Genetic analysis reveals essential and non-essential amino acids within the telomeric DNA-binding interface of Cdc13p

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Cdc13p is a specific single-stranded telomeric DNA-binding protein of *Saccharomyces cerevisiae*. It is involved in protecting telomeres and regulating telomere length. The telomere-binding domain of Cdc13p is located between residues 497 and 693, and its structure has been resolved by NMR spectroscopy. A series of aromatic, hydrophobic and basic residues located at the DNA-binding surface of Cdc13p are involved in binding to telomeres. Here we applied a genetic approach to analyse the involvements of these residues in telomere binding. A series of mutants within the telomere-binding domain of Cdc13p were identified that failed to complement *cdc13* mutants *in vivo*. Among the amino acids that were isolated, the Tyr⁵²², Arg⁶³⁵, and Ile⁶³³ residues were shown to locate at the DNA-binding surface. We further demonstrated that Y522C and R635A mutants failed to bind telomeric DNA *in vitro*,

indicating that these residues are indeed required for telomere binding. We did not, however, isolate other mutant residues located at the DNA-binding surface of Cdc13p beyond these three residues. Instead, a mutant on Lys⁵⁶⁸ was isolated that did not affect the essential function of Cdc13p. The Lys⁵⁶⁸ is also located on the DNA-binding surface of Cdc13p. Thus these results suggested that other DNA-binding residues are not essential for telomere binding. In the present study, we have established a genetic test that enabled the identification of telomere-binding residues of Cdc13p *in vivo*. This type of analysis provides information on those residues that indeed contribute to telomere binding *in vivo*.

Key words: Cdc13, DNA replication, telomere binding, telomere structure, yeast two-hybrid.

INTRODUCTION

In eukaryotic cells, the end of the linear chromosomes are composed of special nucleoprotein complexes known as telomeres, which are essential for chromosome integrity by protecting the chromosome ends from fusion and nuclease degradation, and by facilitating complete chromosome replication [1]. In addition, in some organisms, such as *Saccharomyces cerevisiae*, the expression of genes near telomeres is repressed. This is a phenomenon known as the telomere position effect [2]. The telomeric DNA consists of a short-tandem-repeat duplex DNA with one strand which is rich in guanosine running 5' toward telomeres. For example, the telomeres in *S. cerevisiae* consist of 300 ± 75 bp of C₁₋₃A/TG₁₋₃ DNA [3]. In addition to duplex telomeric DNA, the G-rich strand extends to form a single-stranded G-tail at the very ends of the telomeres. The length of the telomeres is maintained by telomerase, a specialized reverse transcriptase composed of two core components, the *TLC1* RNA template and the Est2p catalytic protein subunit [4–7].

Proteins binding to the single-stranded G-tail of telomeres have been identified in several organisms. For example, in the hypotrichous ciliate *Oxytrichia nova*, the heterodimeric telomere-binding proteins specifically recognize the single-stranded DNA tail [8,9]. In *Schizosaccharomyces pombe* and in humans, the Pot1 protein can specifically bind and protect the end of telomeres [10]. In *S. cerevisiae*, the Cdc13p is a single-stranded telomeric DNA-binding protein that has multiple functions in telomere replication and protection [11–14]. These telomeric DNA-binding proteins utilize a similar structure motif, the OB (oligonucleotide/oligosaccharide binding)-fold, to bind to DNA, although they share limited sequence similarities [15].

CDC13 is an essential gene that is involved in cell cycle control. A mutant allele of *CDC13*, *cdc13-1*, causes cell-cycle arrest in the G₂/M phase at a non-permissive temperature. This cell cycle defect is caused by a failure to cap telomeres by Cdc13p [11,16]. The telomeric capping function of Cdc13p is probably mediated through its interaction with Stn1p and Ten1p [17]. Cdc13-1p fails to interact with Stn1p, yet still maintains its telomere-binding ability at non-permissive temperatures [12,18–20]. Cdc13p also affects telomere length maintenance, as several *CDC13* mutant alleles affect telomere length. For example, *cdc13-2* cells gradually lose telomere DNA and eventually cause cell death, whereas a deletion allele of *CDC13*, *cdc13-5*, extends the G-rich DNA in telomeres [13,21]. This evidence indicates that Cdc13p has a dual role in maintaining telomere length.

CDC13 encodes a protein of 924 amino acids. Domain mapping studies of Cdc13p have defined telomeric DNA-binding activity to a fragment in the range of amino acids 451–693 [18,22], and further to within amino acids 497–693 [23]. The amino acid region from 190 to 340 is involved in the recruiting of telomerase on to telomeres [24]. We have demonstrated previously [25] that the N-terminal fragment ranging from amino acids 1–251 of Cdc13p interacted with Pol1p, Imp4p, Zds2p and Sir4p and is required for telomere maintenance and cell growth. The structure of the telomeric DNA-binding domain within Cdc13p has been solved [23]. A conserved OB-fold is found in several telomere-binding proteins in other organisms [15,26]. Structural analysis revealed that DNA contact residues include five aromatic (Tyr⁵²², Tyr⁵⁵⁶, Tyr⁵⁵⁸, Trp⁵⁶⁵ and Tyr⁶²⁶), three hydrophobic (Ala⁵³⁸, Ile⁵⁷⁸ and Ile⁶³³) and five basic amino acids (Lys⁵³⁶, Lys⁵⁶⁸, Lys⁵⁷⁶, Lys⁶²⁹ and Arg⁶³⁵) [23]. Among these residues, mutation of residues Tyr⁵²², Ile⁶³³ or Arg⁶³⁵ severely affects binding activity, suggesting

Abbreviations used: 3-AT, 3-aminotriazole; EMSA, electrophoretic mobility-shift assay; 5-FOA, 5-fluoro-orotic acid; GAL4^{DBD}–Cdc13p, GAL4 DNA-binding domain fused to Cdc13p; OB, oligonucleotide/oligosaccharide binding; YC, yeast complete; YEFD, yeast extract peptone dextrose.

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that these residues are important for Cdc13p binding to telomeric DNA [27]. Mutations on other residues also affect the telomere binding activity moderately, for example, the Lys⁵⁶⁸ mutant decreased the binding affinity approx. 8-fold [27]. Although these residues were shown to make contact with telomeric DNA, the role of these residues on telomere binding *in vivo* is not clear.

Telomere binding appears to be important for its function, because the lethality of a *cdc13* null mutant can be nullified by delivering CDC13^{DBD}-Stn1p (Stn1p fused to the DNA-binding domain of Cdc13p) to telomeres. Moreover, CDC13^{DBD}-Est1p (Est1p fused to the Cdc13p DNA-binding domain) is sufficient to recruit telomerase to telomeres [24]. Utilizing this property, in the present study, we established a genetic screen to identify amino acid residues within the DNA-binding region of Cdc13p that are required for the essential functions of Cdc13p. Our genetic analysis also provided a functional test for the residues involved in telomere binding *in vivo*. Several mutations within the DNA-binding region of Cdc13p were also identified which affected the essential nature of Cdc13p to yeast cells. Among them, mutation of Tyr⁵²², Arg⁶³⁵ and Ile⁶³³ showed that these residues were located at the DNA-binding surface of Cdc13p. We did not, however, identify other mutants that failed to complement *cdc13* mutants and bind to telomeric DNA. Instead, we have isolated a mutant that did not affect the essential function of Cdc13p, although the residue is located on the DNA-binding surface of Cdc13p. These results provided functional evidence for the involvement of Tyr⁵²², Arg⁶³⁵ and Ile⁶³³ residues in telomere binding *in vivo*, and also suggested that other residues are not required for telomere binding, even though they are located at the DNA-binding surface of Cdc13p.

MATERIALS AND METHODS

Yeast strains

Yeast strain YJL503 (*MATa ura3-52 lys3-5 ade2-10 trp1-Δ63 his3-Δ200 leu2-Δ1 ade3 cdc13Δ::HIS3/pRS314-CDC13-ADE3*) strain was used to isolate *cdc13* mutants. Strain 2758-8-4b (*MATa cdc13-1 his7 leu2-3, 112 ura3-52 trp1-289*) was used in a complement assay of the *cdc13-1* mutant. Strain YJL501 (*MATa ura3-52 lys3-5 ade2-10 trp1-Δ63 his3-Δ200 leu2-Δ1 cdc13Δ::HIS3 rad52Δ::TRP1/ YEP24-CDC13*) strain was used in a complement assay of *cdc13* null mutant. STY264 cells originated from YPH499, with the chromosomal locus of *CDC13* tagged with nine Myc epitopes in its C-terminus [28]. This was used to detect Cdc13p expression of *cdc13* mutants. Y190 (*MATa ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3, 112 gal4Δ gal80Δ cyh2 LYS2::GAL1_{UAS}-HIS3_{TATA}-HIS3 URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ*) was used in the yeast two-hybrid assays.

Plasmids

Plasmid pRS315ΔB-CDC13 was also used in the construction of a loss-of-function allele of *CDC13*. To construct pRS315ΔB-CDC13, the *Apal* *CDC13* DNA-containing fragment from YEP24-CDC13 [12] was ligated into the *Apal*-digested pRS315ΔB (pRS315 with the *Bam*HI site deleted) [29]. Plasmid pAS-CDC13, which was used in the yeast two-hybrid assays has been described previously [18].

Construction of a loss-of-function allele of *CDC13*

PCR mutagenesis was used to generate random mutations in the telomeric DNA-binding region of Cdc13p. Two oligonucleo-

tides, 5'-GCCATGGCTAGTGATGGCTCAGTT-3' and 5'-ACG-CGTCGACTGACGCTGTAAGTAGGC-3', complementary with sequences 291 bp upstream and 381 bp downstream of the DNA-binding region respectively, were used to amplify *CDC13* mutant fragments. The PCR was performed using Taq DNA polymerase in the presence of 0.1 mM dITP and other four dNTPs, 0.4 mM dGTP, 0.4 mM dCTP, 0.4 mM dTTP and 0.3 mM dATP, for 25 cycles. The PCR products were purified by agarose gelelution, and transformed, together with pRS315ΔB-CDC13 linearized with *Bam*HI and *Nru*I, into YJL503 yeast cells. Cells were plated on to medium lacking leucine and incubated at 30°C until colonies formed. Approx. 6000 transformants were screened. Red-coloured colonies were selected and plasmids were recovered from these cells. The 729 bp *Bam*HI and *Nru*I digested fragments from these plasmids were subcloned into wild-type plasmid pRS315ΔB-CDC13 to replace the wild-type fragment. The resulting plasmids were transformed into YJL503 to confirm the phenotypes. The mutation sites were then determined by DNA sequencing.

Complementation assay

Two types of complementation assays were conducted to evaluate the function of *cdc13* mutants, *cdc13* null and *cdc13-1*. First, a plasmid-loss assay was used to test if the *cdc13* mutants were capable of complementing the lack of cell viability caused by the *cdc13Δ* mutant. The *cdc13* mutants in pRS315ΔB-CDC13 were introduced into yeast YJL501 *rad52* (*cdc13Δ::HIS3 rad52Δ::TRP1/YEP24-CDC13*) cells. The resulting cells were spotted in 10-fold serial dilutions on to plates containing 5-FOA (5-fluoro-orotic acid) and incubated at 30°C until colonies formed. Secondly, yeast 2758-8-4b (*cdc13-1*) cells (provided by Dr L. Hartwell, Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A.) were used to test the complementation of the temperature sensitivity of the *cdc13-1* allele. Plasmids pRS315ΔB, pRS315ΔB-CDC13, or pRS315ΔB-*cdc13* carrying different mutants of *CDC13* were introduced into 2758-8-4b cells and the resulting transformants were spotted in 10-fold serial dilutions on to YC (yeast complete) – Leu (lacking leucine) agar plates and grown at 25°C, 30°C or 37°C until colonies formed.

Expression and purification of His₆-tagged Cdc13(451–693)p

The *Escherichia coli* expression system was used for Cdc13(451–693)p expression. Plasmid pET6H-cdc13(451–693) carrying different mutations of *CDC13* were constructed by ligating the 0.73 kbp *Bam*HI–*Nru*I fragment of pRS315ΔB-CDC13 with pET6H which was linearized with the same enzymes. *E. coli* BL21(DE3) pLysS cells were used as hosts for Cdc13(451–693)p expression. The purification procedure was as described previously [22].

EMSA (electrophoretic mobility-shift assay)

Oligonucleotide TG15 (5'-TGTGTGGGTGTGGTG-3') was labelled with [γ -³²P]ATP (3000 mCi/mM; New England Nuclear) using T4 polynucleotide kinase (New England Biolabs) and subsequently purified from a 10% sequencing gel following electrophoresis. This assay was performed in buffer A (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl and 1 mM dithiothreitol). Wild-type or mutant Cdc13(451–693)p was mixed with 5 nM of ³²P-labelled TG15 DNA in a total reaction volume of 15 μ l. The reactions were carried out at room temperature (25°C) for 10 min and the reaction products were analysed by 8% gel

Figure 1 Genetic screening strategy and loss of sectoring phenotypes of *cdc13* mutants

(A) The genetic screening strategy for telomere-binding mutants of *CDC13*. The mutated PCR products were co-transformed with linearized pRS315 Δ B-*CDC13* [where its *CDC13*-BD (binding domain) was deleted by BamHI and NruI digestion] into yeast strain YJL503 (*ade2 ade3 cdc13 Δ ::HIS3*/ pRS314-*CDC13*-*ADE3*). Homologous recombination (marked by X) between the two transformed DNA molecules generates a plasmid harbouring the *LEU2* gene and mutated *CDC13* (*). Screening of mutations that affect *CDC13* function is based on the presence of plasmid pRS314-*CDC13*-*ADE3*. Yeast cells carrying wild-type *CDC13* on pRS315 Δ B-*CDC13* do not require the existence of pRS314-*CDC13*-*ADE3* for viability. These cells form sectorial colonies, whereas mutations that affect the essential function of *CDC13* means that the cells maintain pRS314-*CDC13*-*ADE3* and form red colonies. (B) The sectoring phenotype of *cdc13* mutants on YEPD (yeast extract peptone dextrose) plates. Yeast strain YJL503 harbouring plasmid pRS315 Δ B (vector), pRS315 Δ B-*CDC13* wild-type (wt), or pRS315 Δ B-*cdc13* that were carrying mutations in the telomere-binding region of *CDC13* were plated on YEPD agar plates and incubated at 30°C until colonies formed.

electrophoresis in TBE (89 mM Tris/borate and 2 mM EDTA) at 125 V for approx. 100 min. The gels were dried and subjected to autoradiography.

Yeast two-hybrid assay

Plasmids pACT2 and pACT-*STN1* [18] were separately transformed into yeast strain Y190. The resulting strains were then transformed with pAS2-1, pAS-*CDC13* or pAS-*cdc13* carrying mutations in the telomeric DNA binding region. The *HIS3* reporter gene was used to evaluate the interaction between each mutant Cdc13p and Stn1p. In this assay, freshly transformed colonies from each transformation were spotted in 10-fold serial dilution onto YC plates lacking tryptophan and leucine with or without 3-AT (3-aminotriazole). Plates were incubated at 30°C until colonies formed.

RESULTS

Establishing a genetic screening scheme for the identification of *CDC13* mutants within the telomere-binding region

Cdc13p is an essential protein for *S. cerevisiae*. It is a multiple function protein that can act through binding to telomeric DNA and/or recruiting other telomere-associated proteins, such as

Stn1p, Ten1p, Est1p, Pol1, Imp4p, Sir4p or Zds2p to telomeres [25]. To identify amino acids that are required for telomere binding, we designed a genetic screening strategy to find mutants that had lost their essential functions. Since we were interested in testing the essential function of telomere-binding activity, the mutations were generated within the region ranging from amino acids 451 to 693 of Cdc13p [18,22]. As shown in Figure 1(A), the *cdc13* mutants were isolated using PCR mutagenesis combined with the gap-repair method [30]. The mutated PCR products were mixed with a linearized pRS315 Δ B-*CDC13* plasmid with the telomeric DNA-binding region of *CDC13* being deleted and co-transformed into yeast cells. Recombination between the PCR products and the linearized pRS315 Δ B-*CDC13* plasmid fragment within the yeast cells would generate pRS315 Δ B-*CDC13* plasmids carrying mutations in the telomere-binding region of *CDC13*. Yeast strain, YJL503 (*ade2 ade3 cdc13 Δ ::HIS3*), carrying pRS314-*CDC13*-*ADE3* plasmid was used in the present study. The plasmid-borne *CDC13* is required to maintain the viability of host cells, whereas the *ADE3* in plasmids rendered a red coloration to the colonies. Yeast cells would keep the pRS314-*CDC13*-*ADE3* plasmid to maintain their viability if the *cdc13* mutant in pRS315 Δ B-*CDC13* plasmids affected its essential function. On the contrary, the pRS314-*CDC13*-*ADE3* plasmid would gradually be lost during cell divisions if the *cdc13* mutant did not affect its essential function. The presence of pRS314-*CDC13*-*ADE3* plasmid within

Table 1 Summary of *cdc13* mutants

Mutations	Complement <i>cdc13Δ</i>		Complement <i>cdc13-1</i> §	Protein expression¶	Single-strand TG ₁₋₃ binding <i>K_d</i> (app) (nM)	Interaction with Stn1p⊥
	Red/Sector†	5-FOA‡				
Wild-type	S	Y	Y	+	79 ± 9	+
Y522C*	R	N	N	+	> 363	+
I552F	R	N	N	±	Aggregate	+
S611P	R	N	N	—	Aggregate	—
R635C*	R	N	N	+	> 363	+
T458P/D505G	R	N	N	+	Aggregate	+
I508T/L513S	R	N	N	—	Low expression	—
Y522C*/K622N	R	N	N	+	Aggregate	+
N620D/F660S	R	N	N	—	Aggregate	—
K624R/I633T*	R	N	N	+	Aggregate	+
N572S/K581E	R	Y/N	Y/N	+	253 ± 49	+
K568M*	S	Y	Y	+	Low expression	ND

* Amino acids that made contact with single-stranded telomeric DNA from NMR analysis [23].

† *ADE2*–*ADE3*-based reporter system for plasmid-loss assay (as shown in Figure 1A). R, solid red colonies; S, sector colonies.

‡ *URA3*-based reporter system for plasmid-loss assay. Y, grew on 5-FOA; N, no growth on 5-FOA; Y/N, a fraction of the cells grew on 5-FOA.

§ Complementation tests using *cdc13-1* cells. Y, complementation at 37 °C; N, no growth at 37 °C.

¶ The level of mutant protein expression in yeast strain STY264 (YPH499 *CDC13-9Myc*) was determined by Western blotting using polyclonal antibodies against Cdc13p. +, Mutant protein overexpression compared with the wild-type; ±, decreased expression relative to the wild-type; —, no expression.

|| The apparent telomeric DNA-binding constants of these mutants were determined. Aggregate, the recombinant protein formed aggregated inclusion bodies in *E. coli*.

⊥ Interaction with Stn1p was judged by yeast two-hybrid assays (Figure 4). ND, not determined.

cells could be easily assayed by the colour of the colonies. Gradual loss of pRS314-*CDC13-ADE3* in YJL503 cells would lead to the formation of sector colonies.

Isolation of *cdc13* mutants that lose *CDC13* essential functions

Approx. 6000 colonies were screened, of which 50 red coloured colonies were selected (Figure 1B). Several sector colonies were also randomly selected as controls. After confirming the coloration phenotype of these mutants, the sequences of the mutants were determined. Among them, 32 of the mutations fell within the telomere-binding region of Cdc13p. Sequence analysis of these mutated sites indicated that several of the mutations were isolated multiple times. Thus we did not attempt to isolate more mutants. Among these mutations, four were single-point mutants and six were double-point mutants. A summary of these mutations is listed in Table 1. Among these mutations, the Tyr⁵²², Arg⁶³⁵ and Ile⁶³³ residues were shown to locate in the DNA–protein interface of Cdc13p [23].

To demonstrate that the complementation is not limited to the *ADE2*–*ADE3* reporter system, the analysis was also tested using *URA3* as a reporter. The yeast strain we used was *cdc13Δ::HIS3*, *rad52Δ::TRP1*, YJL501, which required the YEP24-*CDC13* plasmid (2 μ, *URA3* marker) for viability. Here the presence of YEP24-*CDC13* could be monitored by growing the cells on agar plates containing 1 mg/ml 5-FOA. As shown in Figure 2(A), 5-FOA resistant cells could be observed in YJL501 cells when transformed with a plasmid expressing wild-type Cdc13p. However, with the exception of the N572S/K581E mutant, transforming YJL501 cells with vector pRS315ΔB or pRS315ΔB-*CDC13* plasmids expressing *cdc13* mutants did not yield any 5-FOA resistant cells. The N572S/K581E mutant appeared to rescue the *cdc13Δ* defect with an efficiency approx. 10-fold less than that of the wild-type Cdc13p. It is also apparent that the *URA3*-reporter system is a more sensitive system, which can discriminate between different levels of complementation. Complementation analysis was also conducted in *cdc13-1* cells to determine the allelic specificity of these mutants. Consistent with the observations in *cdc13Δ* cells, most of the mutants could not complement the *cdc13-1* at 30 °C and 37 °C (Figure 2B). Mutant N572S/K581E

complemented the *cdc13-1* with approx 10- to 100-fold less efficiency than that of the wild-type Cdc13p.

Since loss of *CDC13* essential functions in *cdc13* mutants might have been due to abnormal Cdc13p expression, the expression of the Cdc13p mutants was determined. These experiments were conducted in yeast strain STY264 (YPH499 *CDC13-9Myc*) in which the endogenous Cdc13p was tagged with nine Myc epitopes to its C-terminus. The level of expression of the Cdc13p mutants was determined by transforming plasmids carrying *CDC13* mutations into STY264 cells and analysing them by Western blotting using polyclonal antibodies against Cdc13(1–924)p. As shown in Figure 2(C), migration of Myc-tagged Cdc13p was significantly different from Cdc13p mutants, and their levels of expression could be easily detected. The expression of R635C, Y522C, Y522C/K622N, K624R/I633T, T458P/D505G, N572S/K581E and K568M mutant proteins did not appear to be different from that of wild-type Cdc13p. The I552F mutation had a reduced expression level, whereas S611P, N620D/F660S and I508T/L513S mutations resulted in almost no Cdc13p expression. Thus loss of essential function in S611P, N620D/F660S and I508T/L513S mutants is probably due to failed expression of these mutant proteins, whereas the intermediate phenotype presented by the I552F mutation might be due to the reduced level of protein expression.

Failure to bind telomeric DNA by R635C, Y522C and N572S/K581E mutants

To evaluate the telomere-binding property of these mutants, purified recombinant mutant proteins were obtained and analysed by EMSA. The DNA fragments containing the *CDC13* mutations were subcloned into an *E. coli* expression vector and the recombinant proteins were purified. Of these mutants, we were able to obtain soluble recombinant proteins for the R635C, Y522C and N572S/K581E mutants (Figure 3A). The rest of the mutants caused either a low level of expression of proteins or protein was in an aggregated form that could not be analysed further (Table 1). The telomeric DNA-binding activities of these three mutant proteins were determined (Figure 3B and Table 1). Mutations of residues Arg⁶³⁵ and Tyr⁵²² caused severe loss of telomere-binding

Figure 2 Identification of *cdc13* mutants that lost essential functions

(A) Complementation of a *cdc13* null mutant by *cdc13* mutants. Yeast strain YJL501(*cdc13Δ::HIS3 rad52Δ::TRP1/YEP24-CDC13*) carrying plasmids pRS315ΔB (vector), pRS315ΔB-*CDC13* (wt), or different mutations of *CDC13* in plasmid pRS315ΔB-*cdc13* were spotted in 10-fold serial dilutions on YC -Leu, YC -Ura, or YC with the addition of 5-FOA and incubated at 30°C until colonies formed. (B) Complementation of the *cdc13-1* temperature-sensitive mutant by *cdc13* mutants. Yeast 2758-8-4b (*cdc13-1*) carrying plasmids pRS315ΔB, pRS315ΔB-*CDC13* or pRS315ΔB-*cdc13* mutants were spotted in 10-fold serial dilutions on YC -Leu and grown at 25°C, 30°C or 37°C until colonies formed. (C) Expression of *cdc13* mutants in yeast cells. Total cell-free extracts (50 μg) from STY264 (*CDC13-9Myc*) cells carrying plasmids pRS315ΔB, pRS315ΔB-*CDC13* or pRS315ΔB-*cdc13* mutants were resolved by SDS/PAGE (6% gels) and subjected to Western blotting. Polyclonal antibodies against full-length Cdc13p were used to detect Cdc13p wild-type and mutant proteins.

activities and their binding constants could not be determined precisely under our assay conditions ($K_d > 363$ nM). Loss of telomeric DNA-binding activity of these two mutants did not appear to be caused by global structural alteration of the mutant proteins, because they were soluble, expressed at the same level as the wild-type protein and showed similar chromatographic activity (results not shown). Thus mutations of these two residues in Cdc13p caused a severe defect in telomere-binding activity and loss of essential function *in vivo*. The double mutants N572S/K581E showed a 3-fold reduction in their binding affinity ($K_d = 253 \pm 49$ nM). Reduction of telomeric DNA-binding activity in

Figure 3 R635C and Y522C mutations of *CDC13* fail to bind single-stranded TG₁₋₃ DNA *in vitro*

(A) Purification of wild-type and mutant His₆-tagged Cdc13(451-693)p proteins. The wild-type (lane 2) and mutant proteins (lanes 3-5) were expressed as His₆-tagged Cdc13(451-693)p in *E. coli* and purified on nickel-affinity resins. Purified proteins (2 μg) were analysed by SDS/PAGE (12% gel), and a Coomassie Blue-stained gel is shown. (B) The single-stranded TG₁₋₃ DNA-binding activity of Cdc13(451-693)p and its mutants. ³²P-labelled TG15 (5 nM) was mixed with several concentrations of the purified wild-type Cdc13(451-693)p and mutant proteins. EMSA was then performed. The protein concentrations used in each set of experiments were 33, 100 and 300 nM. A representative autoradiogram is shown.

the N572S/K581E mutant might contribute to its decreased level of complementation function *in vivo*.

Interaction with Stn1p is affected in some Cdc13 DNA-binding domain mutants

Cdc13p is shown to interact with Stn1p and the interaction is required for the essential functions of Cdc13p [18,31]. The region responsible for interacting with Stn1p was loosely mapped to amino acids 252-924 of Cdc13p using yeast two-hybrid assays [18]. Since the telomere-binding region of Cdc13p overlaps with the Stn1p-interaction region, it is possible that the mutants we isolated were also unable to interact with Stn1p. To test this possibility, *CDC13* fragments containing the mutations were subcloned into the pAS2-1 plasmid so that these *cdc13* mutants were fused to the DNA-binding domain of *GAL4* (*GAL4^{DBD}*-Cdc13p). The plasmids were transformed into the yeast strain Y190 carrying a plasmid where *STN1* was fused to the activation domain of *GAL4* (pACT-*STN1*). The ability to grow on an agar plate lacking

histidine in the medium was used to evaluate the interaction between *STN1* and the Cdc13 mutants. The compound 3-AT was added to the selection medium to block basal *HIS3* gene expression. As shown in Figure 4(A), His⁺ colonies could be observed in cells harbouring pACT-*STN1* and pAS-*CDC13*. Similarly, His⁺ colonies grew to the wild-type level in several *cdc13* mutants including Y522C, I552F, R635C, Y522C/K622N, K624R/I633T, T458P/D505G and N572S/K581E, indicating that the presence of mutations on these residues of Cdc13p did not affect its interaction with Stn1p. The S611P, N620D/F660S and I508T/L513S mutants appeared to reduce their interaction with Stn1p, as the His⁺ colonies were reduced approx. 100-fold, suggesting that at least a portion of the defects in these mutants were the result of their reduced interaction with Stn1p. Interestingly, even though the expression level of these GAL4^{DBD} Cdc13p mutants were similar to the wild-type protein (Figure 4B), mutants that failed to interact with Stn1p were not expressed as well as the non-fusion forms (Figure 2 and Table 1). These results suggested that alteration of protein structures also contributes to the loss of Stn1p interactions in these mutants. Clearly our results indicate that the loss of essential function in the Y522C, I552F, R635C, Y522C/K622N, K624R/I633T, T458P/D505G and

The region responsible for the telomeric DNA-binding of Cdc13p *in vivo* and *in vitro* has been mapped to amino acids 497–693 [18,20,22,23,33]. Structural analysis indicated that a series of basic, hydrophobic and aromatic amino acids are responsible for contacts with single-stranded telomeric DNA [23]. A site-directed mutagenesis analysis of these contact residues indicated that mutations of residues Tyr⁵²², Ile⁶³³ and Arg⁶³⁵ had a deleterious effect on the binding affinity of Cdc13p *in vitro* [27]. These three residues were clustered in the same region of the binding domain. Consistent with the structure and mutagenesis results, our genetic screening strategy also identified that the mutations including Y522C or R635C caused a loss of Cdc13p's essential function and telomere-binding activities. Our result for Ile⁶³³ is less certain, because the only relevant mutant we identified was K624R/I633T. However, since the Lys⁶²⁴ is a conserved replacement of the arginine residue, this alteration might not have made a major contribution to the function of Cdc13p. It was to our surprise that mutations were not identified on other telomeric DNA-contacting residues that affect the essential function of *CDC13* beyond Tyr⁵²², Arg⁶³⁵ and Ile⁶³³. Instead, we have also identified a mutant K568M

that did not affect the apparent function of Cdc13p. This residue was shown to make contact with single-stranded telomeric DNA in NMR analysis and was shown to reduce its telomeric DNA-binding affinity in mutants using *in vitro* DNA-binding analysis. Thus even though there are a total of 13 amino acids residues within Cdc13p that make contact with telomeric DNA, only a limited number of residues are important for forming stable complexes with telomeric DNA *in vitro* [27] and performing its function *in vivo*. Our *in vivo* genetic assays thus provide a functional test for telomeric DNA-binding residues of Cdc13p. Our analysis also provides a definite evaluation of whether a DNA-binding residue is indeed essential for the function of Cdc13p on telomeres *in vivo*.

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