOylation often works by targeting many physically interacting proteins together. This may be executed by the local recruitment of SUMO ligases to preassembled, co-localized protein groups. SUMOylation thus may increase their interactions, acting to increase their local concentration or effective interaction (Jentsch & Psakhye, 2013). SUMOylation of telomeric proteins seems to be one of those cases (Garg et al., 2014; Hang et al., 2011; Matmati et al., 2018; Miyagawa et al., 2014). In our model, PCNA, Cdc13 and possibly Stn1 undergo SUMOylation to ensure the proper sequence of events in the coordination between telomerase activity and chromosomal replication. Cdc13 binds Pol12, a subunit of Polymerase Alpha (Grossi et al., 2004; Petreaca et al., 2006; Puglisi et al., 2008), allowing the synthesis of the lagging strand on the newly synthesized ssDNA. Thus, the arrival of the replisome to a short telomere at the end of S-phase brings PCNA, whose SUMOylation can elicit both telomerase activity, and its subsequent repression when PCNA is unloaded by Elg1. This in turn primes synthesis of the complementary strand, thus coordinating telomere elongation with chromosomal replication.

Materials and methods

Yeast strains, plasmids and Media

All yeast strains used are described in **Table 2**. All plasmids are described in **Table 3**. Yeast extract-peptone-dextrose (YPD) medium was prepared with a ready-to-use mixture (Formedium). Synthetic complete (SC) minimal medium was prepared with 2% dextrose (Formedium), yeast

nitrogen base without amino acids (Difco), and all necessary amino acids. 2% agar (Difco) was added for solid medium. Standard yeast genetic procedures were used to create single and double mutants. Unless stated otherwise, all experiments were carried out at 30°C.

PCNA overexpression

The high copy number pGAD424 plasmid alone, or carrying the wild type *POL30*, the mutant *pol30-KK127,164RR* (*pol30-RR*), or the *pol30-RR* fused to ubiquitin or to SUMO (Parker et al., 2007; Takahashi et al., 2020) were transformed into *pol30-RR* strains and grown under selective conditions.

Southern Teloblots

(Harari & Kupiec, 2018; Harari, Romano, Ungar, & Kupiec, 2013; Harari, Zadok-Laviel, & Kupiec, 2017; Rubinstein et al., 2014): For each genotype, at least 3 independently created strains were tested after 10 consecutive passages on YPD plates. Genomic DNA was isolated by pelleting 15 ml saturated overnight yeast culture grown in YPD, then beating the cells using 0.5 mm glass beads in 500 µl lysis buffer and 500 µl phenol chloroform. Cells were spun down for 10 min at 14 000 rpm, and ~500 µl of the top clear solution, containing the genomic DNA, was carefully taken out. DNA was precipitated using 1000 µl of 95% ethanol and then pelleted. The supernatant was discarded, and 500 µl of 70% ethanol was added. Pellets were left to dry before resuspension in 300 µl TE with RNaseA for 10 minutes at 65°C. The DNA was precipitated by adding 130 µl of 7.5 M ammonium acetate (pH 7) and 1 ml of 95% ethanol at −20°C, spun, and resuspended in 50 µl of TE. About 10 µl of genomic DNA was digested using XhoI overnight at 37°C. Digested gDNA and ladder were loaded onto a 0.8% agarose gel and electrophoresed overnight at ~67V in 1X TBE. The gel was denatured for 30 min in a rocking shaker (1.5 M NaCl, 0.5 M NaOH) and was neutralized for 15 min (1.5 M NaCl, 0.5M Tris, pH 7.0). The DNA on the gel was transferred onto a hybond nylon membrane (GE Healthcare GERPN303B) with 10X SSC (1.5 M NaCl 0.17 M NaCitrate, dihydrate) and UV cross-linked before blocking in Church buffer (0.5 M Na_2HPO_4, pH7.2, 7% SDS, 1 mM EDTA, 1% BSA) for ~2 h at 65°C. $_{32}$P radiolabeled PCR fragments were added onto the membrane and left to incubate overnight. The membrane was washed 3–4 times for 15 min each, with 1X SSC 0.1%SDS buffer before drying and exposing it to X-ray film. The teloblots blots were exposed for 1-8 days at -70$_0$C before the film was developed.

Telomeric probe

S. cerevisiae-specific telomeric probe was labeled by random priming of 20 -25 ng of a telomeric fragment and of 20-25 ng of size-control fragments using the DNA labeling mix from Biological Industries. Both fragments were generated by PCR using the primers indicated below. For the telomeric probe, a specific region of the left telomere of chromosome VII was amplified to generate a product 370 bp long. As size-control probe, a specific region of chromosome II was amplified to generate a product 1100 bp long. Since it contains an XhoI site, the size-control probe detects two bands in the sizes of 2044bp, 779bp in the Southern blot.

The primers for the Y' element product are:

Y' elementForward: GTTGGAGTTTTTCAGCGTTTGC Y' element Reverse: TGTGAACCGCTACCATCAGC The Y' element PCR product is ~370bp

The primers for the Control product are:

Tel Control Forward: TTGTAGGGGCCTTTTGTAATGT Tel Control Reverse: GTGCGCCCAGTAAGGGGT

The Control PCR product is ~1100bp

Strain Number	*Name*	*Genotype*
13237	BY4741	*MATa his3del, leu2del, met15del, ura3del*
12480	*BY elg1Δ::HygMX*	*MATa his3del, leu2del, met15del, ura3del, elg1::HYG*
14421	*BY pol30-K127R*	*MATa his3del, leu2del, met15del, ura3del, pol30-K127R::LEU2*
14425	*BY pol30-K164R*	*MATa his3del, leu2del, met15del, ura3del, pol30-K164R::LEU2*
14423	*BY pol30-KK127,164RR*	*MATa his3del, leu2del, met15del, ura3del, pol30-RR::LEU2*
14426	*BY pol30-K127R elg1Δ*	*MATa his3del, leu2del, met15del, ura3del, pol30-K127R::LEU2, elg1::HYG*
14430	*BY pol30-K164R elg1Δ*	*MATa his3del, leu2del, met15del, ura3del, pol30-K164R::LEU2, elg1::HYG*
14428	*BY pol30-KK127,164RR elg1Δ*	*MATa his3del, leu2del, met15del, ura3del,pol30-RR::LEU2,elg1::HYG*
14398	*BY rad18Δ:: KanMX*	*MATa his3del, leu2del, met15del, ura3del, rad18::KanMX.*
14401	*BY rad18D::KanMX elg1D::HygMX*	*MATa his3del, leu2del, met15del, ura3del, rad18::KanMX, elg1::HYG*
19606	*BY pol30-KK127,164RR Elg1-HA*	*MATa his3del, leu2del, met15del, ura3del ELG1-HA-NAT pol30-KK127,164RR:HIS3*
20622	*BY bar1 CDC13-HA ELG1-HA*	*MATa his3del, leu2del, met15del, ura3del, bar1::LEU2, CDC13:3HA:HISMX, ELG1-HA-KanMX*
18418	*BY STN1-Myc Elg1-HA*	*MATa his3del, leu2del, met15del, ura3del stn1::HYG CEN LEU2 STN1-(G)9-(myc)7 ELG1-HA-KanMx*

Table 2

Yeast strains used in this study.

18790	*BY bar1 STN1-Myc ELG1-HA POL3-FLAG*	*MATa his3del, leu2del, met15del, ura3del bar1::NatMX stn1::HYG CEN LEU2 STN1-(G)9-(myc)7 ELG1-HA-KanMx POL3-FLAG-URA3*
19552	*BY bar1 CDC13-HA POL30-FLAG*	*MATa his3del, leu2del, met15del ura3del, bar1:: NatMX CDC13-3HA::HISMX, POL30-FLAG::KanMX,*
20625	*BY CDC13-HA STN1-Myc*	*MATa his3del, leu2del, met15del, ura3del, CDC13-3HA::HISMX, stn1::HYG CEN LEU2 STN1-(G)9-(myc)7,*
20626	*BY CDC13-HA STN1-Myc elg1D*	*MATa his3del, leu2del, met15del, ura3del,CDC13-3HA::HISMX, stn1::HYG ,CEN LEU2 STN1-(G)9-(myc)7,elg1::KanMx*
20623	*BY TEN1-FLAG*	*MATa his3del, leu2del, met15del, ura3del trp1del Lys2del can1:: STE2pr-Sp_HIS5 ten1::KanMX, Elg1-HA-NAT CEN URA3 TEN1-(G)8-(FLAG)3*
17611	W303-1a	*MATa leu2-3, 112 ura3-1 his3-11,15, trp1-1, ade2-1, can1- 100*
9842	*W303 elg1Δ::HygMX*	*MATa leu2-3, 112 ura3-1 his3-11,15, trp1-1, ade2-1, can1- 100, elg1::KanMx*
9551	*W303 stn1-13*	*MATa leu2-3, 112 ura3-1 his3-11,15, trp1-1, ade2-1, can1- 100, stn1-13*
9848	*W303 elg1Δ::HygMX stn1-13*	*MATa leu2-3, 112 ura3-1 his3-11,15, trp1-1, ade2-1, can1- 100 elg1::KanMx, stn1-13*
12357	*W303 stn1-164*	*MATa leu2-3, 112 ura3-1 his3-11,15, trp1-1, ade2-1, can1- 100, stn1Δ::Hyg + pRS-stn1-L164A (pVL3571)*
12358	*W303 stn1-164 elg1Δ: KanMX*	*MATa leu2-3, 112 ura3-1 his3-11,15, trp1-1, ade2-1, can1- 100, elg1::KanMx, stn1Δ::Hyg+pRS-stn1-L164A (pVL3571)*

Table 2 (continued)

12062	PJ69-4	*MAT@ trp1-901 leu2-3,112 ura3-52 his3-200 gal4del gal80del GAL2-ADE2 LYS2:: GAL1-HIS3 met2::GAL7-lacZ*
15017	*PJ elg1D*	*MAT@ trp1-901 leu2-3,112 ura3-52 his3-200 gal4del gal80del GAL2-ADE2 LYS2:: GAL1-HIS3 met2::GAL7-lacZ elg1::HYG*
19774	*PJ siz1 siz2*	*MAT@ trp1-901 leu2-3,112 ura3-52 his3-200 gal4del gal80del GAL2-ADE2 LYS2:: GAL1-HIS3 met2::GAL7-lacZ siz1::KanMX siz2::HYG*
11069	*PJ pol30-RR*	*MAT@ trp1-901 leu2-3,112 ura3-52 his3-200 gal4del gal80del GAL2-ADE2 LYS2:: GAL1-HIS3 met2::GAL7-lacZ POL30-RR :: leu2 :: KANMX*
20624	*PJ STN1-Myc*	*MAT@ trp1-901 leu2-3,112 ura3-52 his3-200 gal4del gal80del GAL2-ADE2 LYS2:: GAL1-HIS3 met2::GAL7-lacZ elg1-sim::KanMx, stn1::HYG,CENLEU2 STN1-(G)9-(myc)7*
19916	*PJ elg1-sim-Myc*	*MAT@ trp1-901 leu2-3,112 ura3-52 his3-200 gal4del gal80del GAL2-ADE2 LYS2:: GAL1-HIS3 met2::GAL7-lacZ elg1-SIM-MYC-KANMX*
18798	*PJ elg1-DDMyc*	*MAT@ trp1-901 leu2-3,112 ura3-52 his3-200 gal4del gal80del GAL2-ADE2 LYS2:: GAL1-HIS3 met2::GAL7-lacZ elg1-DD-MYC-KANMX*
19917	*PJ elg1-DD+sim-Myc*	*MAT@ trp1-901 leu2-3,112 ura3-52 his3-200 gal4del gal80del GAL2-ADE2 LYS2:: GAL1-HIS3 met2::GAL7-lacZ elg1-DDsim-MYC-KANMX*
19915	*PJ elg1-Walker A*	*MAT@ trp1-901 leu2-3,112 ura3-52 his3-200 gal4del gal80del GAL2-ADE2 LYS2:: GAL1-HIS3 met2::GAL7-lacZ, elg1 KK343/4DD:MYC:KanMX*
19486	*PJ cdc13-snm*	*MAT@ trp1-901 leu2-3,112 ura3-52 his3-200 gal4del gal80del GAL2-ADE2 LYS2:: GAL1-HIS3 met2::GAL7-lacZ cdc13-snm*

Table 2 (continued)

Strain Number	Genotype	Source or Reference
4239	pGad424 (LEU2)	Kupiec lab
4238	pGad424-POL30	Helle Ulrich lab
4237	pGad424-pol30-k127r,k164r	Helle Ulrich lab
4236	pGad424-pol30-k127r,k164r-SMT3	Helle Ulrich lab
4235	pGAD424-pol30-k127r,k164r-UBI	Helle Ulrich lab
4147	CEN LEU2 STN1-(G)9-(myc)7	Victoria Lundblad lab
4144	CEN URA3 TEN1-(G)8-(FLAG)3	Victoria Lundblad lab
2201	pCN181 pACT2-STN1 (LEU2)	Constance Nugent lab
2205	pVL855 pACT2-CDC13	Constance Nugent lab
2168	pACT2 - TEN1	Michel Charbonneau lab
1775	pGBU9 (URA3)	Kupiec lab
1973	pGBU9-ELG1-NTD(1-234)	This Study
3301	pGBU9-ELG1-CTD(541-791)	This Study
2260	pGBU9-ELG1-NTDsim(1-234)	This Study
2241	pGBU9-SMT3	This Study
2419	pGAD424-Stn1-Nt(1-282)	David Shore lab
2420	pGAD424-Stn1-Ct(282-494)	David Shore lab
2169	pGAD424-Stn1-13	Michel Charbonneau lab
2418	pGAD424-Stn1-63	David Shore lab
4287	pACT-cdc13-snm	This Study

Table 3

Plasmids used in this study

Telomere length was determined using TelQuant (Rubinstein et al., 2014).

Yeast Two-Hybrid

The yeast two-hybrid assays were performed using PJ69-4A (James, Halladay, & Craig, 1996) strains cotransformed with a *LEU2*-marked plasmid containing genes fused to the *GAL4* activating domain (pACT or pGAD424) and a *URA3*- marked plasmid containing genes fused to the *GAL4* DNA binding domain (pGBU9). Strains containing the test plasmids were grown for 24 h at 30°C in SC-Ura-Leu liquid medium, and were plated on the reporter maker SC-His medium.

Protein extraction and immunoprecipitation (IP) assays

Cells were grown to mid-logarithmic phase, washed once with water, and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM DTT, 125 mM potassium acetate, 12.5% glycerol, 0.1% Triton X-100, protease inhibitor mixture, and 1 mM phenylmethylsulfonyl fluoride). Cells were broken for 45 min with glass beads, centrifuged for 10 min at 10,000 × *g* and the supernatant was collected. 20-30 µg of total protein extract was resolved on SDS-PAGE using 10% acrylamide gels. For immunoprecipitation, 1,000 µg of proteins were prepared and pre-cleared with 20 µl of protein A-Sepharose and protein G-Sepharose beads mixture (GE Healthcare). 2 µl of (HA, Santa Cruz Biotechnology (sc7392; 1:1,000) or MYC, Santa Cruz Biotechnology (9E10, SC-40; 1:1,000)) antibodies were added to the cleared extract and incubated overnight at 4 $_0$C. The beads were washed once with lysis buffer, once with lysis buffer containing 0.5 M NaCl, and twice with buffer A (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol). The resulting immunoprecipitates were used for *in vitro* kinase assays.

Western blotting

Cells were collected by centrifugation, re-suspended in 600 ul of phosphate-buffered saline with 1% Triton X-100 (PBST), supplemented with a protease inhibitor cocktail (Roche) and subjected to mechanical rupture using glass beads. The cell debris were removed by centrifugation, and the supernatants were applied onto 0.1 M dithiothreitol, and incubated at 80◦C for 10 min before sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Resolving gel: 30% Acrylamide, 1.5 M Tris-HCl pH8.8, 10% SDS (pH 7.2), 9.7 ml H2O, 100 ul 10% APS and 10 ul TEMED. Stacking gel: 30% Bis/Acrylamide, 1 M Tris-HCl pH 6.8, 10% SDS (pH 7.2), 5.5 ml H2O, 800 ul 10% APS and 8 ul TEMED). The samples were run with SDS-PAGE buffer at 100 V until the samples have passed the stacking gel and then at 160 V until the samples have been fully separated. Transfer to nitrocellulose was done in transfer buffer (200 ml Methanol, 3.03 gr Tris Base, 14.4 gr Glycine) at 500 mAmp and verified by staining with Ponceau-S dye. The blot was blocked with Milk for at least 60 min at room temperature. Primary antibody was added for 12 h at 4◦C. The blot was washed 3 × 5 min with TBST (Tris-Buffered Saline Tween-20) and secondary antibody was added for 1 h. The blot was washed 3 × 5 min with TBST and subjected to electro-chemi-luminiscence (ECL).

Cell cycle synchronization

To assay Elg1 and Stn1 interaction during the cell cycle, the triple tagged strain was grown in YPD to OD_{600} 0.6-0.8, followed by the addition of 500ng/ml α factor and was grown until ~90% of the cells appeared unbudded or exhibit Shmoo formation (for ~2 hrs). The α-factor was removed by centrifugation and washing cells 2-3 times with warm YPD. Cells were released into YPD at OD_{600} 0.6-0.8 with addition of pronase at a final concentration of 0.1mg/ml. Cells were collected for both FACS and Western blot analysis at different time points after release. Cells for FACS analysis were fixed in 70% ethanol, digested with RNase overnight, washed again and stained with propidium iodide (15 µg/mL), and analyzed by flow cytometry.

Flow cytometry

200 µl of a logarithmic cell culture (0.6 OD600) were harvested, resuspended in 60 µl of 50 mM Tris pH 7.5 and 140 µl of ethanol was added; cells were then kept overnight at 4 °C. Fixed cells were centrifuged and washed once in 200 µl of 50 mM Tris pH 7.5 bufer and resuspended in 100 µl RNAse (0.2 mg/ml in 50 mM Tris pH 7.5) for 2 h at 37 °C. In addition, proteinase-K (0.2 mg/ml in 50 mM Tris pH 7.5) was added to each tube and cells were incubated for 60 additional minutes at 50 °C. 20 µl of the sample was taken into a new tube and a 180 µl of 18 µg/ml propidium iodide 50 mM Tris pH 7.5 was added. The samples were kept in the dark at 4 °C overnight, sonicated twice at low setting (20% power) for 3–5 s and stored in the dark at 4 °C. The fow cytometry MACSQuant system was used for reading. Results were analyzed using either the Flowing Software or the FlowJo program.

Chromatin Immunoprecipitation

50 ml of each strain were grown to OD600 ≈ 1 in YPD. 1.5ml formaldehyde (37% solution) was added for 15 minutes and the formaldehyde was quenched with 2.5ml of 2.5M glycine for five minutes. Cells were harvested, washed once with 15ml cold PBS and broken down for 10 minutes with glass beads in 600 µl lysis buffer (50mM HEPES-KOH pH7.5, 140mM NaCl, 1mM EDTA, 1% triton X100, 0.1% Na-Deoxycholic acid). The supernatant (lysate) was removed to a new tube. The glass beads were washed with 500 µl lysis buffer, centrifuged and the supernatant was added to the lysate. The lysate was sonicated six to eight times for 10-15 seconds at 80% amplitude with one minute on ice between each time. The sonicated material was centrifuged for 20 minutes at 2500 rpm. The supernatant was used for immunoprecipitations (IP). The sonicated proteins were pre-cleared with a 25µl protein A sepharose and protein G sepharose beads mixture (GE Healthcare) and the appropriate antibodies were added to the cleared extract and incubated overnight at 4°C. PCNA were immunoprecipitated with 2–5µg of anti-PCNA antibody (Sigma). A total of 10% of the extract was saved as input. The beads after the IP were washed once with lysis buffer, once with lysis buffer with 360 mM NaCl, once with washing buffer (10mM Tris/HCl pH8, 0.25M LiCl, 0.5% NP40, 0.5% Na-Deoxycholic acid, 1mM EDTA) and once with TE (10mM Tris/HCl pH8 and 10mM EDTA). The washed beads and the input were treated with elution buffer (50mM Tris/HCl pH8, 10mM EDTA, 1% SDS) overnight at 65°C. The DNA was precipitated, re-suspended in water and used for PCR real-time analysis(ABI StepOnePlusTM Real-Time PCR System); primer concentration and cycles number were calibrated individually for each reaction. All experiments are plotted as the average of at least three independent biological repeats and each biological repeat is the average of three technical PCR repeats. The oligonucleotides used are:

Y'-element 5'-GGCTTGATTTGGCAAACGTT-3', and

5'-GTGAACCGCTACCATCAGCAT-3'.

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Figure Legends

Figure S1: Weak interaction between Elg1 and Ten1. Results of YTH experiments.

Figure S2: Cdc13 interacts with PCNA, and mutations that prevent PCNA SUMOylation (*pol30-RR*) also impair its interaction with Cdc13.

Figure S3: All *elg1* alleles used are expressed at similar levels. Western blot results.

Figure S4: A. The *sumo-no-more* allele of Cdc13 has no effect on the YTH interactions between wt Cdc13 or Stn1 and the N-terminus of Elg1. **B.** The C-terminus of Stn1 interacts with the N-terminus of Elg1 in wild type and *elg1*Δ strains.

Figure S5: Whole cell extract showing the level of Elg1, Stn1 and Pol3 in the cell cycle experiment shown in **Figure 5**.

References

1. Arbel M., Choudhary K., Tfilin O., Kupiec M (2021) **PCNA Loaders and Unloaders-One Ring That Rules Them All** *Genes (Basel* **12** https://doi.org/10.3390/genes12111812

2. Askree S. H., Yehuda T., Smolikov S., Gurevich R., Hawk J., Coker C., McEachern M. J. (2004) **A genome-wide screen for Saccharomyces cerevisiae deletion mutants that affect telomere length** *Proc Natl Acad Sci U S A* **101**:8658–8663 https://doi.org/10.1073/pnas.0401263101

3. Bell D. W., Sikdar N., Lee K. Y., Price J. C., Chatterjee R., Park H. D., Myung K. (2011) **Predisposition to cancer caused by genetic and functional defects of mammalian Atad5** *PLoS Genet* **7** https://doi.org/10.1371/journal.pgen.1002245

4. Ben-Shitrit T., Yosef N., Shemesh K., Sharan R., Ruppin E., Kupiec M (2012) **Systematic identification of gene annotation errors in the widely used yeast mutation collections** *Nat Methods* **9**:373–378 https://doi.org/10.1038/nmeth.1890

5. Bonnell E., Pasquier E., Wellinger R. J (2021) **Telomere Replication: Solving Multiple End Replication Problems** *Front Cell Dev Biol* **9** https://doi.org/10.3389/fcell.2021.668171

6. Chandra A., Hughes T. R., Nugent C. I., Lundblad V (2001) **Cdc13 both positively and negatively regulates telomere replication** *Genes Dev* **15**:404–414 https://doi.org/10.1101/gad.861001

7. Chen H., Xue J., Churikov D., Hass E. P., Shi S., Lemon L. D., Lei M. (2018) **Structural Insights into Yeast Telomerase Recruitment to Telomeres** *Cell* **172**:331–343 https://doi.org/10.1016/j.cell.2017.12.008

8. Chen L. Y., Lingner J (2013) **CST for the grand finale of telomere replication** *Nucleus* **4**:277–282 https://doi.org/10.4161/nucl.25701

9. de Lange T. (2018) **Shelterin-Mediated Telomere Protection** *Annu Rev Genet* **52**:223–247 https://doi.org/10.1146/annurev-genet-032918-021921

10. Evans S. K., Lundblad V (1999) **Est1 and Cdc13 as comediators of telomerase access** *Science* **286**:117–120 https://doi.org/10.1126/science.286.5437.117

11. Evans S. K., Lundblad V (2000) **Positive and negative regulation of telomerase access to the telomere** *J Cell Sci* **113**:3357–3364 https://doi.org/10.1242/jcs.113.19.3357

12. Gao H., Cervantes R. B., Mandell E. K., Otero J. H., Lundblad V (2007) **RPA-like proteins mediate yeast telomere function** *Nat Struct Mol Biol* **14**:208–214 https://doi.org/10.1038/nsmb1205

13. Garg M., Gurung R. L., Mansoubi S., Ahmed J. O., Dave A., Watts F. Z., Bianchi A (2014) **Tpz1TPP1 SUMOylation reveals evolutionary conservation of SUMO-dependent Stn1 telomere association** *EMBO Rep* **15**:871–877 https://doi.org/10.15252/embr.201438919

14. Gatbonton T., Imbesi M., Nelson M., Akey J. M., Ruderfer D. M., Kruglyak L., Bedalov A. (2006) **Telomere length as a quantitative trait: genome-wide survey and genetic mapping of telomere length-control genes in yeast** *PLoS Genet* **2** https://doi.org/10.1371/journal.pgen.0020035

15. Ge Y., Wu Z., Chen H., Zhong Q., Shi S., Li G., Lei M. (2020) **Structural insights into telomere protection and homeostasis regulation by yeast CST complex** *Nat Struct Mol Biol* **27**:752–762 https://doi.org/10.1038/s41594-020-0459-8

16. Grandin N., Reed S. I., Charbonneau M (1997) **Stn1, a new Saccharomyces cerevisiae protein, is implicated in telomere size regulation in association with Cdc13** *Genes Dev* **11**:512–527 https://doi.org/10.1101/gad.11.4.512

17. Grossi S., Puglisi A., Dmitriev P. V., Lopes M., Shore D. (2004) **Pol12, the B subunit of DNA polymerase alpha, functions in both telomere capping and length regulation** *Genes Dev* **18**:992–1006 https://doi.org/10.1101/gad.300004

18. Hang L. E., Liu X., Cheung I., Yang Y., Zhao X. (2011) **SUMOylation regulates telomere length homeostasis by targeting Cdc13** *Nat Struct Mol Biol* **18**:920–926 https://doi.org/10.1038/nsmb.2100

19. Harari Y., Kupiec M (2018) **Mec1(ATR) is needed for extensive telomere elongation in response to ethanol in yeast** *Curr Genet* **64**:223–234 https://doi.org/10.1007/s00294-017-0728-

20. Harari Y., Romano G. H., Ungar L., Kupiec M (2013) **Nature vs nurture: interplay between the genetic control of telomere length and environmental factors** *Cell Cycle* **12**:3465–3470 https://doi.org/10.4161/cc.26625

21. Harari Y., Zadok-Laviel S., Kupiec M (2017) **Long Telomeres Do Not Affect Cellular Fitness in Yeast** *mBio* **8** https://doi.org/10.1128/mBio.01314-17

22. Hoege C., Pfander B., Moldovan G. L., Pyrowolakis G., Jentsch S (2002) **RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO** *Nature* **419**:135–141

23. Hughes T. R., Weilbaecher R. G., Walterscheid M., Lundblad V (2000) **Identification of the single-strand telomeric DNA binding domain of the Saccharomyces cerevisiae Cdc13 protein** *Proc Natl Acad Sci U S A* **97**:6457–6462 https://doi.org/10.1073/pnas.97.12.6457

24. Itzkovich Z., Choudhary K., Arbel M., Kupiec M (2023) **Effects of Defective Unloading and Recycling of PCNA Revealed by the Analysis of ELG1 Mutants** *Int J Mol Sci* **24** https://doi.org/10.3390/ijms24021568

25. James P., Halladay J., Craig E. A (1996) **Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast** *Genetics* **144**:1425–1436

26. Jentsch S., Psakhye I (2013) **Control of nuclear activities by substrate-selective and protein-group SUMOylation** *Annu Rev Genet* **47**:167–186 https://doi.org/10.1146/annurev-genet-111212-133453

27. Johnson C., Gali V. K., Takahashi T. S., Kubota T (2016) **PCNA Retention on DNA into G2/M Phase Causes Genome Instability in Cells Lacking Elg1** *Cell Rep* **16**:684–695 https://doi.org/10.1016/j.celrep.2016.06.030

28. Kang M. S., Ryu E., Lee S. W., Park J., Ha N. Y., Ra J. S., Myung K. (2019) **Regulation of PCNA cycling on replicating DNA by RFC and RFC-like complexes** *Nat Commun* **10** https://doi.org/10.1038/s41467-019-10376-w

29. Kim S. J., Wie M., Park S. H., Kim T. M., Park J. H., Kim S., Lee K. Y. (2020) **ATAD5 suppresses centrosome over-duplication by regulating UAF1 and ID1** *Cell Cycle* **19**:1952–1968 https://doi.org/10.1080/15384101.2020.1785724

30. Kubota T., Katou Y., Nakato R., Shirahige K., Donaldson A. D (2015) **Replication-Coupled PCNA Unloading by the Elg1 Complex Occurs Genome-wide and Requires Okazaki Fragment Ligation** *Cell Rep* **12**:774–787 https://doi.org/10.1016/j.celrep.2015.06.066

31. Kubota T., Nishimura K., Kanemaki M. T., Donaldson A. D (2013) **The Elg1 replication factor C-like complex functions in PCNA unloading during DNA replication** *Mol Cell* **50**:273–280 https://doi.org/10.1016/j.molcel.2013.02.012

32. Kuchenbaecker K. B., Ramus S. J., Tyrer J., Lee A., Shen H. C., Beesley J., Brca (2015) **Identification of six new susceptibility loci for invasive epithelial ovarian cancer** *Nat Genet* **47**:164–171 https://doi.org/10.1038/ng.3185

33. Lim C. J., Barbour A. T., Zaug A. J., Goodrich K. J., McKay A. E., Wuttke D. S., Cech T. R (2020) **The structure of human CST reveals a decameric assembly bound to telomeric DNA** *Science* **368**:1081–1085 https://doi.org/10.1126/science.aaz9649

34. Lin J. J., Zakian V. A (1996) **The Saccharomyces CDC13 protein is a single-strand TG1-3 telomeric DNA-binding protein in vitro that affects telomere behavior in vivo** *Proc Natl Acad Sci U S A* **93**:13760–13765 https://doi.org/10.1073/pnas.93.24.13760

35. Maicher A., Gazy I., Sharma S., Marjavaara L., Grinberg G., Shemesh K., Kupiec M. (2017) **Rnr1, but not Rnr3, facilitates the sustained telomerase-dependent elongation of telomeres** *PLoS Genet* **13** https://doi.org/10.1371/journal.pgen.1007082

36. Maleva Kostovska I., Wang J., Bogdanova N., Schurmann P., Bhuju S., Geffers R., Dork T. (2016) **Rare ATAD5 missense variants in breast and ovarian cancer patients** *Cancer Lett* **376**:173–177 https://doi.org/10.1016/j.canlet.2016.03.048

37. Matmati S., Vaurs M., Escandell J. M., Maestroni L., Nakamura T. M., Ferreira M. G., Coulon S. (2018) **The fission yeast Stn1-Ten1 complex limits telomerase activity via its SUMO-interacting motif and promotes telomeres replication** *Sci Adv* **4** https://doi.org/10.1126/sciadv.aar2740

38. Mersaoui S. Y., Wellinger R. J (2019) **Fine tuning the level of the Cdc13 telomere-capping protein for maximal chromosome stability performance** *Curr Genet* **65**:109–118 https://doi.org/10.1007/s00294-018-0871-3

39. Miyagawa K., Low R. S., Santosa V., Tsuji H., Moser B. A., Fujisawa S., Tanaka K. (2014) **SUMOylation regulates telomere length by targeting the shelterin subunit Tpz1(Tpp1) to modulate shelterin-Stn1 interaction in fission yeast** *Proc Natl Acad Sci U S A* **111**:5950–5955 https://doi.org/10.1073/pnas.1401359111

40. Nugent C. I., Hughes T. R., Lue N. F., Lundblad V (1996) **Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance** *Science* **274**:249–252 https://doi.org/10.1126/science.274.5285.249

41. Parker J. L., Bielen A. B., Dikic I., Ulrich H. D (2007) **Contributions of ubiquitin- and PCNA-binding domains to the activity of Polymerase eta in Saccharomyces cerevisiae** *Nucleic Acids Res* **35**:881–889 https://doi.org/10.1093/nar/gkl1102

42. Parnas O., Amishay R., Liefshitz B., Zipin-Roitman A., Kupiec M (2011) **Elg1, the major subunit of an alternative RFC complex, interacts with SUMO-processing proteins** *Cell Cycle* **10**:2894–2903 https://doi.org/10.4161/cc.10.17.16778

43. Parnas O., Zipin-Roitman A., Pfander B., Liefshitz B., Mazor Y., Ben-Aroya S., Kupiec M. (2010) **Elg1, an alternative subunit of the RFC clamp loader, preferentially interacts with SUMOylated PCNA** *Embo J* **29**:2611–2622 https://doi.org/10.1038/emboj.2010.128

44. Petreaca R. C., Chiu H. C., Eckelhoefer H. A., Chuang C., Xu L., Nugent C. I (2006) **Chromosome end protection plasticity revealed by Stn1p and Ten1p bypass of Cdc13p** *Nat Cell Biol* **8**:748–755 https://doi.org/10.1038/ncb1430

45. Petreaca R. C., Chiu H. C., Nugent C. I (2007) **The role of Stn1p in Saccharomyces cerevisiae telomere capping can be separated from its interaction with Cdc13p** *Genetics* **177**:1459–1474 https://doi.org/10.1534/genetics.107.078840

46. Puglisi A., Bianchi A., Lemmens L., Damay P., Shore D (2008) **Distinct roles for yeast Stn1 in telomere capping and telomerase inhibition** *Embo J* **27**:2328–2339 https://doi.org/10.1038/emboj.2008.158

47. Rice C., Skordalakes E (2016) **Structure and function of the telomeric CST complex** *Comput Struct Biotechnol J* **14**:161–167 https://doi.org/10.1016/j.csbj.2016.04.002

48. Rubinstein L., Ungar L., Harari Y., Babin V., Ben-Aroya S., Merenyi G., Kupiec M. (2014) **Telomere length kinetics assay (TELKA) sorts the telomere length maintenance (tlm) mutants into functional groups** *Nucleic Acids Res* **42**:6314–6325 https://doi.org/10.1093/nar/gku267

49. Shemesh K., Sebesta M., Pacesa M., Sau S., Bronstein A., Parnas O., Kupiec M. (2017) **A structure-function analysis of the yeast Elg1 protein reveals the importance of PCNA unloading in genome stability maintenance** *Nucleic Acids Res* **45**:3189–3203 https://doi.org/10.1093/nar/gkw1348

50. Shiomi Y., Nishitani H (2013) **Alternative replication factor C protein, Elg1, maintains chromosome stability by regulating PCNA levels on chromatin** *Genes Cells* **18**:946–959 https://doi.org/10.1111/gtc.12087

51. Soudet J., Jolivet P., Teixeira M. T (2014) **Elucidation of the DNA end-replication problem in Saccharomyces cerevisiae** *Mol Cell* **53**:954–964 https://doi.org/10.1016/j.molcel.2014.02.030

52. Stelter P., Ulrich H. D (2003) **Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation** *Nature* **425**:188–191 https://doi.org/10.1038/nature01965

53. Takahashi T. S., Wollscheid H. P., Lowther J., Ulrich H. D (2020) **Effects of chain length and geometry on the activation of DNA damage bypass by polyubiquitylated PCNA** *Nucleic Acids Res* **48**:3042–3052 https://doi.org/10.1093/nar/gkaa053

54. Wang M. J., Lin Y. C., Pang T. L., Lee J. M., Chou C. C., Lin J. J (2000) **Telomere-binding and Stn1p-interacting activities are required for the essential function of Saccharomyces cerevisiae Cdc13p** *Nucleic Acids Res* **28**:4733–4741 https://doi.org/10.1093/nar/28.23.4733

55. Yang K., Moldovan G. L., Vinciguerra P., Murai J., Takeda S., D'Andrea A. D (2011) **Regulation of the Fanconi anemia pathway by a SUMO-like delivery network** *Genes Dev* **25**:1847–1858 https://doi.org/10.1101/gad.17020911

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Editors

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Reviewer #1 (Public Review):

In this manuscript, the authors investigated the role of Elg1 in the regulation of telomere length. The main role of the Elg1/RLC complex is to unload the processivity factor PCNA, mainly after completion of synthesis of the Okazaki fragment in the lagging strand. They found that Elg1 physically interacts with the CST (Cdc13-Stn1-Ten1) and propose that Elg1 negatively regulates telomere length by mediating the interaction between Cdc13 and Stn1 in a pathway involving SUMOylation of both PCNA and Cdc13. Accumulation of SUMOylated PCNA upon deletion of ELG1 or overexpression of RAD30 leads to elongated telomeres. On the other hand, the interaction of Elg1 with Sten1 is SIM-dependent and occurs concurrently with telomere replication in late S phase. In contrast Elg1-Cdc13 interaction is mediated by PCNA-SUMO, is independent on the SIM of Elg1 but still dependent on Cdc13 SUMOylation. The authors present a model containing two main messages 1) PCNA-SUMO acts as a positive signal for telomerase activation 2) Elg1 promotes Cdc13/Stn1 interaction at the expense of Cdc13/Est1 interaction thus terminating telomerase action.

The manuscript contains a large amount of data that make a major inroads on a new type of link between telomere replication and regulation of the telomerase. Nevertheless, the detailed choreography of the events as well as the role of PCNA-SUMO remain elusive and the data do not fully explain the role of the Stn1/Elg1 interaction. The data presented do not convincingly support the claim that SUMO-PCNA is a positive signal for telomerase activation. This was partially addressed in the current version.

Reviewer #2 (Public Review):

This paper purports to unveil a mechanism controlling telomere length through SUMO modifications controlling interactions between PCNA unloader Elg1 and the CST complex that functions at telomeres. This is an extremely interesting mechanism to understand, and this paper indeed reveals some interesting genetic results, leading to a compelling model, with potential impact on the field. Overall, however, the data do not provide sufficient support for the claims. The model may be correct but it is not yet convincingly demonstrated.

The current version addressed some of the issues regarding language describing conclusions and more experimental detail has been provided. However, the authors have not provided new data supporting the model, so the overall evaluation is that the work remains inconclusive.

Reviewer #3 (Public Review):

This paper reveals interesting physical connections between Elg1 and CST proteins that suggest a model where Elg1-mediated PCNA unloading is linked to regulation of telomere length extension via Stn1, Cdc13, and presumably Ten1 proteins. Some of these interactions appear to be modulated by sumolyation and connected with Elg1's PCNA unloading activity. The strength of the paper is in the observations of new interactions between CST, Elg1, and PCNA. These interactions should be of interest to a broad audience interested in telomeres and DNA replication.

What is not well demonstrated from the paper is the functional significance of the interactions described. The model presented by the authors is one interpretation of the data shown, and proposes that the role of sumolyation is temporally regulate the Elg1, PCNA and CST interactions at telomeres. This model makes some assumptions that are not demonstrated by this work (such as Stn1 sumolyation, as noted) and are left for future testing. Alternative models that envision sumolyation as a key in promoting spatial localization could also be proposed based on the data here (as mentioned in the discussion), in addition to or instead of a role for sumolyation in enforcing a series of switches governing a tightly sequenced series of interactions and events at telomeres. Critically, the telomere length data from the paper indicates that the proposed model depicts interactions that are not necessary for telomerase activation or inhibition, as telomeres in pol30-RR strains are normal length and telomeres in elg1Δ strains are not nearly as elongated as in stn1 strains. One possibility mentioned in the paper is the PCNAS and Elg1 interactions are contributing to the negative regulation of telomerase under certain conditions that are not defined in this work. Could it also be possible that the role of these interactions is not primarily directed toward modulating telomerase activity? It will be of interest to learn more about how these interactions and regulation by Sumo function intersect with regulation of telomere extension.

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

In this manuscript, the authors investigated the role of Elg1 in the regulation of telomere length. The main role of the Elg1/RLC complex is to unload the processivity factor PCNA, mainly after completion of synthesis of the Okazaki fragment in the lagging strand. They found that Elg1 physically interacts with the CST (Cdc13-Stn1- Ten1) and propose that Elg1 negatively regulates telomere length by mediating the interaction between Cdc13

and Stn1 in a pathway involving SUMOylation of both PCNA and Cdc13. Accumulation of SUMOylated PCNA upon deletion of ELG1 or overexpression of RAD30 leads to elongated telomeres. On the other hand, the interaction of Elg1 with Sten1 is SIM-dependent and occurs concurrently with telomere replication in late S phase. In contrast Elg1-Cdc13 interaction is mediated by PCNA-SUMO, is independent on the SIM of Elg1 but still dependent on Cdc13 SUMOylation. The authors present a model containing two main messages 1) PCNA- SUMO acts as a positive signal for telomerase activation 2) Elg1 promotes Cdc13/Stn1 interaction at the expense of Cdc13/Est1 interaction thus terminating telomerase action.

The manuscript contains a large amount of data that make a major inroad on a new type of link between telomere replication and regulation of the telomerase. Nevertheless, the detailed choreography of the events as well as the role of PCNA- SUMO remain elusive and the data do not fully explain the role of the Stn1/Elg1 interaction. The data presented do not sufficiently support the claim that SUMO- PCNA is a positive signal for telomerase activation.

We thank the reviewer for her/his review efforts and opinion. We have re-submitted a new version of the manuscript in which we clarify some of the criticisms presented. In a point-by-point letter we respond to all the specific queries.

Reviewer #2 (Public Review):

This paper purports to unveil a mechanism controlling telomere length through SUMO modifications controlling interactions between PCNA unloader Elg1 and the CST complex that functions at telomeres. This is an extremely interesting mechanism to understand, and this paper indeed reveals some interesting genetic results, leading to a compelling model, with potential impact on the field. The conclusions are largely supported by experiments examining protein-protein interactions at low resolution and ambiguous regarding directness of interactions like co-IP and yeast two-hybrid (Y2H) combined with genetics. However, some results appear contradictory and there's a lack of rigor in the experimental data needed to support claims. There is significant room for improvement and this work could certainly attain the quality needed to support the claims. The current version needs substantial revision and lacks the necessary experimental detail. Stronger support for the claims would add detail to help distinguish competing models.

We thank the reviewer for her/his positive opinion. We have re-submitted a new version of the manuscript in which we clarify some of the criticisms presented by thereferees, and added all the missing experimental details. In a point-by-point letter we respond to all the specific queries.

Reviewer #3 (Public Review):

This paper reveals interesting physical connections between Elg1 and CST proteins that suggest a model where Elg1-mediated PCNA unloading is linked to regulation of telomere length extension via Stn1, Cdc13, and presumably Ten1 proteins. Some of these interactions appear to be modulated by sumolyation and connected with Elg1's PCNA unloading activity. The strength of the paper is in the observations of new interactions between CST, Elg1, and PCNA. These interactions should be of interest to a broad audience interested in telomeres and DNA replication.

We thank the reviewer for her/his positive opinion. We have re-submitted a new version of the manuscript in which we clarify some of the criticisms presented. In a point-by-point letter we respond to all the specific queries.

What is not well demonstrated from the paper is the functional significance of the interactions described. The model presented by the authors is one interpretation of the data shown, and proposes that the role of sumolyation is temporally regulate the Elg1, PCNA and CST interactions at telomeres. This model makes some assumptions that are not demonstrated by this work (such as Stn1 sumolyation, as noted) and are left for future testing. Alternative models that envision sumolyation as a key in promoting spatial localization could also be proposed based on the data here (as mentioned in the discussion), in addition to or instead of a role for sumolyation in enforcing a series of switches governing a tightly sequenced series of interactions and events at telomeres. Critically, the telomere length data from the paper indicates that the proposed model depicts interactions that are not necessary for telomerase activation or inhibition, as telomeres in pol30-RR strains are normal length and telomeres in elg1Δ strains are not nearly as elongated as in stn1 strains. One possibility mentioned in the paper is the PCNAS and Elg1 interactions are contributing to the negative regulation of telomerase under certain conditions that are not defined in this work. Could it also be possible that the role of these interactions is not primarily directed toward modulating telomerase activity? It will be of interest to learn more about how these interactions and regulation by Sumo function intersect with regulation of telomere extension.

We present compelling evidence for a role of SUMOylated PCNA in telomere length regulation. Figure 1 shows that this modification is both necessary and sufficient to elongate the telomeres, indicating that PCNA SUMOylation plays a positive role in telomere elongation. The model we present is consistent with all our results. There are, of course, possible alternative models, but they usually fail to explain some of the results. We agree that the fact that pol30-RR presents normal-sized telomeres implies that SUMO-PCNA is not required for telomerase to solve the "end replication problem", but rather is needed for "sustained" activity of telomerase. Since elongated telomeres (by absence of Elg1 or by over-expression of SUMO-PCNA) was the phenotype monitored, this may require sustained telomerase activity. Similar results were seen in the past for Rnr1 (Maicher et al., 2017), and this mode depends on Mec1, rather than Tel1 (Harari and Kupiec, 2018). Telomere length regulation is complex, and we may not yet understand the whole picture. It appears that for normal “end replication problem” solution, very little telomerase activity may be needed, and spontaneous interactions at a low level may suffice. Future work may find the conditions at which telomerase switches from "end replication problem" to "sustained" activity. We have added further explanations on this subject to the Discussion section.

We suspect, but could not prove, a role for Stn1 SUMOylation in the interactions. SUMOylation is usually transient, and notoriously hard to detect, and despite the fact that many telomeric proteins are SUMOylated, Stn1 SUMOylation could not be shown directly by us and others (Hang et al, 2011).

Reviewer #1 (Recommendations For The Authors):

Suggestions for improved or additional experiments, data or analyses.

- *My main concern is the claim that SUMOylated PCNA acts as a positive signal for telomerase activation. Yet the pol30-RR mutant has no impact on telomere length. The explanation of the authors is not entirely convincing.*

We are aware that the regulation of telomere length is complex, and we may not fully understand it yet. Just consider the fact that ~500 genes participate in determining the final telomere length of a yeast (Askree et al., 2004). Since mutation in EACH of these genes has a phenotype, the implication is that the joint action of 500 players determines the outcome (a

dialogue of 500 participants). Having said this, we clearly show in figure 1 that mutations that prevent PCNA SUMOylation prevent telomere length elongation in cells lacking Elg1, and overexpressing SUMOylated PCNA is enough to elongate the telomeres. Thus, SUMOylation of PCNA does act as a positive signal for elongation.

However, it appears that to fulfill the minimal requirement of dealing with the "end-replication problem", PCNA SUMOylation is not required, and only a "sustained activity" mode requires the S-PCNA signal (as we have also shown, surprisingly, for RNR1, Maicher et al. 2017). This sustained activity mode depends on Mec1, rather than Tel1 (Harari and Kupiec, 2018). Since elongated telomeres (by absence of Elg1 or by over-expression of SUMO-PCNA) was the phenotype monitored, this may require sustained telomerase activity. Telomere length regulation is complex, and we may not yet understand the whole picture. It appears that for normal “end replication problem” solution, very little telomerase activity may be needed, and spontaneous interactions at a low level may suffice (for example, unmodified PCNA may promote telomerase activity at a lower level than that of SUMO-PCNA. Future work may find the conditions at which telomerase switches from "end replication problem" to "sustained" activity.

We have added further explanations on this subject to the Discussion section.

> - *The model is entitled « Elg1 negatively regulates the telomere length by forming an interaction with the CST complex ». Nevertheless, expression of PCNA-RR completely reversed the long telomere phenotype of elg1Δ cells. Thus it appears that although the interaction between Stn1 and Cdc13 is reduced in the absence of Elg1, Elg1/Stn1 interaction is not instrumental in the formation of the CST complex and thus in the termination of telomerase activity. Does the elg1ΔSIM mutant that does not interact with Stn1 impact telomere length?*
>
> - *In the model part (lane 318), it is argued that the complex Elg1-Stn1 unloads SUMOylated PCNA. Elg1-Stn1 interaction depends on the SIM of Elg1. This SIM is however not required for Elg1's function in genome-wide SUMO-PCNA unloading, is it required specifically at telomeres?*

The interactions between Elg1 and SUMOylated PCNA are carried out through both the SIM and the Threonines 386 and 387 (Shemesh et al, 2017). Consistently, the single elg1-SIM mutant has telomeres of normal length, and its effects on telomere length can only be seen when combined with mutations in the Threonines (elg1- TT386/7AA or elg1-TT386/7DD). Although the unloading of SUMOylated PCNA by Elg1 is important, the gene is not essential, and PCNA is either eventually unloaded by RFC, or spontaneously dis-assembles. This explains why the telomere length does not reach the same length in the absence of Elg1 as in the absence of, say, Stn1.

> - *The model suggests that Elg1 promotes the interaction between Cdc13 and Stn1. This is based on the data presented in Figure 5 E and F. This is an important result. Because the experiment has been done on cells synchronized in S phase and the Elg1/Stn1 interaction occurs specifically at the end of S-phase, the FACS profile should be shown or a control provided to show that the two conditions are comparable.*

The FACS profile for this experiment is shown in Figure 5C.

> - *Does the interaction between Cdc13 and Pol30 depend on the SUMOyaltion of POL30 ?*

Yes. We have added this as new Figure S2, and presented the results together with Figure 3 (Figure 3 is already too crowded).

> *Others points :*
>
> - *Fig 1 : it should be mentioned in the Materials and Methods or in the figure legend how the average telomere lengths (horizontal bar) were calculated from the teloblot, as the position of the bar is not always intuitive*

We estimate telomere length by using TelQuant (Rubinstein et al., 2014). We have added this to the Methods section.

> *-Fig 2 : Owing to the large span of telomere length in the stn1 mutants, the epistatic relationship between elg1Δ and stn1 mutants is poorly illustrated by the teloblot.*

We repeated this experiment several times, and stn1 mutants consistently gave a very spread telomere length. In ALL the blots, however, the double mutants elg1 stn1 showed a telomere length similar to that of the single stn1 mutant, and never longer.

> - *It is mentioned that other mutants in the collection showed epistasis. Are any of these mutants related to telomere replication or the proposed model?*

Since we used the collection of non-essential mutants (so far), it was quite devoid of genes involved in DNA replication, which are mostly essential. An exception was siz1□, which showed epistasis with elg1Δ.

> - *The section entitled « Elg1's functional activity is essential for its interaction with Cdc13 » (lane 205) is difficult to follow. The hierarchy between the different mutants of Elg1 on their capacity to unload PCNA is not totally in agreement with the data published in Itzkovich et al 2023 and Shemesh et al. 2017. In particular it appears to me from these papers that elg1-WalkerA 238 (KK343/4AA) mutant did not show a defect in contrast to elg1-WalkerA 238(KK343/4DD).*

We are sorry for the typo in the results. We used the elg1-WalkerA (KK343/4DD) allele, which has a normal SIM but no activity. In a nutshell, we used mutants that either did or did not show unloading activity and/or SIM. The results clearly show that you need to unload PCNA in order for the N-ter of Elg1 to interact with Cdc13.

> - *Are the synchronization done at 30{degree sign}C ?*

Yes. We have added the information to the Methods section.

> - *ChIP experiments are not described in the Materials and Methods*

We apologize for this. They are now described.

> - *In the figure 6, the PCNA rings are curiously placed at the beginning of the Okasaki fragments.*

We thank the referee for noticing, we have corrected the figure.

Reviewer #2 (Recommendations For The Authors):

This paper purports to unveil a mechanism controlling telomere length through SUMO modifications controlling interactions between PCNA unloader Elg1 and the CST complex that functions at telomeres. This is an extremely interesting mechanism to understand, and this paper indeed reveals some interesting genetic results, leading to a compelling model, with potential impact on the field. The conclusions are largely supported by experiments examining protein-protein interactions at low resolution and ambiguous regarding directness of interactions like co-IP and yeast two-hybrid (Y2H) combined with genetics. However, some results appear contradictory and there's a lack of rigor in the experimental data needed to support claims. There is significant room for improvement and this work could certainly attain the quality needed to support the claims. The current version needs substantial revision and lacks necessary experimental detail. Stronger support for the claims would add detail to help distinguish competing models.

Specific comments:

Insufficient technical detail: I could find no explanation of how overexpression was achieved. No description of how teloChIP is performed, either for the PCNA IP or how the sequence analysis is performed. Too limited details on growth like exact temperatures for the cell cycle time course.

We have significantly expanded the Methods section to include all the technical information.

> *Please do not bold and underline text for emphasis-EVER*

We have removed those from the text.

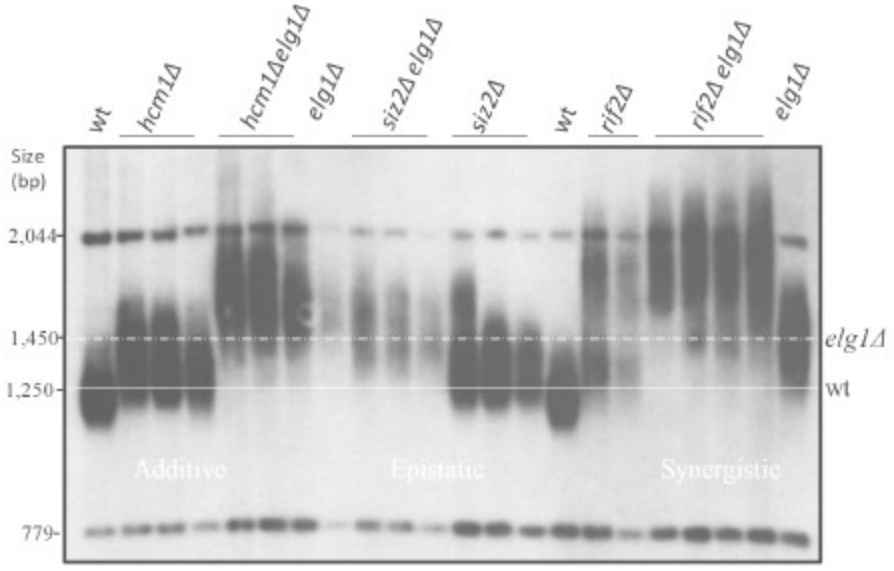
> *Lines 130-132: they have not shown "accumulation of SUMOylated PCNA" anywhere; this is an inference.*

We have modified the text, it says: ”show that SUMOylated PCNA, and not unmodified or ubiquitinated PCNA, is both necessary and sufficient for telomere elongation in the presence or in the absence of Elg1.”

> *Fig 2A Can authors show any other very long-telomere mutant like stn1 that does show enhancement in combination with elg1Δ to show feasibility of such phenotype?*

We don't think it is appropriate for the paper, but we have systematically created double mutants with elg1Δ and found many additive and even synergistic interactions. Here is an example. in Author response image 1, taken from the PhD thesis of Taly Ben-Shitrit, a PhD student in the lab.

Author response image 1.



> *What about cdc13 or ten1? Epistatic?*

We did not test telomere length in combination with Ten1. Combining elg1Δ with cdc13-50 resulted in synergistic elongation. Given the complex genetic relationship between Stn1/Ten1 and Cdc13, it is hard to interpret this result.

> *Seems tenuous to use Y2H to decipher protein-protein interactions occurring out of context (i.e., not at telomere but at reporter gene promoter)*

Y2H is a great method to detect interactions, even if they are transient. Whenever possible, we confirm our findings using co-IP or telo-ChIP.

> *Lines 268-270: It would be more accurate to state "can be" instead of "becomes" or "is" as they have not shown that SUMOylation or PCNA unloading have occurred.*

We agree, and have changed the text.

> *Cdc13snm protein level?*

Unfortunately our Western blot is not presentable, but the level of Cdc13snm was similar to that of the wt Cdc13, and this result has been already published by Hang et al., 2011.

> *Fig S3A: If SUMOylated Cdc13 mediates the Stn1-Elg1 interaction, why is Stn1-Elg1 interaction maintained in cdc13snm strain? This result seems to directly contradict the premise and overall conclusion of this section that Cdc13-SUMO mediates the (Y2H) interaction of Elg1 and Stn1.*

According to our model, the interaction between Stn1 and Elg1 takes place upstream, and only then this complex interacts with SUMOylated Cdc13. Hence, if Cdc13 cannot be SUMOylated, the interaction Elg1-Stn1 is not lost, although Stn1 fails to interact with Cdc13, leading to a telomeric phenotype.

> *Line 279: which data establishes Stn1-Elg1 interaction as direct? Fig 2B co-Ip indicates physical but not necessarily direct interaction, but later the authors suggest that the interaction requires a SUMOylated intermediary, and Y2H in Fig. S3B doesn't demonstrate direct interaction.*

We have changed the text, taking out the word "direct".

> *Co-Ip shows that interaction of Elg1 with Stn1 occurs mainly during later Sphase and with an overall delay compared to initial Elg1-Pol3 interaction.Co-IP Interaction between Cdc13 and Stn1 is reduced in the absence of Elg1*
>
> *The subsection title: "The interaction of Elg1 with Stn1 takes place at telomeres only at late S-phase" is not well supported by the data. I agree the data are consistent with the idea of the interactions occurring at telomeres but there's no direct evidence of this.*

We have changed the subsection title. It now reads: " The interaction of Elg1 with Stn1 takes place only at late S-phase"

> *Model: Is unloading happening at the fork? Doesn't PCNA unloading have to follow its loading which occurred behind the fork particularly on the lagging strand? Model now suggest that Stn1 itself is SUMOylated.*

Yes, according to the model Elg1 moves with the fork, unloading PCNA from the lagging strand. Once Elg1 reaches the telomeres, it interacts with Stn1 (Figure 5). This interaction requires SUMOylation of Stn1 or of some other protein, which is not PCNA (Figure 3D) nor Cdc13 (Figure S3A) and could be Stn1 itself or another telomeric protein (Hang et al., 2011)

> *Title is rather vague.*

We think it summarizes what we present in the paper.

> *Abstract:*
>
> *"We report that SUMOylated PCNA acts as a signal that positively regulates telomerase activity."*
>
> *I don't think this is supported or a good description of what they find*

Figure 1B clearly shows that SUMO-PCNA is both necessary and sufficient for telomere elongation.

> *"and dissected the mechanism by which Elg1 and Stn1 negatively regulates telomere elongation, coordinated by SUMO."*
>
> *Again, I don't think this is sufficiently supported and the model invokes SUMOylation events not demonstrated like Stn1, which might be a significant step forward.*
>
> *On the positive side, their model makes several predictions that they could test much more directly and rigorously: for example, examining the impact of the relevant mutations in the recruitment of proteins to the telomere.*

We have dissected the mechanism, and future work will be devoted to examining the impact of the relevant mutations in the recruitment of proteins to the telomere.

Reviewer #3 (Recommendations For The Authors):

Comments:

1. *The telomere length analysis data presented here is consistent with an interpretation that Stn1 and Elg1 play roles in a similar telomere maintenance pathway because the telomere restriction fragment pattern in the double mutants are not longer than the stn1 single mutants. No comment is made with respect to the yellow bars in Figure 2 that presumably measure telomere length appearing to be slightly shorter than in the stn1 single mutants. It may be interesting and informative if the double mutants do in fact have some phenotype distinct from the single stn1 mutants. Is there an impact on viability in the double mutant?*

Given the variable telomeric phenotype of the single stn1 mutants, slight variations in the measurement of the median telomere size are expected. The difference observed is not likely to be significant. What is important is that the double mutants with elg1Δ do not show longer telomeres. In terms of fitness, the stn1 mutants grow slightly slowly, but the elg1Δ mutation does not slow them down further.

1. *It is somewhat surprising that no additional telomere length analysis is included that actually tests the proposed model, including whether this path could be operational only under certain conditions. Maybe this is a topic of the next paper?*

Indeed, future work will explore the conditions under which PCNA SUMOylation is essential, and those under which is only needed.

1. *Were the error bars in Figure 5F determined only from the experiment in E? Does this represent error in measuring the data from one biological replicate? The type of error should be made clear to avoid readers assuming the data represents measurements from more than one sample in more than one experiment. The data would be stronger if it represented measurements from multiple experiments.*

The graph was made with data from three biological replicates. We show the best blot in Figure 5E. We have now stressed this in the Figure Legend.

1. *Why was only one two hybrid reporter shown? Having the multiple reporters can give confidence in interactions. (Not a big deal here given the nice co-IP data.)*

We thought that it is enough to show one reporter, as the results with a different reporter (B-gal assay) led to the same conclusions. since this did not add information and made the paper too lengthy (and boring), we took them out. In any case all data was verified by co-IP.

1. *Line 414 - what are the 32P-radio labeled PCR fragments? Are these solely comprised of TG1-3 repeats of some length? A bit more detail in this aspect of the method could be helpful.*

We have added an explanation on the probe in the Methods section.

1. *Line 432-433 - which anti-HA or anti-My antibodies are these? (very minor detail)*

We have added the details.