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SUMOylation regulates telomere length homeostasis by targeting Cdc13

Lisa E Hang^{1,2,5}, Xianpeng Liu^{1,4,5}, Iris Cheung^{3,4}, Yan Yang^{1,4}, and Xiaolan Zhao^{1,2}

¹Molecular Biology Program, Memorial Sloan Kettering Cancer Center, New York, New York, USA

²Programs in Biochemistry, Cell and Molecular Biology, Weill Graduate School of Medical Sciences, Cornell University, New York, New York, USA

³Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA

Abstract

Telomere length homeostasis is an important aspect of telomere biology. Here we show that SUMOylation limits telomere length and targets multiple telomere proteins in *Saccharomyces cerevisiae*. A main target is Cdc13, which both positively and negatively regulates telomerase and confers end protection. We demonstrate that Cdc13 SUMOylation restrains telomerase functions by promoting Cdc13 interaction with the telomerase inhibitor Stn1, without affecting end protection. Mutation of the Cdc13 SUMOylation site (*cdc13-snm*) lengthens telomeres and reduces the Stn1 interaction, whereas Cdc13-SUMO fusion has the opposite effects. *cdc13-snm*'s effect on telomere length is epistatic with *stn1*, but not with *yku70*, *tell* or *est1* alleles, and is suppressed by Stn1 overexpression. Cdc13 SUMOylation peaks in early to mid S phase, prior to its known Cdk1-mediated phosphorylation, and the two modifications act antagonistically, suggesting that the opposite roles of Cdc13 in telomerase regulation can be separated temporally and regulated by distinct modifications.

Telomeres are essential for the stability and complete replication of the genome. In the budding yeast *S. cerevisiae*, telomeric sequences are composed of ~300 base pairs of TG₁₋₃/CA₁₋₃ repeats followed by short single-stranded 3' extensions of TG₁₋₃ sequences, called G-overhangs. Telomere DNA is bound by more than a dozen proteins that collaborate in telomere maintenance. Important functions of these proteins include telomere length homeostasis, G-overhang protection, nuclear positioning and transcriptional repression. Each function can be regulated by multiple pathways. In particular, maintenance of a constant telomere length unique to each organism requires a balance of positive and negative regulations of telomerase.

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Correspondence should be addressed to X.Z. (zhaox1@mskcc.org).

⁵These authors contributed equally to this work.

⁴Present addresses: Department of Biochemistry and Molecular Biology, Louisiana State University, Health Sciences Center, Shreveport, Louisiana, USA (X.L.); Eurofins MWG Operon, Huntsville, Alabama, USA (I.C.); New York University Medical School, New York, New York, USA (Y.Y.).

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In budding yeast, three positive regulators recruit telomerase to telomeres. These include the essential protein Cdc13 that binds to the Est1 subunit of the telomerase, the DNA end-binding Ku complex and the Tel1 kinase (reviewed in refs. 1,2). Concurrently, three negative regulators restrain telomerase, including the Rif1 and Rif2 proteins, the Pif1 helicase, and Cdc13 when bound to the Stn1 subunit of the Stn1–Ten1 telomerase inhibitory complex (reviewed in refs. 1,2). This multilayered regulatory network possibly confers robustness to telomere length homeostasis.

It is becoming increasingly clear that dynamic protein modifications have important roles in telomere length regulation, possibly by modifying some or all of the telomerase regulators. For instance, Cdk1 is critical for telomere synthesis during late S to G2 phases and is thought to target multiple telomere proteins^{3–7}. One such protein substrate is Cdc13, which, besides regulating telomerase positively and negatively in S phase, also prevents telomere end degradation^{1,2,8–15}. Cdk1 phosphorylates Cdc13 at Thr308 in its Est1 binding domain during late S and G2 phases, leading to enhanced telomerase recruitment at telomeres without affecting end protection⁶. This regulation explains one facet of Cdc13's activation of telomerase and provides a clue to the conundrum of how Cdc13 exerts opposite regulations of telomerase within the same cell-cycle stage^{1,2}.

Another type of protein modification implicated in telomere metabolism is SUMOylation, which entails the covalent linkage of the small ubiquitin-like modifier (SUMO) to the lysine residues of target proteins^{16–22}. SUMOylation is executed by the sequential action of SUMO E1, E2 and E3 proteins. Most organisms possess one E1, one E2 and multiple E3s, which facilitate conjugation to a wide range of substrates. Like phosphorylation, SUMOylation exerts diverse effects on its targets and is reversible in nature²³. It has been shown that mutating SUMO E3s in fission yeast leads to longer telomeres in a telomerase-dependent manner, although it has not been determined which, if any, telomere proteins are directly SUMOylated¹⁹.

In this study, we examined how SUMOylation affects telomere length homeostasis in the budding yeast *S. cerevisiae*. We found that defective SUMOylation in this organism also leads to telomere lengthening. We identified five telomeric proteins as SUMO targets and we demonstrated that SUMOylation of the telomerase regulator Cdc13 limits telomere length by strengthening the Cdc13–Stn1 interaction that is required for telomerase inhibition. Examination of the relationship between SUMOylation and Cdk1-mediated phosphorylation of Cdc13 suggests that the two modifications act antagonistically to promote the opposing roles of Cdc13 in telomere homeostasis.

RESULTS

Sumoylation mutants have longer telomeres

To know whether SUMOylation contributes to telomere length regulation in budding yeast, we examined *ubc9-1*, a temperature-sensitive allele affecting the SUMO E2 enzyme Ubc9 (ref. 23), and found that telomeres in *ubc9-1* cells were approximately 145 base pairs (bp) longer than those in wild-type cells, even at a semi-permissive temperature of 30 °C (Fig. 1a). Two other *ubc9* alleles also resulted in longer telomeres, albeit to a lesser degree than did *ubc9-1* (Fig. 1a). These observations suggest that SUMOylation is required for limiting telomere length in budding yeast.

To determine which SUMO E3s are involved in this process, we examined mutants of the nonessential Siz1 and Siz2 E3s and the essential Mms21 E3^{16,24}. Telomeres of *siz1Δ siz2Δ* and *siz2Δ mms21-CH* double mutants (*mms21-CH* has mutations in the critical residues of the SUMO E3 domain²⁵) were approximately 60 bp and 90 bp longer than wild-type

telomeres, respectively (Fig. 1b). No obvious telomere length alteration was seen in *siz1Δ mms21-CH* or single SUMO E3 mutants, compared to wild-type cells (Fig. 1b,c). The E3 triple mutant *siz1Δ siz2Δ mms21-CH* was lethal, preventing the examination of telomere length in those cells. These results suggest that all three SUMO E3s contribute to telomere length regulation with partly redundant roles, consistent with the observation that these E3s share a considerable number of targets (ref. 26 and see below).

Multiple telomere-binding proteins are SUMOylated

Next, we examined whether any of the known telomere proteins are SUMOylated. We tested thirteen proteins: the telomerase subunits, the Ku complex, Cdc13, Stn1, Ten1, Tel1, Rap1, Rif1, Rif2 and Pif1. Each protein was tagged with either TAP (composed of protein A (ProA) and calmodulin-binding peptide) or Myc epitope at its own genomic locus. Protein extracts were made under denaturing conditions to prevent the co-purification of interacting proteins and to preserve SUMOylated forms; each protein was immunoprecipitated and examined by western blot analysis with an antibody specific to the tag and another recognizing the budding yeast SUMO¹⁶ (Fig. 1d). Because a low percentage of substrate is typically SUMOylated²³, the anti-tag antibody detects only the unmodified form at the exposures shown. Bands preferentially recognized by the anti-SUMO antibody and specifically decreased in SUMO E3 mutants (*siz1Δ siz2Δ* and *mms21*) represent SUMOylated forms (Fig. 1d and Supplementary Fig. 1a,b).

In accordance with previous reports, Rap1 and Yku70 were SUMOylated under normal growth conditions^{16,27}. In addition, we identified a previously unknown SUMO substrate, Cdc13, and verified its SUMOylation using a tagged version of SUMO that resulted in a shift of the SUMOylated band (Fig. 1d and Supplementary Fig. 1c).

Because SUMOylation is known to respond to environmental stimuli, we examined the panel of telomere proteins under DNA-damage and high-temperature (37 °C) conditions, which also influence telomere metabolism or telomere proteins^{28–30}. Consistent with our previous report, methylmethanesulfonate (MMS) treatment enhanced Yku70 SUMOylation¹⁶. MMS also stimulated the SUMOylation of Rap1 and Cdc13 and allowed clear detection of the SUMOylation of Pif1 and Yku80 (Fig. 1d). Moreover, SUMOylation of four of these proteins (Rap1, Yku70, Cdc13 and Pif1) also increased at 37 °C (Fig. 1e). For comparison, SUMOylation of the recombination protein Rad52 was induced after MMS treatment but not at 37 °C (ref. 31) (Fig. 1e).

It is noteworthy that the SUMOylation patterns of these five telomere proteins vary, with Cdc13 showing mostly a monoSUMO form and the other proteins showing poly- or multiSUMO forms. In addition, protein SUMOylation is differentially dependent on the three SUMO E3s: whereas the Siz proteins were responsible for the bulk of Cdc13, Yku80 and Rap1 SUMOylation, *mms21* partly affected that of Pif1 and Yku70 (Supplementary Fig. 1a,b,d; ref. 16). In the case of Cdc13, *siz1Δ siz2Δ*, but not the single *sizΔ* mutants, eliminated most of its SUMOylation (Supplementary Fig. 1e). These observations indicate that the three E3s have both unique and overlapping roles in the SUMOylation of telomere proteins, a notion consistent with their effects on telomere length (Fig. 1b,c).

Cdc13 is SUMOylated primarily in early to mid S phase at Lys909

We next examined how SUMO regulates the function of Cdc13. We chose to focus on Cdc13 because it functions solely at telomeres and is SUMOylated in all conditions tested. We first examined the cell cycle stage during which Cdc13 is SUMOylated. Cdc13 SUMOylation was barely detectable in G1 cells, appeared when cells entered S phase, peaked during early to mid S phase and decreased in late S and G2 phases (Fig. 2a). This

pattern is different from what is seen in telomerase enrichment and activation at telomeres, which is low in early to mid S phase and peaks in late S phase (refs. 1,2,13 and Supplementary Fig. 2). These observations indicate that SUMOylation may regulate Cdc13 in early to mid S phase before telomerase is activated.

Next, we sought to generate a non-SUMOylatable *cdc13* mutant. We identified a SUMOylation consensus sequence Ψ KXE/D (Ψ : bulky hydrophobic residue) within the Stn1-binding domain of Cdc13 that is highly conserved among orthologs in the *Saccharomyces* clade^{23,32} (Fig. 2b). Indeed, mutating the lysine to arginine within this consensus sequence abolished Cdc13 SUMOylation *in vivo* (Fig. 2c). This *cdc13-K909R* allele (hereafter referred to as *cdc13-snm*, SUMO no more) affected neither Cdc13 protein levels before or after MMS treatment nor cell cycle progression (Fig. 2d and Supplementary Fig. 3a), indicating that Cdc13-snm retains the essential function of Cdc13 and that SUMOylation does not affect its protein stability. The *cdc13-snm* allele thus provides a useful genetic tool to assess the role of Cdc13 SUMOylation in telomere metabolism.

***cdc13-snm* leads to telomerase-mediated telomere lengthening**

We then examined *cdc13-snm*'s effects on telomere length and observed a reproducible 60-bp increase in both W303 and YPH strains (Fig. 3a and Supplementary Fig. 3b). This lengthening was dependent on telomerase, as it did not occur in the absence of telomerase components (*tlc1Δ* and *est1Δ*, Fig. 3b and Supplementary Fig. 3c). The epistatic relationship of *cdc13-snm* with telomerase deletions extended to senescence as well (Fig. 3c and Supplementary Fig. 3d). We also assessed whether *cdc13-snm* affects end protection but found no detectable changes in G-overhang abundance at either 30 °C or 37 °C in the *cdc13-snm* strains (Supplementary Fig. 4a). These results demonstrate that *cdc13-snm* affects telomerase-mediated telomere elongation without affecting end protection.

A logical interpretation of the above observations is that lack of SUMOylation underlies the telomere length defect of *cdc13-snm*. To further test this idea, we examined the genetic relationship between *cdc13-snm* and *siz1Δ siz2Δ*, which abolishes the majority of Cdc13 SUMOylation (Supplementary Fig. 1e). If the above hypothesis is correct, *cdc13-snm* should not further increase telomere length in *siz1Δ siz2Δ* cells. Indeed, we found that the telomere length of *cdc13-snm siz1Δ siz2Δ* cells was similar to that of *siz1Δ siz2Δ* and of *cdc13-snm*, and all were approximately 60-bp longer than wild type (Supplementary Fig. 4b). This epistatic relationship supports the above interpretation and indicates that Cdc13 is a main shared substrate for the Siz SUMO E3s in telomere length regulation.

***cdc13-snm* weakens interaction with Stn1**

The Lys909 SUMOylation site is located in Cdc13's Stn1-binding domain but not in its Est1, Pol1 or DNA binding domains (refs. 12,32–36 and M. Lei, University of Michigan Medical School, personal communication) (Fig. 2b). We asked whether SUMOylation at Lys909 could alter Cdc13's interaction with Stn1. The Cdc13-Stn1 interaction is difficult to measure: several labs have detected it by yeast two-hybrid (Y2H) assay but not by co-immunoprecipitation of the proteins expressed at endogenous levels^{6,8,11,15,34,37}. Using Y2H assay, we found that when combined with Stn1, wild-type Cdc13 activated the Ade+ reporter to a greater degree than Cdc13-snm (Fig. 4a). Similarly, the Stn1 and Cdc13 pair showed a more robust activation of the His+ and LacZ reporters than the Stn1 and Cdc13-snm pair (Fig. 4a,b). As a control, Cdc13 and Cdc13-snm showed the same level of interaction with Pol1 (Fig. 4b). These results indicate that the *cdc13-snm* allele specifically weakens the interaction with Stn1. To test if this weakened Stn1 interaction is due to deficient SUMOylation, we examined wild-type Cdc13-Stn1 interaction in Y2H strains lacking the Siz E3 proteins, which are responsible for Cdc13 SUMOylation (Supplementary

Fig. 1e). The Cdc13-Stn1 interaction, but not control interactions, was reduced in *siz1Δ siz2Δ* cells (Supplementary Fig. 4c). Taken together, these results indicate that lack of Cdc13 SUMOylation achieved by either the *K909R* mutation or by the *siz1Δ siz2Δ* mutation weakens the Cdc13-Stn1 interaction.

***cdc13-snm* lengthens telomeres in *yku70Δ*, *tel1Δ* and *est1* cells**

The above results suggest that Cdc13-snm may lead to diminished Stn1-dependent telomerase inhibition. To test this idea, we investigated whether *cdc13-snm* could suppress mutants defective in other telomerase regulatory pathways. Cells lacking the Ku complex, a positive regulator of telomerase and end protection, have shorter telomeres and are inviable at 37 °C; this temperature sensitivity, due to faulty telomere structures, can be suppressed by the overexpression of telomerase subunits^{38–42}. If *cdc13-snm* leads to increased telomerase function by weakening Stn1-mediated inhibition, but not by affecting the Ku pathway, then *cdc13-snm* should suppress *kuΔ* temperature sensitivity and telomere length defects. Indeed, *yku70Δ cdc13-snm* and *yku80Δ cdc13-snm* cells grew at 37 °C and had longer telomeres than *kuΔ* cells (Fig. 4c and Supplementary Fig. 5a,b). This suppression is unlikely to be the result of rescuing defective end protection in *kuΔ* cells, as *cdc13-snm* did not change G-overhang levels in *yku70Δ* cells (Supplementary Fig. 5c).

We next tested Cdc13-snm's effect in cells lacking Tel1, another positive telomere length regulator^{1,43}, or containing a partially defective Est1, which binds Cdc13 to achieve telomerase recruitment^{9,11–14}. Again, if telomere lengthening in *cdc13-snm* mutants occurs through misregulation of Stn1, then *cdc13-snm* should increase telomere length in *tel1Δ* cells or in cells with defective Est1. As with *yku70Δ*, *cdc13-snm* increased telomere length in both types of cells (Fig. 4d,e). These results indicate that *cdc13-snm* enhances telomerase function independently of Ku and Tel1 and can occur in cells with hypomorphic Est1.

***stn1* is epistatic with *cdc13-snm* and affects Cdc13 SUMOylation**

We then conducted epistasis tests with *stn1* alleles, which show longer telomeres with increased heterogeneity^{6,8,10,11,15,37}. We used two *stn1* alleles: a mild one generated by the addition of a Myc tag (*stn1-myc*), and *stn1ΔC199*, which lacks the C-terminal region required for Cdc13 binding and telomerase inhibition^{6,10,11,15,37}. In both cases, *cdc13-snm* did not further increase telomere length, indicating an epistatic relationship (Fig. 5a,b). We then examined how Stn1 overexpression affects telomere length in *cdc13-snm* cells. If *cdc13-snm* weakens the Stn1 interaction, increased levels of Stn1 may compensate for this deficiency and consequently suppress the *cdc13-snm* defect. Indeed, Stn1 overexpression reduced the telomere length of *cdc13-snm* cells to approximately wild-type levels (Supplementary Fig. 6). These genetic results support the idea that *cdc13-snm* influences telomere length by means of the Stn1 pathway.

We also investigated whether Stn1 influences Cdc13 SUMOylation and found that both *stn1* alleles led to increased Cdc13 SUMOylation (Fig. 5c). In particular, *stn1ΔC199* resulted in both a large increase in mono-SUMOylated Cdc13 and the appearance of polySUMOylated Cdc13. All SUMOylated Cdc13 species in *stn1ΔC199* cells depended on the Lys909 residue, as the Cdc13-snm protein was not SUMOylated in these cells (Fig. 5c). The increased level of Cdc13 SUMOylation in *stn1* cells was not due to a general enhancement of telomere protein SUMOylation, as *stn1ΔC199* did not affect Rap1 and Yku70 SUMOylation (Fig. 5d). It was also not due to increased telomere length, as *rif1Δ* mutants that also have longer telomeres did not stimulate Cdc13 SUMOylation (Fig. 5e). Nor was the Stn1's effect due to increased G-overhang levels, as *yku70Δ* cells with excessive G-overhangs had normal levels of Cdc13 SUMOylation (Fig. 5f). Lastly, Cdc13 SUMOylation was unchanged in *tel1Δ* or *est1-myc* cells (Fig. 5f), suggesting that defective telomerase

function is not sufficient to affect Cdc13 SUMOylation. Therefore, *stn1* alleles exert a unique effect on Cdc13 modification, further strengthening the connection between Cdc13 SUMOylation and Stn1.

CDC13-SUMO has opposite effects as *cdc13-snm*

To analyze the role of Cdc13 SUMOylation from another angle, we used a 'constitutively SUMOylated' Cdc13 allele generated by fusing Cdc13 with SUMO. Because Lys909 is only 15 amino acids away from the Cdc13 stop codon, we reasoned that fusing SUMO to the C terminus might resemble a constitutively SUMOylated protein and that this protein and *cdc13-snm* should express opposite phenotypes. Indeed, the Cdc13-SUMO fusion led to shortened telomeres (Fig. 6a). We then examined the physical interaction between Cdc13-SUMO and Stn1. The Y2H analysis is not suitable for this test, as SUMO can bind a large number of proteins in the assay^{44,45}. We therefore did coimmunoprecipitation using extracts with overexpressed Stn1 because this approach can detect the Cdc13-Stn1 interaction⁶. We observed a moderate yet reproducible increase in the amount of Cdc13-SUMO co-immunoprecipitated with Stn1, when compared to Cdc13 (Fig. 6b). These results with Cdc13-SUMO further support the notion that SUMO favors Cdc13's interaction with Stn1, leading to stronger inhibition of telomerase function.

Interplay of the two modifications of Cdc13

Whereas SUMOylation of Cdc13 acts as a negative regulator of telomere length, Cdk1-phosphorylation of Cdc13 at Thr308 (in Cdc13's Est1 binding domain) promotes telomerase recruitment⁶. We sought to understand the interplay of these two modifications of Cdc13. First, we found that the non-phosphorylatable *cdc13-T308A* did not affect Cdc13 SUMOylation (Fig. 6c). Conversely, *cdc13-snm* did not alter the levels of Cdc13-Thr308 phosphorylation (Fig. 6d). Thus, globally assessed, each modification is not a prerequisite for the other, nor are they competitive. Next, telomere length in cells lacking both modifications (*cdc13-T308A snm*) was examined and found to be between that of *cdc13-T308A* and *cdc13-snm*, suggesting that the two modifications act antagonistically and that neither effect is dominant (Fig. 6e). Finally, we examined the combined effect of T308A and SUMO fusion and found that the telomere-shortening effects of *CDC13-SUMO* and *cdc13-T308A* were approximately additive in *cdc13-T308A-SUMO* cells, consistent with the independent effects of the two modifications (Fig. 6a).

DISCUSSION

Here we demonstrate that SUMOylation limits telomere length by directly modifying telomere proteins in budding yeast. The observation that the abolition of Cdc13 SUMOylation led to longer telomeres, whereas Cdc13-SUMO fusion resulted in shorter telomeres, indicates that SUMO negatively regulates telomere length, in part by targeting Cdc13 (Figs. 3a and 6a). We show that Cdc13 is a main target of Siz SUMO E3s in telomere length regulation, though SUMOylation of other identified substrates probably contribute to the full inhibitory effect of SUMO, because telomere lengthening in *cdc13-snm* or *siz1Δ siz2Δ* cells was not as severe as in *ubc9-1* cells (Fig. 1 and Supplementary Fig. 4b). It is noteworthy that a similar multisubstrate strategy has been suggested for Cdk1 kinase, with Cdc13 being one of several targets³⁻⁷.

As some of the identified SUMO substrates modulate transcriptional silencing and telomere positioning, their SUMOylation could affect these aspects of telomere metabolism. Indeed, Siz2-mediated Ku SUMOylation was found to influence telomere positioning⁴⁶. Our finding of MMS- and high temperature-induced SUMOylation may suggest that SUMO can also modulate telomerase behavior both at native telomeres and/or at DNA breaks under these

conditions (Fig. 1d,e). For example, SUMO may prevent telomere addition at the DNA breaks in favor of DNA repair, similar to the effect of Mec1 checkpoint kinase-mediated phosphorylation of telomere proteins^{29,30}. Our identification of telomere SUMO substrates opens up opportunities for further analysis of SUMO's roles in telomere metabolism under various conditions.

SUMOylation specifically affects the actions of Cdc13 in telomerase regulation but not in end protection, as shown by our finding that *cdc13-snm* lengthened telomeres in a telomerase-dependent manner without altering end protection in either wild-type or *kuΔ* cells (Fig. 3 and Supplementary Figs. 3, 4a, 5c). Several lines of evidence support the possibility that Cdc13 SUMOylation affects the Stn1 pathway of telomerase inhibition. Abolition of Cdc13 SUMOylation by *cdc13-snm* or *siz1Δ siz2Δ* weakened the Cdc13-Stn1 interaction in Y2H assays (Fig. 4a,b and Supplementary Fig. 4c). Genetically, *cdc13-snm* was epistatic with *stn1* alleles, but not with *tel1Δ*, *kuΔ* and hypomorphic *est1* alleles, and its telomere defect was suppressed by Stn1 overexpression (Figs. 4c–e, 5a,b and Supplementary Fig. 6). Moreover, Cdc13-SUMO was enriched in the Stn1 immunoprecipitate and led to shorter telomeres (Fig. 6a,b). These results strongly support the idea that SUMO promotes the Cdc13-Stn1 interaction to restrain telomerase function. The specific effect of increased Cdc13 SUMOylation by *stn1* alleles also points to a functional interaction between Cdc13 SUMOylation and Stn1 and raises the possibility of an inhibitory feedback mechanism (Fig. 5c). As SUMO does not interact with Stn1 or Ten1 by Y2H, it is unlikely that SUMO enhances the Cdc13-Stn1 interaction by simply binding to these proteins (data not shown). Rather, SUMO may affect other properties of Cdc13 such as conformation or DNA binding. Further investigation of these ideas requires the development of an *in vitro* assay for the Cdc13-Stn1 interaction.

We showed that SUMOylation and phosphorylation of Cdc13 operate independently and act antagonistically (Fig. 6a,c–e). This result and those of others⁶ suggest that each role of Cdc13 can be facilitated by a different modification, which helps explain how Cdc13 acts both positively and negatively to regulate telomerase during S phase. Because Cdc13 SUMOylation peaks in early to mid S phase (Fig. 2a), whereas Cdc13 phosphorylation and telomerase recruitment peak in late S to G2 phase (refs. 1,2,13 and Supplementary Fig. 2), these modifications may help to restrict telomere addition to a narrow window within the cell cycle (Fig. 6f). As Cdc13 was also proposed to inhibit telomerase in late S to G2 phase, Cdc13 may exert a dual-phase inhibition of telomerase¹¹. Other models, which are not mutually exclusive, may also help to explain the interplay of the two modifications. For example, because telomerase is preferentially recruited to short telomeres⁴⁷, phosphorylation and SUMOylation of Cdc13 may predominantly occur at short and long telomere ends, respectively, to promote or restrain telomerase action (Fig. 6f). Future studies will be needed to test these models and to examine the coordination of these modifications with other layers of telomerase regulation.

Because SUMOylation was also found to limit telomere length in fission yeast¹⁹, this modification can have an evolutionarily conserved role in inhibiting telomerases. Considering that Cdk1 and Tel1 kinases and their homologs are typically positive regulators of telomerase, SUMOylation and phosphorylation may provide balancing mechanisms in telomere length homeostasis. Further understanding of their interplay could provide new insights into telomere-related human diseases.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nsmb/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Defects in SUMOylation enzymes lead to longer telomeres, and multiple telomere proteins are SUMOylated. (a–c) *ubc9* mutants (a) and two SUMO E3 double mutants (b), but not SUMO E3 single mutants (c), show longer telomeres. *ubc9-MYC* and *ubc9-HA* are the Myc- and hemagglutinin (HA)-tagged *UBC9* genes, respectively. *mms21-CH* (*mms21*) contains two point mutations in the SUMO E3 domain²⁵. (d) Rap1, Yku70, Cdc13, Pif1 and Yku80 are SUMOylated. Proteins were tagged with TAP (not labeled) or Myc and were immunopurified before (–) and after (+) MMS treatment. Unmodified proteins were detected on immunoblots by anti-ProA or anti-Myc antibodies (below) and SUMOylated forms of the proteins by anti-SUMO antibody (above). Asterisks label the positions of SUMOylated proteins before MMS treatment. The occasional difference in signals on the anti-tag blots reflects variation in loading rather than protein levels. (e) SUMOylation of Rap1, Yku70, Cdc13 and Pif1, but not Rad52, increases at 37 °C.

Figure 2.

SUMOylation of Cdc13 at a single lysine peaks in early to mid S phase. (a) Cdc13 SUMOylation peaks in early to mid S phase. Alpha-factor–arrested G1 cells (0 min) containing Cdc13-TAP were released and samples at indicated time points were examined for protein SUMOylation (left) and by FACS analysis (right). The position of SUMOylated Cdc13 is labeled as Cdc13-S. (b) Schematic map of Cdc13. Stn1- and Pol1-interacting domains as well as telomerase recruitment (RD) and DNA binding (DBD) domains are labeled^{12,32–36}. The dot represents the Cdk1 phosphorylation site. The SUMOylation consensus site and the position of the SUMOylated lysine are depicted. (c) *K909R* abolishes Cdc13 SUMOylation. Wild-type Cdc13 (WT) or Cdc13-K909R proteins tagged with TAP were examined as in Figure 1d. (d) *cdc13-snm* does not affect protein levels. Protein extracts before (–) and after (+) MMS treatment were examined by immunoblots using anti-ProA antibody. Cdc13 and Cdc13-snm were tagged with TAP. Equal loading is shown by amido black staining.

Figure 3. *cdc13-snm* leads to longer telomeres in a telomerase-dependent manner. (a) *cdc13-snm* cells show increased telomere length. (b) Deleting the telomerase component *TLC1* abolishes telomere lengthening caused by *cdc13-snm*. (c) *cdc13-snm* and *tlc1Δ* show no synthetic defects in senescence. Senescence experiments were conducted by repeatedly streaking strains (number of times streaked is indicated for each).

Figure 4. *cdc13-snm* weakens Stn1 interaction and increases telomere length in *yku70 Δ* , *tel1 Δ* and *est1* cells. **(a,b)** Cdc13-snm affects Stn1 interaction in yeast two-hybrid assays. Two-hybrid strain containing pairs of indicated Gal4 DNA-binding domain (GBD) and Gal4 activation domain (GAD) plasmids were grown on medium lacking leucine and tryptophan (–LEU–TRP). The activation of reporters was scored by replica plating onto –LEU–TRP–ADE and –LEU–TRP–HIS media **(a)** and by assaying the activity of β -galactosidase **(b)**. Cdc13-snm specifically weakens interaction with Stn1 ($P = 0.0018$) but not with Pol1 **(b)**. Error bars represent s.d. of six trials. **(c–e)** *cdc13-snm* leads to longer telomeres in *yku70 Δ* , *tel1 Δ* and hypomorphic *est1* cells. Hypomorphic *est1* (*est1-myc*) was generated by tagging Est1 at its N terminus with 13MYC.

Figure 5.

stn1 alleles are epistatic to *cdc13-snm* and specifically enhance Cdc13 SUMOylation. (a,b) *stn1* alleles are epistatic to *cdc13-snm*. The double mutants of *cdc13-snm* with *stn1-myc* (a) or *stn1-ΔC199* (b) have telomere lengths similar to those of the *stn1* single mutants. (c,d) *stn1* alleles enhance the SUMOylation of Cdc13 but not that of Rap1 and Yku70. The effects of *stn1-myc* (-myc) and *stn1-ΔC199* (ΔC) on SUMOylation of Cdc13-TAP (c) and of Rap1 and Yku70 (d) were examined. (e,f) Cdc13-TAP SUMOylation is not altered in *rif1Δ* (e) or in *yku70Δ*, *tel1Δ* and *est1-myc* cells (f).

Figure 6. *CDC13-SUMO* leads to shorter telomeres and enhanced Stn1 interaction, and the two modifications of Cdc13 act antagonistically. **(a)** *CDC13-SUMO* leads to shorter telomeres and *cdc13-T308A-SUMO* results in a greater degree of telomere shortening than either *cdc13-T308A* or *CDC13-SUMO*. **(b)** *CDC13-SUMO* fusion enhances Stn1 interaction. Untagged and Myc-tagged Stn1 cells both contain Cdc13-HA. Protein extracts from these cells were co-immunoprecipitated with anti-Myc antibody, and Cdc13 and Cdc13-SUMO were examined in the immunoprecipitated fraction with anti-HA antibody. The numbers indicate the relative amounts of co-purified Cdc13 in wild-type and *CDC13-SUMO* strains based on the average of four trials (mean $\pm$ s.d.). Cdc13 and Cdc13-SUMO input levels are similar (data not shown). **(c,d)** Abolition of Cdk1-mediated phosphorylation of Cdc13 does not affect its SUMOylation, and vice versa. HA-tagged wild-type and mutant Cdc13 were examined for SUMOylation **(c)** and phosphorylation **(d)** after immunoprecipitation. **(e)** Lack of Cdk1-mediated Cdc13 phosphorylation and lack of Cdc13 SUMOylation affect telomere length in an antagonistic manner. Telomere length was examined in wild-type (WT) and *cdc13* mutant cells, and telomere length of *cdc13-T308A snm* cells was found to be inbetween those of *cdc13-T308A* and *cdc13-snm* cells. **(f)** Models for the roles of the two protein modifications on Cdc13. See text for details.