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The mechanisms of *K. lactis* Cdc13 in telomere DNA-binding and telomerase regulation

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Abstract

Eukaryotic chromosome ends, or telomeres, are essential for genome stability and are protected by an intricate nucleoprotein assembly. Cdc13, the major single-strand telomere-binding protein in budding yeasts, mediates critical functions in both telomere protection and telomere elongation by telomerase. In particular, the interaction between *S. cerevisiae* Cdc13 and telomerase subunit Est1 has long served as a paradigm for telomerase regulation. However, despite extensive investigations, the role of this interaction in regulating telomerase recruitment or activation remains controversial. In addition, budding yeast telomere repeat sequences are extraordinarily variable and how Cdc13 orthologs recognize diverse repeats is not well understood. In this report, we examined these issues using an alternative model, *K. lactis*. We reconstituted a direct physical interaction between purified *K. lactis* Cdc13 and Est1, and by analyzing point mutations, we demonstrated a close correspondence between telomere maintenance defects *in vivo* and Cdc13-Est1 binding defects *in vitro*, thus supporting a purely recruitment function for this interaction in *K. lactis*. Because mutations in well aligned residues of Cdc13 and Est1 in *S. cerevisiae* and *K. lactis* do not cause identical defects, our results also point to significant evolutionary divergence in the Cdc13-Est1 interface. In addition, we found that *K. lactis* Cdc13, unlike previously characterized orthologs, recognizes an unusually long and non-G-rich target sequence, underscoring the flexibility of the Cdc13 DNA-binding domain. Analysis of *K. lactis* Cdc13 and Est1 thus broadens understanding of telomere and telomerase regulation in budding yeast.

Graphical abstract

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Keywords

Telomere; Telomerase; Cdc13; Est1; Survivors

1. Introduction

Telomeres, the ends of linear eukaryotic chromosomes, are essential for genome stability and are protected by a plethora of proteins [1–3]. Aberrations in the telomere nucleoprotein assembly can trigger DNA degradation, end-to-end fusion, excessive recombination, and other detrimental reactions. Owing to the “end-replication” problem, telomere DNA is progressively lost with each cell division. In most organisms, this loss is counter-balanced by telomerase, which extends telomeres by copying an integral RNA template that specifies the telomere repeat sequence [4, 5]. Telomerase is thus a specialized reverse transcriptase dedicated to telomere maintenance. Because the lengths of telomere DNA impact critically on the replicative potential of cells, the activity of telomerase has to be tightly regulated by telomere proteins in order to promote normal growth as well as to suppress aberrant proliferation [6, 7]. Thus, telomere proteins serve critical functions in both telomere protection and telomerase regulation.

An especially well studied telomere protein with diverse functions is Cdc13 in the budding yeast *S. cerevisiae*. It binds with high affinity and sequence specificity to single-stranded telomere DNA, and is critical for both telomerase regulation and telomere protection [8, 9]. The telomerase regulatory function of Cdc13 is thought to be mediated primarily through its interaction with Est1, a budding yeast telomerase subunit [10, 11]. The physical interaction between Cdc13 and Est1 has been detected in cell extracts, as well as between purified proteins [12, 13], and its functional relevance is strongly supported by a pair of allele-specific, charge-swap suppressor mutations. Specifically, the *cdc13^{E252K}* (named *cdc13-2*) and the *est1^{K444E}* (named *est1-60*) alleles each triggers senescence and progressive telomere shortening, whereas the combination of the two mutations restores normal growth and telomere homeostasis [10]. However, despite extensive investigations over the past two decades, there remain substantial unresolved arguments concerning the mechanism and function of Cdc13-Est1 interaction. In particular, whether this interaction serves exclusively to recruit telomerase to telomere ends or mediates an additional activating function has been controversial, owing to disparate results on the effects of charge-swap mutations on Cdc13-Est1 binding affinity. If as reported in one study [12], there is a close correspondence between effects of mutations on Cdc13-Est1 binding affinity and telomere maintenance, then

a function in recruitment alone would be sufficient to account for the data. Alternatively, if the mutations have no effect on affinity [13], then it may be necessary to invoke an allosteric or activating function (besides recruitment) for Cdc13-Est1 binding [14]. (Notably, precedent for a single protein-protein interface being responsible for both telomerase recruitment and activation is well established in mammals, where both functions are mediated by an interaction between the shelterin subunit TPP1 and the telomerase catalytic protein TERT [15].)

Another interesting and unresolved question concerning Cdc13 is raised by the exceptional variability in telomere sequence in the Saccharomycotina subphylum of budding yeasts, to which *S. cerevisiae* belongs [9]. The telomere sequences in some members of this subphylum are short and irregular (e.g., TG₁₋₃ in *S. cerevisiae*), whereas others are long and regular (e.g., a 25-bp regular repeat in *K. lactis* (see Fig. 1)). How the DNA-binding domains of Cdc13 orthologs can recognize such diverse repeats is not well understood. Previous analysis of Cdc13s from *S. cerevisiae* and *S. castellii* identified relatively short and G-rich target sites [16–19], leading to the proposal that all Cdc13 orthologs may recognize a core G-rich sequence shared by fungi with diverse repeat units (e.g., GTGTN[C/T]GG as suggested in [16]). However, whether this applies to all Cdc13 orthologs is unclear.

In the current study, we sought to address the mechanisms of Cdc13 in DNA-binding and telomerase regulation in *K. lactis*, a closely related Saccharomycotina yeast. While the great majority of telomere features are conserved between *S. cerevisiae* and *K. lactis*, the latter has an unusually long (25 bp) and regular telomere repeat unit that offered experimental advantages in certain analyses, e.g., in the cloning of telomerase RNA gene (*TER1*) and in analyzing the translocation mechanism of telomerase [20, 21]. *K. lactis* also has a reduced set of telomere proteins because, unlike *S. cerevisiae*, it has not undergone whole genome duplication. In an early study, we characterized the *K. lactis* Est1 ortholog, uncovering the molecular basis of its interaction with telomerase RNA [22]. In addition, we showed that *K. lactis* Est1 mediates functions in both telomere maintenance and rapamycin-resistance, which in *S. cerevisiae* are performed separately by Est1 and Ebs1, two paralogs that resulted from whole genome duplication. We now extend our investigation of *K. lactis* to Cdc13. Using purified recombinant proteins isolated from *E. coli*, we reconstituted a direct physical interaction between Cdc13 and Est1, and characterized the effects of charge swap mutations on Cdc13-Est1 interaction *in vitro* and telomere maintenance *in vivo*. Our results are consistent with an exclusively recruitment function for this interaction and revealed species-specific differences in the Cdc13-Est1 interface. We also investigated DNA-binding by *K. lactis* Cdc13 and uncovered an unusually long target site that is 75% non-G, thus underscoring the versatility of the Cdc13 DNA-binding domain in evolving diverse sequence specificities. Finally, we found serendipitously that *K. lactis est1* strains can generate type I survivors (characterized by amplification of subtelomeric DNA) after prolonged passage, which has not been described before in this yeast. Investigation of the *K. lactis* telomere machinery thus provided broader insights on multiple issues in yeast telomere regulation and evolution.

2. Materials and Methods

2.1 *K. lactis* strains and growth

The *K. lactis* strain 7B520 (*ura3-1 his2-2 trp1*) was used as the parental strain [23]. The construction of the *est1* mutants bearing *EST1* allele on the pCXJ18 plasmid have already been described [22]. The strains expressing FLAG-tagged Cdc13 or Cdc13-EK from the endogenous locus were generated as follows. First, we amplified a FLAG₃-tagged *CDC13* (aa 131 to 907, with an artificial *PvuII* site from nt 655 to 660 that does not alter the protein sequence) fragment with the *HIS3* marker through overlapping PCR amplification of two fragments: (i) a CDC13-FLAG₃ fragment amplified from pSMT3-*KCDC13*-FLAG, and (ii) a FLAG₃-HIS3 fragment amplified from pFA6A-FLAG₃-HIS3 plasmid. This “gene substitution” fragment was then transformed into *K. lactis est1* bearing pCXJ18-KIEST1_{NTD}-TAP plasmid. Following selection on synthetic defined (SD) agar plates lacking histidine and uracil, candidate clones were checked to confirm the integration of the FLAG₃-tagged *CDC13* by PCR, *PvuII* digestion, and DNA sequencing. Strains were passaged at 30°C in either YPD or SD-HIS-URA medium.

2.2 Telomere length analysis

Chromosomal DNAs were isolated from 5 ml of saturated *K. lactis* culture by Smash and Grab method [24], digested with *EcoRI*, and fractionated in 0.6 to 0.8% agarose gels. Following transfer to nylon membranes, the telomere restriction fragments were detected as previously described using an oligonucleotide probe that contains two copies of the *K. lactis* telomere repeat (5′-ACG GAT TTG ATT AGG TAT GTG GTG TAC GGA TTT GAT TAG GTA TGT GGT GT-3′). The hybridization was performed at 50°C to 60°C.

2.3 Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed using an untagged strain and one that carries the *CDC13-FLAG₃* allele (integrated at the endogenous locus). The crosslinking, immunoprecipitation, and PCR were performed as described [25] with the following modifications. First, the crosslinked samples were subjected to immunoprecipitation using anti-FLAG agarose resin (Genscript, Inc.), and the resin-bound protein-nucleic conjugates eluted using 0.4 mg/ml FLAGx3 peptide (ApexBio Technology). Second, the PCR reactions were performed using two primer pairs designed to amplify an ~240 bp *K. lactis* subtelomeric fragment (forward primer: KIsutb1F, TCC CCG CGG GAA ATC CGT TTC GAG; reverse primer: KIsutb1R, GCT CCT CCC TTT CTG GTA ATT TTA C) and a 380 bp U1 fragment (forward primer: KIU1F, GTT TGT TGA CCG GGT ATG AGG TTT TC; reverse primer: KIU1R: CTC TCA CCC ATA AAC CTG TAA ACA AG).

2.4 Purification of Cdc13 and Est1

The *K. lactis CDC13* and *EST1* coding sequences were amplified from genomic DNA using primer pairs that amplify the wild type genes. In the case of *CDC13*, the reverse primer was further designed to introduce a C-terminal FLAG tag. The PCR fragments were cloned into the pSMT3 vector, and the resulting plasmids introduced into the BL21 codon plus strain [26]. The His₆-SUMO-tagged Est1 and Cdc13-FLAG proteins were then expressed and

purified essentially as previously described [27]. Briefly, extracts were prepared from *E. coli* following IPTG (isopropyl- β -D-thiogalactopyranoside) induction and the fusion proteins were purified using Ni-nitrilotriacetic acid (Ni-NTA) chromatography. The fusion proteins were then cleaved by ULP1 and the untagged Est1 was isolated from the His₆-SUMO tag by a second round of Ni-NTA affinity chromatography, whereas Cdc13-FLAG isolated by FLAG affinity chromatography. In some preparations, the proteins were further purified by sedimentation through 15–30% glycerol gradients as described [28].

2.5 DNA-binding assays

Purified *KlCdc13* was incubated with ³²P-labeled *K. lactis* G-strand probe (7 nM) in EMSA buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM MgCl₂, 1 mM Spermidine, and 1 mM DTT). Following the binding reaction at room temperature for 15 min, the reaction mixtures were further subjected to gel electrophoresis through a 5% polyacrylamide gel in 1 × TBE and analyzed using a Typhoon PhosphorImager and the ImageQuant software (GE Healthcare).

2.6 Anti-FLAG Pull down assays

The FLAG-pull down procedure was based on a previously published method with slight modifications [25]. Purified Cdc13-FLAG (5 μ g) from *E. coli* was first incubated with 20 μ l of FLAG M2 beads (Sigma) in 1.2 ml FLAG-150 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM MgCl₂, 10% glycerol, 1 mM DTT, and 0.1% NP-40). The beads were rotated at 4°C for 1.5 h and the beads were washed five times, each with 300 μ l of FLAG-150 buffer. The M2-bound Cdc13 were then incubated with varying concentrations of purified Est1 in 160 μ l FLAG-100 buffer (same as FLAG-150 except with 100 mM NaCl). After 30 min rotation at 4°C, the beads were washed three times, each with 300 μ l of FLAG-100 buffer. The proteins were eluted by the FLAG peptide (Sigma) and analyzed by SDS-PAGE and Coomassie Blue staining. To quantify the molar ratio of Est1_{NTD} to Cdc13-FLAG, the stained gels were scanned, and the intensities of the proteins estimated by ImageJ. The intensities were normalized against the predicted molecular weights of Est1_{NTD} to Cdc13-FLAG, and then used calculate the molar ratios.

3. Results

3.1 *K. lactis* Cdc13 binds to an unusual target sequence in telomere repeat

The putative *K. lactis* Cdc13 orthologue (XP_456019.1) has not been investigated before. We therefore sought to validate its function at telomeres before engaging in more detailed mechanistic investigations. First, we used chromatin immunoprecipitation (ChIP) to confirm the localization of this putative ortholog to telomere *in vivo*. As predicted, a subtelomeric fragment is preferentially enriched in DNA recovered from anti-FLAG beads when a strain carrying a FLAG₃-tagged Cdc13 was subjected to ChIP analysis (Fig. 1A, third lane). Next, we analyzed the predicted G-strand binding activity of this putative Cdc13 orthologue. Unlike *S. cerevisiae* Cdc13, the *K. lactis* ortholog can be produced in a soluble form at high levels in *E. coli* as a His-SUMO-*KlCdc13*-FLAG fusion protein. We purified the *KlCdc13*-FLAG portion sequentially through Ni-NTA chromatography, ULP1 cleavage (which eliminated the His-SUMO tag), anti-FLAG affinity chromatography, and glycerol gradient

sedimentation. The purified fraction was subjected to EMSA using different variants of the *K. lactis* telomere G-strand repeat sequence as probes (Fig. 1B). Initial pilot assays revealed high affinity binding of Cdc13-FLAG to a 37-nt probe that corresponds to ~ 1.5 copies of the G-strand repeat (1.5R), but not to a 25-nt probe that corresponds to a specific permutation of one repeat (1R). The K_d of Cdc13-FLAG for the 1.5R probe was estimated to be ~10 nM (Fig. 1B and 1D). Accordingly, we performed competition assays using the 1.5R probe and progressively smaller sub-regions of 1.5R as unlabeled competitors (Fig. 1C). This series of analysis revealed a 16-nt sequence (MS) that represents the minimal high affinity site for Cdc13; further trimming of MS from either its 5' or 3' end abolished its binding activity (e.g., see results for MS(-1) and (-2)MS in Fig. 1C and 1D). Notably, in contrast to previously characterized Cdc13 target sites, MS is quite long and non-G-rich (only 25% G). *K. lactis* Cdc13 thus exhibits an atypical DNA recognition specificity that is tailored to the non-G-rich *K. lactis* telomere repeat.

3.2 *K. lactis* Cdc13 binds Est1 with moderate affinity through electrostatic interactions

Next we examined the interaction between purified Cdc13-FLAG and Est1 using a FLAG pull down assay. Because full length Est1 is prone to proteolysis, we purified a 1–598 fragment (named Est1_{NTD}) that had previously been shown to fully complement the telomere function of Est1 *in vivo* [22]. Notably, increasing amounts of Est1_{NTD} can be retained and eluted from Cdc13-FLAG-containing beads but not the control beads in a concentration-dependent fashion (Fig. 2B). With the highest Est1_{NTD} input, the elution contained approximate equal amounts of Cdc13 and Est1, consistent with a 1:1 stoichiometry for binding. Next, we examined the effects of charge swap mutations that had been previously shown to impair telomere maintenance and possibly Cdc13-Est1 interaction in *S. cerevisiae*. Based on the sequence alignments, the *cdc13*^{E252K} and the *est1*^{K444E} alleles in *S. cerevisiae* should be equivalent to *cdc13*^{E233K} and *est1*^{K467E} in *K. lactis* (Fig. 2A). Indeed, we had previously shown that *K. lactis* *est1*^{K467E} caused substantial telomere loss [22]. We therefore purified both mutant proteins (abbreviated as Cdc13-FLAG-EK and Est1_{NTD}-KE) and tested their activities in the pull down assays. Interestingly, in standard assay condition (100 mM NaCl in the binding and washing buffer), the charge swap substitutions in Est1 and Cdc13 each reduced the binding activity by ~50 % over a range of Est1 concentrations (from 2 to 14 μ M), supporting the functional importance of these charged residues (Fig. 2C and 2D). However, in contrast to some reports from *S. cerevisiae*, combining both mutant proteins in the pull down assays did not restore normal binding activity (Fig. 2C and 2D). Instead, the extent the binding was similar to that observed when either Cdc13-FLAG-EK or Est1_{NTD}-KE was combined with the respective wild type binding partner in the pull down assay. Our results thus point to significant differences in the detailed molecular contacts between Cdc13 and Est1 in different budding yeasts.

The detrimental effects of charge substitutions on Cdc13-Est1 interaction suggest that the interface involves at least some electrostatic interactions. Consistent with this interpretation, we observed a strong influence of salt concentration on binding. When 250 mM NaCl was included in the washing buffer, the binding of Est1_{NTD} or Est1_{NTD}-KE to Cdc13-FLAG beads was no greater than that of BSA to Cdc13-FLAG (Fig. 3A). In addition, the Est1 proteins bind equally weakly to Cdc13-containing beads and the control beads. Thus, high

salt completely abolishes specific Cdc13-Est1 interaction. This was also reported by Wu et al. in their analysis of *ScCdc13-ScEst1* interaction [13]. In contrast, when 100 mM and 50 mM NaCl were used in the binding and washing buffer, respectively, the wild type and mutated Est1 proteins bind equally strongly to Cdc13-FLAG (Fig. 3B). Thus, the impact of the charge substitution on Cdc13-Est1 interaction can only be observed at moderate salt concentrations (~100 mM NaCl). As will be discussed later, this observation may help reconcile some of the disparate reports concerning *S. cerevisiae* Cdc13-Est1 interaction.

3.3 The effects of charge swap mutations on telomere maintenance parallel their effects on Cdc13-Est1 interaction

The similar but distinct effects of charge swap mutations in *K. lactis* (vis-à-vis *S. cerevisiae*) suggest significant evolutionary divergence and provide an alternative system to test the mechanistic role of Cdc13-Est1 charge interaction. As discussed in the introduction, if the charged residues work solely to promote recruitment, then one would predict a close correlation between the affinity of Cdc13-Est1 interaction and telomere maintenance. Alternatively, if the charged residues promote an allosteric or other change that is not necessarily reflected in the binding affinity, then the correlation between Cdc13-Est1 interaction and telomere maintenance may be imperfect. We therefore examined telomere maintenance in *K. lactis* strains harboring various combinations of Cdc13 and Est1 substitutions. For this experiment, we used direct gene replacement to substitute the endogenous *CDC13* with a FLAG-tagged wild type *CDC13* or *CDC13-EK*. In addition, we disrupted the chromosomal *EST1* gene, and complemented the strain with a plasmid copy of *EST1* or *EST1-KE* (see Experimental Procedures). The strains were passaged for 10 streaks on plates, and then analyzed for telomere lengths (Fig. 4A). Notably, in contrast to the *S. cerevisiae* *cdc13-2* and *est1-60* mutants, none of the *K. lactis* mutant exhibited senescence (data not shown), indicating that the *K. lactis* mutations do not disrupt telomere maintenance to the same degree. However, Southern analysis revealed in all of the mutants abnormally short telomeres (~200 bp shorter than wild type). Consistent with the lack of senescence, telomeres in these strains did not exhibit the *est* (ever shorter telomeres) phenotype, but were stably maintained through ~250 generations. Note that while the “WT KE” telomeres (i.e., from the strain with WT Cdc13-FG and the KE mutant of Est1_{NTD}-TAP) appear to suffer slightly more severe shortening in later passages, this observation was not observed in other experiments. Moreover, we have passaged these clones for another 5 streaks (i.e., for a total of ~375 generations) without observing senescence or further telomere shortening (data not shown). Most importantly, in close parallel with the Cdc13-Est1 interaction studies, the combination of charge swap mutations in *CDC13* and *EST1* did not suppress the telomere length defect of individual mutations (Fig. 4A). Thus, our observations are fully consistent with an exclusively “recruitment” function for the charged residues in telomere maintenance.

Our interpretation of the charged residues could be confounded by unanticipated effects of mutations on other activities of Cdc13 or Est1 (i.e., activities other than Cdc13-Est1 interaction). Accordingly, we tested for other potential loss of functions. First, we had shown previously that the Est1-KE mutant is expressed at wild type levels *in vivo* and associate normally with telomerase RNA [22]. Similarly, we show in the current study that Cdc13-EK is also expressed at wild type levels (Fig. 4B). Second, we found that the Cdc13-EK

mutation did not impair Cdc13's DNA-binding activity as judged by EMSA analysis (Fig. 5A to 5C). In addition, both Cdc13 and Cdc13-EK migrated as ~200 kDa complexes in glycerol gradients, consistent with normal dimerization, which has been shown to be a conserved property of Cdc13 (Fig. 5D). Finally, we show that the purified Est1-KE mutant protein has normal RNA-binding activity as judged by a previously developed UV-crosslinking assay (Fig. 5E to 5G). Collectively, these assays indicate that the charge swap mutants are not generally defective in protein function, but have a selective reduction in Cdc13-Est1 binding activity.

3.4 Type I survivors from *K. lactis* telomerase null mutant

S. cerevisiae post-senescent survivors are known to employ two distinct recombination pathways to maintain telomeres. One pathway, utilized by Type I survivors, is Rad51-dependent and characterized by dramatic amplification of subtelomeric Y' elements [29, 30]. The other pathway, utilized by Type II survivors, is Rad50-dependent and characterized by heterogeneously sized, often quite lengthy terminal repeat tracts [31]. Interestingly, no *K. lactis* telomerase null mutants have previously been reported to generate Type I-like survivors, raising interesting questions concerning potential differences between different fungi in the regulation of telomere recombination. Notably, in the course of our analysis of the *K. lactis* charge swap mutants, we occasionally observed quite slow growing colonies when the strains were passaged on YPD plates. Further examination revealed the loss of EST1-bearing plasmid in these clones—i.e., they are *est1*⁻. Southern analysis of TRF revealed in some of these clones the amplification of discrete fragments (most predominantly ~1.6 kb and occasionally ~750 bp) that hybridize strongly to the *K. lactis* telomere repeat probe, which is reminiscent of *S. cerevisiae* type I survivors (Fig. 6A). In one series of analysis, 3 out of 10 *K. lactis est1*⁻ clones were shown to exhibit the Type I pattern (Fig. 6B). Notably, the 1.6 Kb restriction fragment is sufficiently abundant to be visible by ethidium bromide staining (Fig. 6C). We therefore cloned and sequenced this fragment and found that it consists of ~1.4 kb of subtelomeric R element and ~200 bp of terminal repeats (Fig. 6D). These observations support the existence of a Type I-like recombination pathway in *K. lactis* telomerase null mutants.

4. Discussion

In this study, we used *K. lactis* as an alternative model organism to address issues concerning Cdc13 mechanisms that remain unresolved despite extensive earlier investigations (primarily in *S. cerevisiae*). Our findings provide insights on the molecular basis of Cdc13-Est1 interaction and support an exclusively recruitment function for this interaction. We also show that contrary to predictions based on the property of other Cdc13 orthologs, the DNA-binding domain of *K. lactis* Cdc13 recognizes an unusually long and non-G-rich target site. Finally, we obtained unanticipated results on the regulation of survivor pathways in *K. lactis*. The implications of these findings are discussed below.

4.1. The role of budding yeast Cdc13-Est1 interaction in telomere maintenance

A long-standing debate in *S. cerevisiae* Cdc13 and Est1 research centers around the effects of charge swap mutations on their mutual interaction, and the implications of such results on

Cdc13 and Est1 mechanisms. In one study, the mutations were found not to affect the binding of purified Cdc13 to Est1 [13]. In another study, the mutations were shown to abolish the association of Cdc13 to Est1 in cell extracts as judged by co-IP [12]. One way to reconcile the discrepancy is to postulate the involvement of other factors in cell extracts in revealing the effects of point mutations. However, we found that the equivalent charge swap mutations in *K. lactis* Cdc13 and Est1 do in fact reduce the binding affinity between purified proteins. Remarkably, the interaction between the *K. lactis* proteins is very salt-sensitive such that the impact of mutations is only observed at moderate (~100 mM) NaCl concentrations. This finding supports the notion that Cdc13-Est1 binding is mediated at least in part through electrostatic interaction, and suggests that the discrepancy in earlier *S. cerevisiae* binding studies may be explained by different salt conditions utilized by different groups. It is worth noting that the interaction between purified *K. lactis* Cdc13 and Est1 is quite weak with an estimated K_d of between 5 and 10 μ M (Fig. 2). This affinity is significantly weaker than that obtained for purified *S. cerevisiae* Cdc13 and Est1 (K_d = ~250 nM). One potential difference is the source of proteins; the *S. cerevisiae* proteins were purified from yeast and may carry post-translational modifications that are not present on the *K. lactis* proteins obtained from *E. coli*. The *S. cerevisiae* Cdc13 is known to be phosphorylated at multiple sites, and some of the sites have been proposed to alter Cdc13-Est1 interaction [32–34].

A feature of the *K. lactis* system that is clearly different from *S. cerevisiae* is the failure to restore Cdc13-Est1 interaction by combining the charge swap mutations in the respective proteins. This result suggests that despite their functional importance and plausible correspondence to *Sc*Cdc13 E252 and *Sc*Est1 K444, the *Kl*Cdc13 E233 and *Kl*Est1 K467 residues probably do not form a direct salt bridge to promote Cdc13-Est1 interaction. Cdc13 is known to be a rapidly evolving protein, and we propose that this rapid evolution has led to significant divergence in the Cdc13-Est1 interface such that aligned residues no longer play identical functions. In support of this idea, we found that the *K. lactis* charge swap mutations neither triggered the *est* phenotype nor induced cellular senescence. In addition, the region flanking *Sc*Cdc13 E252 and *Kl*Cdc13 E233 is only moderately conserved such that multiple positions in this region exhibit little sequence similarity even among closely related budding yeasts (Fig. 2A).

Notably, in the context of the recruitment model for Cdc13-Est1 interaction, the difference between the *S. cerevisiae* and *K. lactis* systems leads to different predictions for the effects of charge swap mutations *in vivo*. As mentioned before, a purely “recruitment” model for Cdc13-Est1 interaction would predict a strong correlation between the strength of Cdc13-Est1 interaction *in vitro* and telomere maintenance *in vivo*. Since in *K. lactis*, combining the *CDC13* and *EST1* charge swap mutations failed to restore normal Cdc13-Est1 interaction, the recruitment model would predict that (in contrast to *S. cerevisiae*) the same combination of mutations should not restore telomere length, which was precisely what we observed. Thus, at least in *K. lactis*, there is no need to invoke an additional “activating” or allosteric function of Cdc13-Est1 interaction in order to account for existing data.

4.2. Recognition of diverse target sequences by Cdc13 orthologs

Another critical and conserved function of Cdc13 is high affinity binding to the telomere G-strand. Because the telomere repeat sequences in Saccharomycotina yeasts are highly variable, a long standing question has been the mechanisms by which the Cdc13 family of proteins recognize diverse G-strand sequences [35]. Previous studies show that the *S. cerevisiae* and *S. castellii* Cdc13s each recognizes a short (~8–11 nt) G-rich high affinity site, leading to the suggestion that all Cdc13s may recognize such a target [17, 18]. Indeed, a short and G-rich “core” motif can be discerned in diverse yeast telomere repeats, and this was proposed to reflect overlapping recognition by Cdc13 and Rap1 [16]. Our identification of a long and non-G-rich target site for *K. lactis* Cdc13 thus necessitates a reappraisal and suggests alternative ideas on the issue of telomere DNA recognition. First of all, the DNA-binding domain of Cdc13 seems quite capable of evolving diverse sequence recognition properties. It is thus risky to infer the DNA target site of a particular Cdc13 based on the recognition properties of other orthologs. Also surprising is that unlike other fungi, there is little overlap between the Cdc13 target site and the Rap1 target site in *K. lactis* (Fig. 1C, the Cdc13 and Rap1 targets are illustrated by the shaded region and the line below the 1.5R sequence, respectively). There is thus no absolute necessity for fungi to retain a core telomere sequence element that can be bound by both Rap1 and Cdc13. Instead, our results highlight the versatility of the DNA-binding properties of Cdc13 homologs and the ability of Cdc13 to co-evolve with the malleable telomere repeats in fungi.

4.3 The regulation of type I telomere recombination pathway in *K. lactis*

An unanticipated outcome of our analysis is the identification of Type I-like survivors in *K. lactis*. Similar to the equivalent *S. cerevisiae* survivors, the *K. lactis* Type I survivors are characterized by the amplification of a restriction fragment(s) that contains subtelomeric as well as telomeric sequences. While the genetic requirements for the *K. lactis* pathway have not been characterized before, it is likely to require Rad51, 52, 54, 55 and 57 by analogy with the *S. cerevisiae* pathway. It is notable that previous characterizations of *K. lactis ter1* (telomerase RNA) mutant revealed exclusively Type II and ALT-like survivors characterized by long and heterogeneously sized terminal repeat tracts. This evidently more common telomere recombination pathway can also be triggered by mutations in telomere sequence as well as in telomere capping proteins such as Stn1 [36]. Thus, the *K. lactis* Type I pathway appears to be under stricter negative regulation. In fact, given the detection of *K. lactis* Type I survivors so far only in *est1* cells, *EST1* itself could be a significant negative regulator. Along this thought, it is worth noting that Tong et al. have earlier shown that *S. cerevisiae* Est1 has a special role in suppressing subtelomeric Y' element recombination [37]. Further analysis of the *K. lactis* survivors could therefore provide broader insights on the regulation of subtelomeric recombination.

4.4 Conclusion

In summary, even though at the outset we used the unresolved issues in *S. cerevisiae* Cdc13 mechanisms to provide motivations and rationales for the study of *K. lactis* Cdc13, our collective findings illustrate the risk of over-extrapolating the conclusions from one budding yeast to another, given the rapid evolution of telomere machinery in this subphylum. Both in

regard to the mechanism of DNA-binding by Cdc13, and that of Cdc13-Est1 interaction, there are enough differences to preclude the use of one organism to serve as a strong paradigm for the other. The larger lessons instead, are (i) the plasticity and adaptability of DNA recognition by Cdc13 OB folds, and (ii) the conserved use of similar but not identical protein-protein interface between Cdc13 and Est1 to mediate telomerase recruitment.

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Highlights

- *K. lactis* Cdc13 recognizes an unusually long and non-G-rich telomere sequence.
- *K. lactis* Cdc13-Est1 interaction serves solely to promote telomerase recruitment.
- The Cdc13-Est1 interface is not fully conserved in budding yeast.
- *K. lactis* telomerase null mutants can yield type I survivors.

Fig 1. *K. lactis* Cdc13-telomere DNA interaction *in vivo* and *in vitro*

(A) An untagged strain and a strain harboring CDC13-FLAG₃ were subjected to chromatin immunoprecipitation using anti-FLAG resin. The input and precipitated nucleic acids were analyzed for the presence of sub-telomeric DNA (Subtel) and U1 DNA (U1 Control) by multiplex PCR.

(B) Purified *K. lactis* Cdc13-FLAG was subjected to EMSA analysis using two different G-strand probes (see Fig. 1C for the sequences of the 1.5R and 1R probes).

(C) Purified Cdc13-FLAG (12 nM) was incubated with P³²-labeled 1.5R probe (10 nM) in the presence of indicated competitor oligos (at 43, 130 and 400 nM for each oligo). Following incubation, the mixtures were analyzed by native gel electrophoresis.

(D) The sequences of the G-strand oligos used as the probes or competitors are listed. The minimal binding site (MS) as defined by a series of competition assays is highlighted with a shaded background. The relative affinity of each oligo for Cdc13 was estimated in competition assays such as those shown in 1B, and the concentration required to inhibit 50% of the complex formation between Cdc13 and the 1.5R probe is listed.

Fig 2. The interactions between wild type and mutated Cdc13 and Est1 *in vitro*

(A) Alignments of Cdc13 and Est1 orthologs near the charge swap mutation positions (designated by asterisks). Positions that are invariant or highly conserved are displayed with shaded background.

(B) Pull down assays were performed using control or Cdc13-FLAG-containing beads, and increasing concentrations of Est1 (0.9, 1.8, 3.5, 7, and 14 μM). The samples were analyzed by SDS-PAGE and Coomassie staining.

(C) Pull down assays were performed using the indicated Cdc13 and Est1 proteins. For each series of assays, four different concentrations of Est1 protein (1.8, 3.5, 7, and 14 μM) were used.

(D) The levels of Est1 and Cdc13 eluted from the FLAG beads in pull down assays were quantified as described in Experimental Procedures. The molar ratios of Est1 to Cdc13 were then calculated (after normalizing the levels against the sizes of the proteins) and plotted. Data (average ± S.D.) were derived from three independent experiments. The two open circles indicate the levels of background binding, i.e., the levels of Est1_{NTD} pulled down by the control beads.

Fig 3. Cdc13-Est1 interaction is strongly influenced by salt concentrations

(A) The pull down assays were performed using the indicated bait and prey proteins. The samples were processed as described in Experimental Procedures except that 250 mM NaCl was included in the washing buffer.

(B) Same as (A) except that the binding and washing buffer included 100 mM and 50 mM NaCl, respectively.

Fig 4. Telomere Southern analysis of *K. lactis* strains harboring various combinations of wild type and mutated *CDC13* and *EST1* alleles

(A) Chromosomal DNAs were isolated from the indicated strains after increasing numbers of passages on plates (2, 4, 6, 8, and 10 re-streaks), digested with *Eco*RI and subjected to Southern analysis of telomere restriction fragments.

(B) Extracts were prepared from the indicated strains and subjected to Western analysis to detect FLAG-tagged Cdc13.

Fig 5. Activities of the charge swap mutants other than Cdc13-Est1 interaction

(A) Purified wild type and mutant Cdc13-FLAG proteins were analyzed by SDS-PAGE and Coomassie staining.

(B) The G-strand binding activities of wild type and mutant Cdc13-FLAG were analyzed in parallel. Successively 2-fold higher concentrations of proteins (6.3, 12.5, 25, 50 and 100 nM) were used in the assays.

(C) The data in (B) were quantified and plotted.

(D) Purified Cdc13-FLAG and Cdc13-FLAG-EK were sedimented through 15–30% glycerol gradients. The level of Cdc13 in each fraction was quantified by SDS-PAGE and Coomassie staining, and plotted. Three size standards (thyroglobulin, 670 kD; aldolase, 160 kD; and BSA, 67kD) were analyzed in a parallel gradient, and their peak positions indicated at the top of the plot.

(E) Purified Est1 and the Est1-KE mutant were analyzed by SDS-PAGE and Coomassie staining.

(F) The RNA-binding activities of wild type and mutated Est1 were tested by UV crosslinking using three different concentrations of Est1 (0.2, 0.4 and 0.8 μ M).

(G) The data in (F) were quantified and plotted.

Fig 6. Type I survivors from *K. lactis est1* mutant

(A) Chromosomal DNAs were isolated from a *CDC13-FLAG est1* clone after 2, 4, 6, 8, and 10 streaks on YPD plates, digested with *EcoRI* and subjected to Southern analysis of telomere restriction fragments. A wild type sample was included as the control. Two amplified fragments (1.6 kb and 750 bp) that hybridize strongly to the telomere repeat probe are highlighted by arrowheads.

(B) Ten subclones of *CDC13-FLAG est1* were each re-streaked ten times on YPD plates. Chromosomal DNAs from each subclone was isolated and subjected to telomere Southern analysis.

(C) The chromosomal DNAs from the parental strain and three Type I survivors were digested with *EcoRI* and analyzed by agarose gel electrophoresis and ethidium bromide staining.

(D) The structure of the 1.6 kb fragment as determined by cloning and sequencing.