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Sua5p a single-stranded telomeric DNA-binding protein facilitates telomere replication

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In budding yeast *Saccharomyces cerevisiae,* telomere length maintenance involves a complicated network as more than 280 telomere maintenance genes have been identified in the nonessential gene deletion mutant set. As a supplement, we identified additional 29 telomere maintenance genes, which were previously taken as essential genes. In this study, we report a novel function of Sua5p in telomere replication. Epistasis analysis and telomere sequencing show that *sua5Δ* cells display progressively shortened telomeres at early passages, and Sua5 functions downstream telomerase recruitment. Further, biochemical, structural and genetic studies show that Sua5p specifically binds single-stranded telomeric (ssTG) DNA *in vitro* through a distinct DNA-binding region on its surface, and the DNA-binding ability is essential for its telomere function. Thus, Sua5p represents a novel ssTG DNA-binding protein and positively regulates the telomere length *in vivo*.

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Introduction

Telomeres are highly organized DNA–protein structures at the ends of linear eukaryotic chromosomes (McEachern *et al*, 2000). They not only help to distinguish chromosome ends from DNA double-strands breaks, but also provide a way to replicate chromosomes completely. The telomeric DNA consists of simple repetitive sequence with the G-rich strand extending in the 3′ direction to form a single-stranded tail (Vega *et al*, 2003) and is bound and protected by specific proteins (Smogorzewska and de Lange, 2004). The single-stranded telomeric (ssTG) G-rich DNA-binding proteins, including *Oxytricha nova* TEBPα/β, mammalian Pot1 and TPP1, fission yeast Pot1, budding yeast Cdc13p and Stn1p (Horvath *et al*, 1998; Baumann and Cech, 2001; Lei *et al*, 2003; Mitton-Fry *et al*, 2004; Gao *et al*, 2007; Wang *et al*, 2007), contain an OB (oligonucleotide/oligosaccharide binding)-fold, which is responsible for ssTG binding (Theobald *et al*, 2003). In addition to telomere protection (Nugent *et al*, 1996; Gao *et al*, 2007), the OB-fold ssTG-binding proteins also participate in telomerase recruitment (Evans and Lundblad, 1999) and/or activity regulation (Wang *et al*, 2007).

Telomere is elongated by the specialized reverse transcriptase telomerase (Blackburn, 1992). In budding yeast *Saccharomyces cerevisiae*, telomeres in each chromosome have approximately 350 bp heterogeneous $TG_{1-3}/C_{1-3}A$ sequence, and telomerase is composed of at least four subunits, Est1p, Est2p, Est3p and *TLC1* RNA (Lundblad and Szostak, 1989; Singer and Gottschling, 1994; Lendvay *et al*, 1996). Est2p, the catalytic subunit of telomerase (Counter *et al*, 1997), and *TLC1*, the RNA subunit (Singer and Gottschling, 1994), are required for the elongation activity of oligo primer *in vitro*, and comprise the catalytic core of telomerase (Counter *et al*, 1997; Lingner *et al*, 1997). Est1p interacts with Cdc13p to recruit and activate telomerase (Evans and Lundblad, 1999; Taggart *et al*, 2002). Est3p is an OB-fold protein (Lee *et al*, 2008; Young Yu *et al*, 2008) and its molecular function is not clear. Telomerase-dependent telomere elongation is mainly achieved through effective telomerase recruitment and activation (Smogorzewska and de Lange, 2004). Telomerase is recruited to telomere through Ku80p–*TLC1* RNA interaction in G1 phase (Peterson *et al*, 2001; Stellwagen *et al*, 2003; Fisher *et al*, 2004), and synergistic action of ssDNA-binding proteins Cdc13p and RPA in late S phase (Evans and Lundblad, 1999; Taggart *et al*, 2002; Schramke *et al*, 2004). Telomere addition is inhibited by Pif1p, a 5′–3′ DNA helicase, which disassociates telomerase from telomeric DNA by unwinding RNA–DNA substrates (Zhou *et al*, 2000; Boule *et al*, 2005). Telomerase preferentially binds shorter telomeres to promote their elongation in a Tel1-dependent manner *in vivo* (Bianchi and Shore, 2007; Chang *et al*, 2007; Hector *et al*, 2007; Sabourin *et al*, 2007). Deregulation of telomerase protein/RNA levels or assembling will affect telomere homeostasis in an indirect manner. For example, deficiency of nonsense mRNA decay (NMD) pathway, which controls mRNA levels of *EST1*, *EST2* and other telomere regulators, shortens telomere lengths (Dahlseid *et al*, 2003).

The genome-wide screenings in the nonessential gene deletion set have revealed a telomere maintenance network of about 280 nonessential genes (Askree *et al*, 2004; Gatbonton *et al*, 2006). The telomere maintenance genes are very diverse in their functions, including DNA and RNA metabolism, chromatin modification and vacuolar traffic-related genes (Askree *et al*, 2004; Gatbonton *et al*, 2006). In a genome-wide characterization of essential genes, we unexpectedly identified additional 64 nonessential genes, which were previously considered as essential genes in genome-wide deletion project (Giaever *et al*, 2002). As a supplement, we examined the telomere lengths of these mutants. Here, we

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document the screening result and describe a new telomere maintenance gene, *SUA5*. *SUA5* (suppressor of upstream ATG) was first identified in a genetic screening of suppressor for translation initiation defect in the leader region of the *CYC1* gene (Na *et al*, 1992). Eight SUA genes were identified in the screening: *SUA7* and *SUA8* encode homologs of transcription factor TFIIB and RNA polymerase II largest subunit B220, respectively, which are required for transcription start site selection (Pinto *et al*, 1992, 1994). *SUA1* and *SUA6*, which latterly were renamed as *NMD2* and *UPF3*, are important factors in NMD pathway (Cui *et al*, 1995). *SUA5* encodes a 46-kDa protein, which has a conserved yrdC domain at the N terminus and a SUA5 domain at the C terminus, and its biological function is still unclear.

Our genetic, biochemical and structural analysis revealed that Sua5p is a conserved non-OB-fold ssTG DNA-binding protein. It positively regulates telomere elongation through affecting telomerase activity, and this positive regulatory function requires its DNA-binding ability.

Results

Deletion of SUA5 causes telomere progressively shortening

In a genome-wide characterization of essential genes in *S. cerevisiae* (FL Meng and JQ Zhou, in preparation), we found that 64 nonessential genes were mis-classified as essential genes in the *Saccharomyces* genome deletion project (Giaever *et al*, 2002) (representative tetrad dissection result is shown in Supplementary Figure 1). As a supplement to the screenings in nonessential gene deletion set, we examined the telomere lengths, and identified 29 additional telomere maintenance genes (Supplementary Table II). The mutant cells were streaked on YPD plate for five times after sporulation. Interestingly, three deletion mutants exhibited progressively shortened telomeres during passages (Figure 1A): *MTR10* has been reported to be required for the normal accumulation of mature *TLC1* RNA and its proper nuclear localization (Ferrezuelo *et al*, 2002); *KAE1* encodes a putative peptidase, which is one component of telomere regulator KEOPS complex (Downey *et al*, 2006); *SUA5* has not been reported to affect telomeres.

The progressively shortened telomere phenotype is reminiscent of the ever shorter telomere (EST) phenotype in telomerase-deficient cells (Lundblad and Szostak, 1989). The *sua5*Δ cells were continuously passaged for approximately 400 generations after sporulation. Telomeres were progressively shortened during early passages (approximately 150 generations after sporulation), and then kept at a short-and-stable state in the successive passages (Figure 1B, *Pst*I digested). The short telomeres could be gradually restored to wild-type length when *SUA5* was re-introduced into *sua5*Δ cells (Figure 1C), indicating that the short telomeres resulted from the *SUA5* deletion rather than other mutations. *SUA5* deletion in YPH499 background displayed a similar progressively shortened telomere phenotype (data not shown).

To investigate how Sua5 affects telomere replication, we examined the genetic interaction of Sua5 with some well-characterized telomere regulators. *SUA5* deletion further shortened telomeres in *yku70*Δ, *yku80*Δ, *rad50*Δ, *rad9*Δ, *dot1*Δ and *tel1*Δ cells (Figure 1D), suggesting that Sua5p plays an independent positive regulatory role in telomere length control. *SUA5* deletion cells express low levels of *CYC1*, and harbour dysfunctional mitochondria, that is, respiratory incompetent (ρ^-) (Na *et al*, 1992; Supplementary Figure 2A). However, telomere length did not show significant change in *cyc1*Δ cells (Supplementary Figure 2B; Askree *et al*, 2004; Gatbonton *et al*, 2006), and loss of mitochondrial DNA did not affect telomere length (Supplementary Figure 2B). In addition, consistent with the earlier report (Cui *et al*, 1995), *SUA5* is not involved in NMD pathway (Supplementary Figure 2C), and *SUA5* deletion further shortened telomeres of *nmd2*Δ and *upf3*Δ cells (Figure 1D). These results indicated that telomere shortening in *sua5*Δ is not caused by either the dysfunctional mitochondria or the defective NMD pathway.

Telomeric DNA in sua5Δ cells is not efficiently elongated

Telomere length is maintained by a dynamic process of lengthening and shortening. The balance between DNA loss and gain ensures telomeres with a constant length, and also defines an average point of divergence (APD) as yeast telomerase adds new variable sequences to telomere ends (Forstemann *et al*, 2000). In the proximal region of the divergence point telomeres have a stable sequence, whereas the distal telomere sequences are divergent (Forstemann *et al*, 2000). To find out the defect(s) of telomere replication caused by Sua5 deletion, Telomere I-L (TEL01L) was cloned and sequenced by Telomere PCR (Forstemann *et al*, 2000; Lee *et al*, 2007). The PCR-amplified TEL01L showed a decreased size during the early passages of *sua5*Δ cells (Figure 2A; Supplementary Figure 3A), which is consistent with the overall telomere length changes shown by telomere blot (Figure 1B). The APD in wild type and the first-restreaked *sua5*Δ cells was 161 nucleotides (nt) and 194 nt into the telomeric tract, respectively (Figure 2B), indicating that the depletion of Sua5p has not enhanced the nucleolytic degradation of telomeric DNA. The temperature sensitivity and quantification of telomeric single-stranded DNA also suggested that Sua5p is not involved in telomere end protection (Supplementary Figure 4A and B). Additionally, depletion of exonuclease Exo1p has no effect on the telomere length of *sua5*Δ cells (Supplementary Figure 4C). Therefore, Sua5p is not a telomere capping factor.

The average length of divergence (ALD) sequences added by telomerase is 101 nt in wild-type cells, whereas the ALDs decreased to 16 and 27 nt in the first- and fifth-restreaked *sua5*Δ cells, respectively, indicating that telomerase activity is greatly reduced in *sua5*Δ cells. To analyse the relationship of telomere elongation and original length in *sua5*Δ cells, we counted the average nucleotide addition length per telomere for the telomeres that are longer and shorter than 170 nt, respectively. Telomeres that are shorter than 100 nt were excluded from the analysis, as the reported high recombination rate for short telomeres at chromosome I-L (Teixeira *et al*, 2004). The average lengths of nucleotides added at the longer telomeres in sua5Δ cells are significantly decreased when the telomere addition length as function of telomere size was plotted (Figure 2C and D). The sequencing result showed that many long telomeres are not elongated. Ten telomeres with close length of nondivergent sequence were grouped into subgroups, and the frequencies of telomere extension as function of telomere size was plotted

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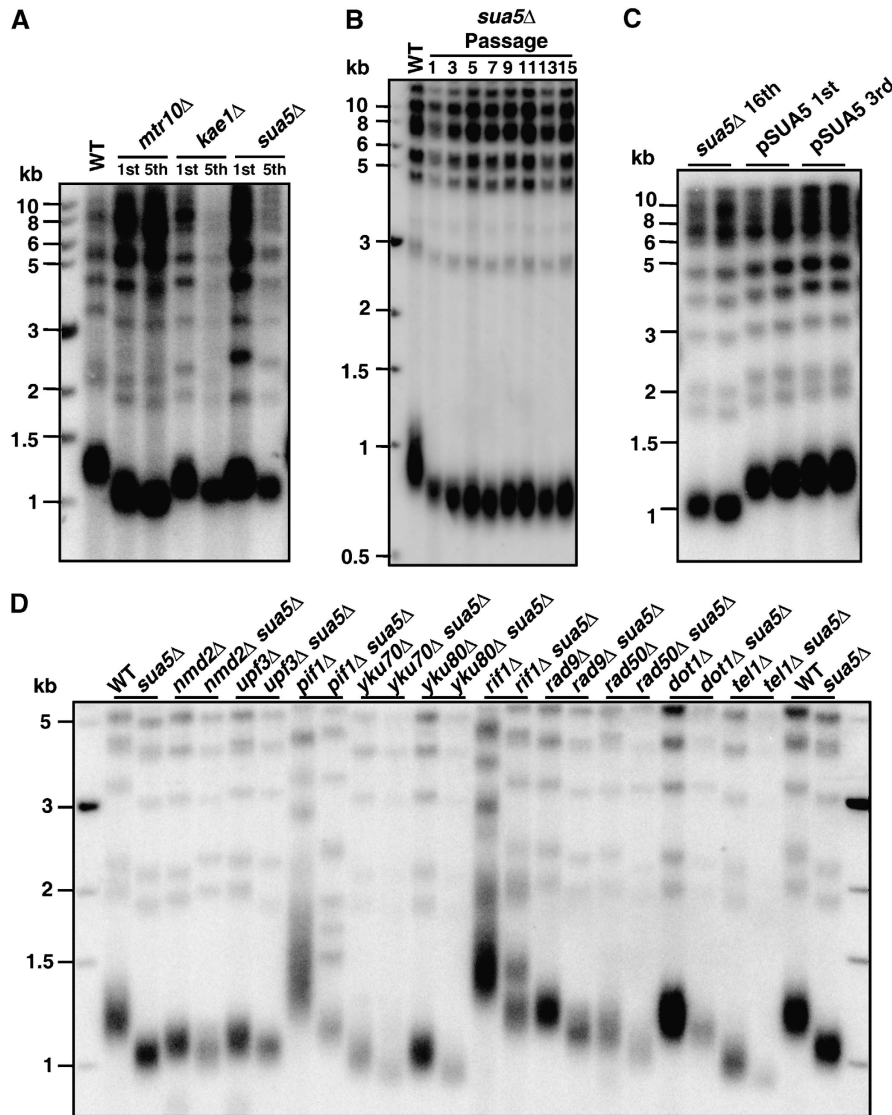

Figure 1 *SUA5* deletion causes telomere shortening. (**A**) Telomere blot shows telomere shortening in early passages (within 165 generations after sporulation) of the *MTR10*, *KAE1* and *SUA5* deletion strains. The '1st' and '5th' indicate the first- and fifth-restreaked cells, respectively. The genomic DNA was digested with *Xho*I, and hybridized with a telomeric TG probe. (**B**) The *sua5*Δ cells were restreaked on YPD plate for 15 times (about 400 generations after sporulation), and the restreaked times are labelled on the top of the panel. The genomic DNA was digested with *Pst*I. *Pst*I and *Xho*I digestions (panel A) of the wild-type yeast genomic DNA result in approximately 0.8 and approximately 1.3 kb terminal telomere signals, respectively. *Pst*I digestion reveals subtle telomere length changes. (**C**) A *CEN* plasmid-borne *SUA5* progressively restores the telomere lengths in *sua5*Δ cells. The numbers indicate the restreaked times. (**D**) Telomere blot analysis of the genetic interactions of *SUA5* and some well-characterized telomere maintenance genes. The isogenic strains are labelled on the top of the panel. The cells were restreaked on YPD plate for three times after sporulation.

(Figure 2E). The frequency of elongation in wild-type cells did not correlate significantly with telomere size after several cell-cycles replication (Figure 2E). This result is different from the telomere addition in one cell cycle revealed by the STEX method (Teixeira *et al*, 2004). In the first-restreaked *sua5*Δ cells, the elongation frequency negatively correlated with the telomere length (Figure 2E), and 93 % of the telomeres that are shorter than 125 nt were elongated (Figure 2B), but only 23 % of the telomeres that are longer than 150 nt were elongated (Figure 2B). Taken together, these data indicated telomerase activity is affected in *sua5*Δ cells and limited telomerase activity is allocated to the shorter telomeres (Chang *et al*, 2007), and longer telomeres are less frequently elongated.

Sua5 functions in telomerase pathway

As Sua5p appears not to be involved in telomere capping, it is possible that Sua5p functions in the telomerase pathway. We then examined the telomere length in the continuously passaged *est1*Δ and *est1*Δ*sua5*Δ cells. The telomere shortening rate of *sua5*Δ*est1*Δ was similar to that of *est1*Δ cells (Figure 3A). The result showed that deletion of *SUA5* does not accelerate the telomere shortening caused by *EST1* deletion, and *SUA5* regulates telomere replication through the telomerase pathway.

To further test whether Sua5p is required for the *in vivo* telomerase activity, we used the gross chromosomal rearrangement (GCR) assay (Myung *et al*, 2001) to measure the rate of telomere healing when telomerase acts on DNA

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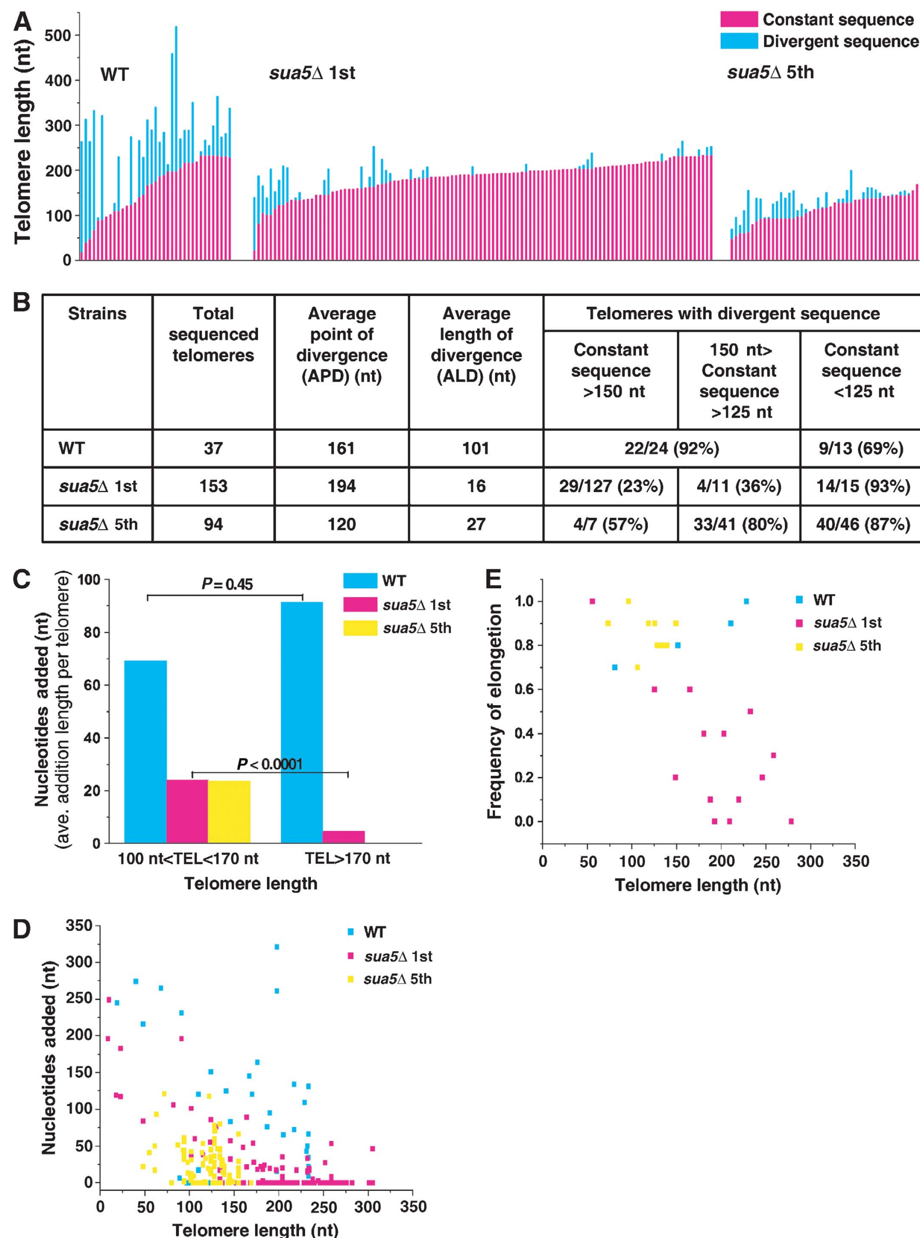

Strains	Total sequenced telomeres	Average point of divergence (APD) (nt)	Average length of divergence (ALD) (nt)	Telomeres with divergent sequence		
				Constant sequence >150 nt	150 nt> Constant sequence >125 nt	Constant sequence <125 nt
WT	37	161	101	22/24 (92%)		9/13 (69%)
*sua5*Δ 1st	153	194	16	29/127 (23%)	4/11 (36%)	14/15 (93%)
*sua5*Δ 5th	94	120	27	4/7 (57%)	33/41 (80%)	40/46 (87%)

Figure 2 Telomere sequencing reveals the defects of telomere replication in *sua5*Δ cells. (**A**) The analysis of Telomere I-L in wild type, first and fifth restreaked *sua5*Δ cells. Each column represents one sequenced telomere. The result of a representative clone of *sua5*Δ strain is shown here. (**B**) Statistics of the telomere sequencing results. The average point of divergence (APD) describes the average length of nondiverging telomere sequence, whereas the average length of divergence (ALD) stands for the average length of diverging telomere sequence. The results were summarized from two independent experiments shown in Supplementary Figure 3B. (**C**) Longer telomeres are not efficiently elongated in *sua5*Δ. The average lengths of the telomere extensions are determined for nondivergent telomeres that are longer or shorter than 170 nt in length. Telomeres shorter than 100 nt are excluded from the analysis because of the reported high recombination rate for short telomeres at TEL01L. The *P*-values associated with two-tailed unpaired *t* tests are shown. (**D**) Length of telomere extension as function of telomere length. The lengths of diverging regions are plotted as a function of the nondivergent sequence, which represents the original telomere sequence. (**E**) Frequency of telomere extension as function of telomere length. Telomere sequences are ordered according to the nondivergent sequence length and pooled into subgroups containing 10 telomeres each. The frequency of elongation in each subgroup is calculated and plotted as a function of telomere length.

double-strand breaks. In *pif1*Δ cells, the GCR rate is dramatically increased through efficient telomere healing (Myung *et al*, 2001). *SUA5* deletion significantly suppressed the GCR rate in *pif1*Δ cells (Figure 3B), suggesting that *SUA5* affects telomerase activity *in vivo*.

Sua5 does not affect telomerase expression and assembling

Earlier studies have suggested that SUA genes might be involved in gene transcription and RNA metabolism (Pinto *et al*, 1992, 1994; Cui *et al*, 1995), we wondered whether the shorter telomeres in *sua5*Δ cells are caused by low level of telomerase or defect of telomerase assembling. With this in mind, we first examined the protein levels of telomerase components (Est2p, Est1p and Est3p) and Cdc13p in *SUA5* and *sua5*Δ cells. Neither telomerase components nor Cdc13p showed any significant changes in *sua5*Δ cells (Figure 3C). Then, we checked the TLC1 RNA. Northern blot analysis showed that TLC1 RNA level in *sua5*Δ cells was comparable to that in wild-type cells (Figure 3D). In addition, Sua5p

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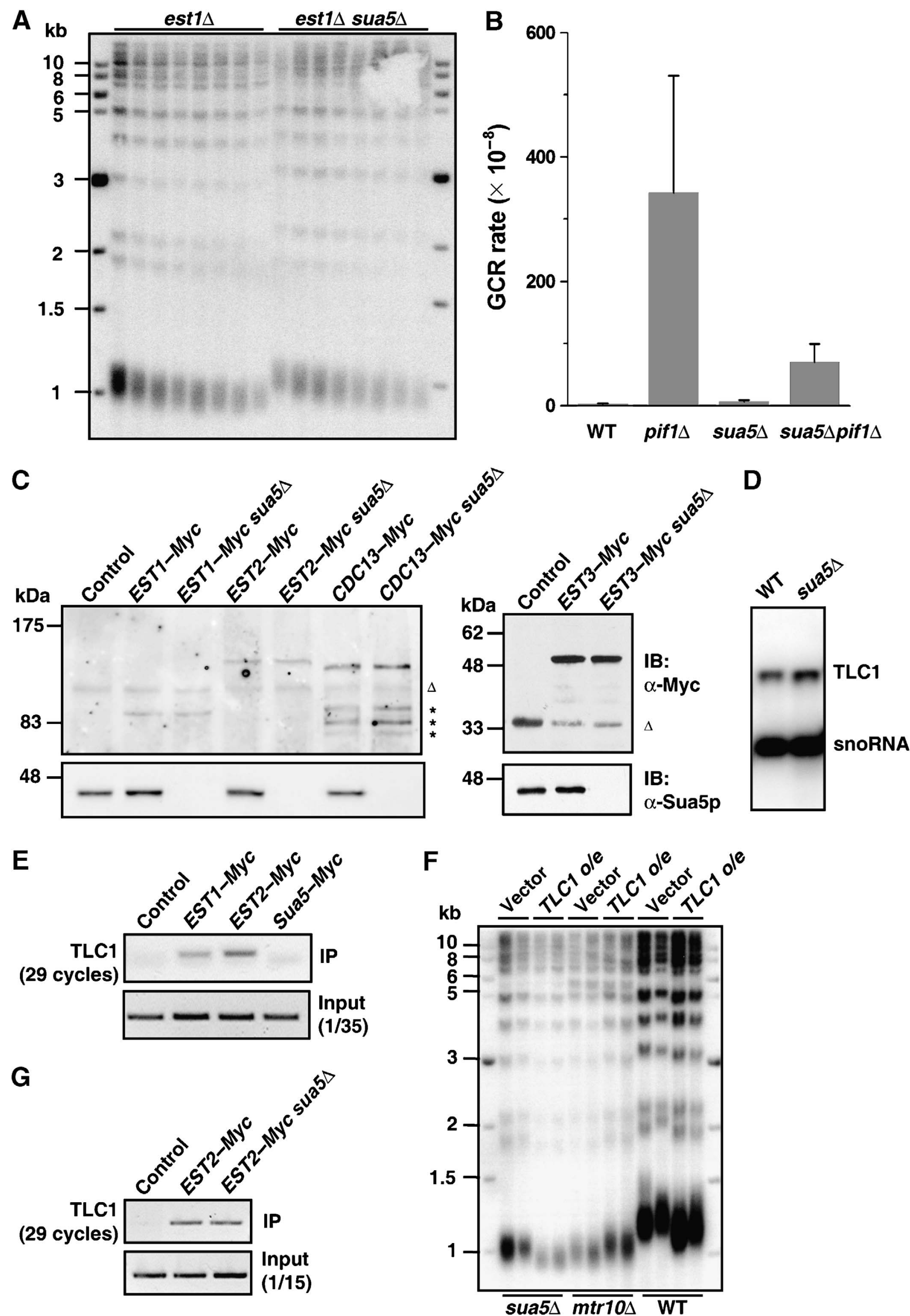

Figure 3 *SUA5* deletion does not affect telomerase expression and assembly. (**A**) *SUA5* deletion does not increase the telomere shortening rate in the telomerase-deficient cells. The isogenic strain of *est1*Δ or *est1*Δ *sua5*Δ was continuously passaged and the genomic DNAs were digested with *Xho*I and subjected to telomere blot. (**B**) *SUA5* is required for the elevated gross chromosomal rearrangements (GCRs) rates observed in *pif1*Δ cells. GCR rates in *pif1*Δ, *sua5*Δ and *pif1*Δ*sua5*Δ strains were analysed as reported (Myung *et al*, 2001). (**C**) The protein levels of telomerase components and Cdc13p are not affected by *SUA5*. The Myc-tagged proteins were examined by western blot with an anti-Myc antibody. The triangle (Δ) indicates unknown proteins cross-reacted with the antibody, which also serve as loading controls. The asterisked (*) bands might be the degradation products of Cdc13p. (**D**) The expression of TLC1 is not affected by *SUA5*. Northern blot analysis of total RNAs from the indicated strains with TLC1 and snoRNA probes. (**E**) Sua5p is not associated with TLC1. The myc-tagged proteins were immunoprecipitated with the anti-myc antibody, and TLC1 was detected with semiquantitative PCR. (**F**) *SUA5* is not involved in TLC1 biogenesis. TLC1 RNA was over-expressed under the control of GPD promoter. The *mtr10*Δ mutant, which has a defect in TLC1 trafficking, serves as a positive control. (**G**) *SUA5* does not affect telomerase assembling. The myc-tagged Est2p was immunoprecipitated, and co-immunoprecipitated TLC1 was detected with semiquantitative PCR.

appeared not to associate with TLC1 *in vivo* as revealed by the co-immunoprecipitation-PCR experiment (Figure 3E). Moreover, over-expression of TLC1 could not elongate the short telomeres in *sua5*Δ cells (Figure 3F; Supplementary Figure 5). Furthermore, the interaction of Est2p and TLC1 is not affected in *sua5*Δ cells (Figure 3G). These data suggested that Sua5p does not affect expression of telomerase subunits and TLC1, nor influences telomerase assembling.

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Sua5 functions downstream telomerase recruitment

Telomerase-dependent telomere elongation is mainly achieved through telomerase recruitment and activation (Smogorzewska and de Lange, 2004). The G1-phase telomere association of telomerase is abolished in Ku–Tlc1 interaction-deficient mutants (*yku80-135i* or *tlc1-Δ48*) (Peterson *et al*, 2001). We examined the telomere lengths of *sua5Δ yku80-135i* and *sua5Δ tlc1-Δ48* double mutants, and found that *SUA5* deletion further shortened the telomeres of the Ku–Tlc1 interaction-deficient mutants (Figure 4A), indicating that *SUA5* functions independent of G1-phase telomerase recruitment. The S-phase telomerase recruitment can be bypassed by the introduction of Cdc13–Est2p or Cdc13–Est1p fusion protein (Evans and Lundblad, 1999; Schramke *et al*, 2004). If Sua5 participates in this or upstream step of telomerase recruitment, it would be bypassed by the presence of

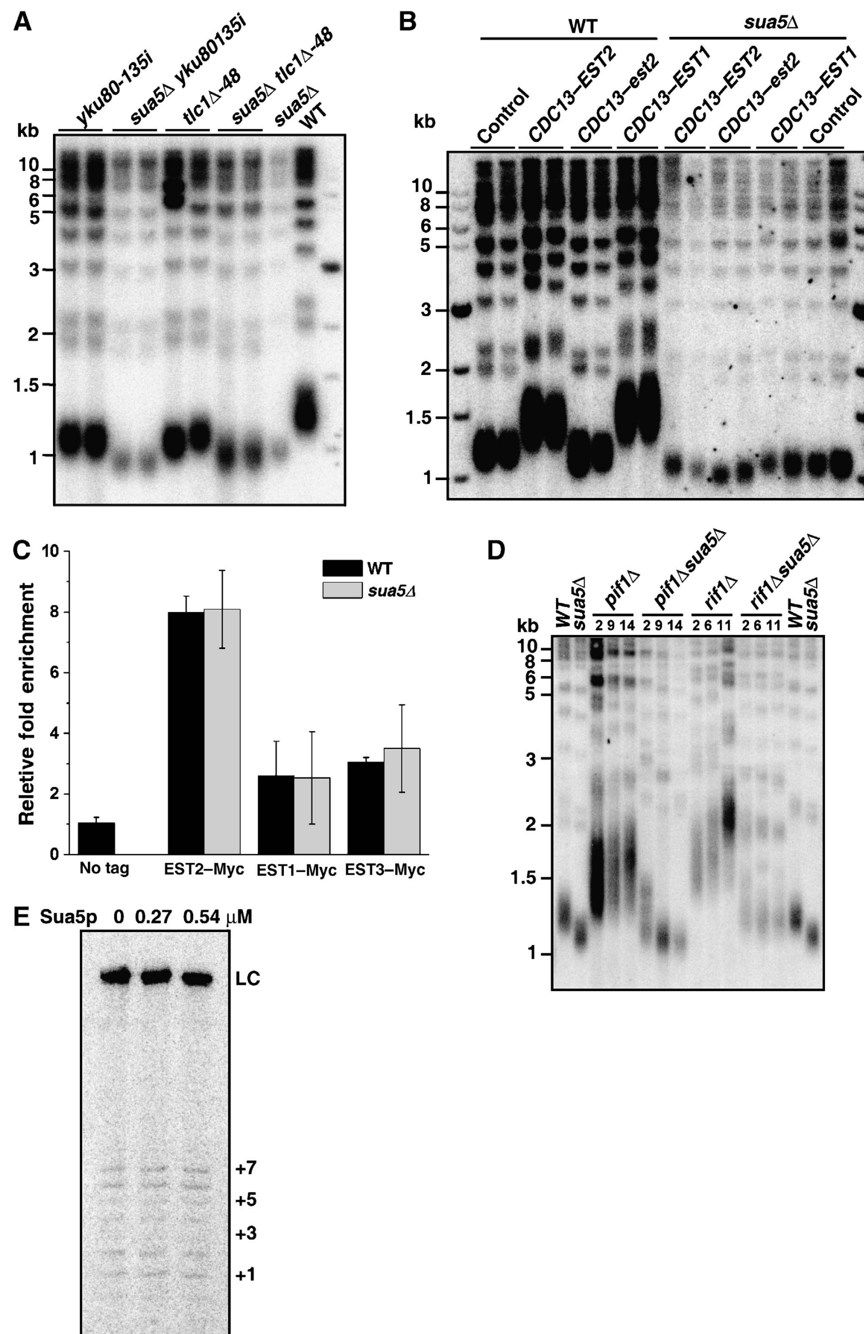

Figure 4 Sua5p does not affect telomerase recruitment. (**A**) Deletion of *SUA5* shortens the telomere lengths of the Ku–Tlc1 interaction-deficient mutants (*tlc1Δ-48* or *yku80-135i*). The genomic DNAs were digested with *Xho*I and subjected to telomere blot. (**B**) Cdc13–Est2p or Cdc13–Est1p fusion protein cannot over-elongate the shortened telomeres in *sua5Δ* cells. (**C**) The association of telomerase to telomeres is not affected. The association of telomerase to telomeres was detected with ChIP-qPCR in *sua5Δ* and wild-type cells. (**D**) Sua5 functions in the same pathway as Pif1, but not Rif1. The isogenic strains are labelled on the top of the panel, and the numbers indicate the restreaked times on YPD plate. (**E**) Sua5p does not affect telomerase activity *in vitro*. Purified telomerase (Liao *et al*, 2005) was incubated with 1 μM Tel15 (5′-TGTGGTGTGTGTGGG-3′) and indicated amount of recombinant Sua5p. LC, loading control.

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Cdc13–Est2p fusion protein. Accordingly, we observed the same dramatic telomere length increase in the wild-type cells, which harbour the fusion Cdc13–Est2p or Cdc13–Est1p proteins (Figure 4B). Surprisingly, telomere over-elongations by fusion proteins were compromised in *sua5Δ* cells (Figure 4B). In addition, the chromatin immunoprecipitation (ChIP) results showed that bindings of telomerase components Est2p, Est1p and Est3p to telomere were not changed in *sua5Δ* cells (Figure 4C). We, therefore, concluded that Sua5p functions downstream telomerase recruitment.

Sua5 functions epistatic to Pif1 in the telomerase pathway

Both Rif1 and Pif1 negatively regulate telomere elongation, and their deletion resulted in longer telomeres (Wotton and Shore, 1997; Zhou *et al*, 2000). Rif1 is an essential component of telomere length counting machinery (Marcand *et al*, 1997), whereas Pif1p inhibits telomerase activity through disassociating telomerase from telomeric DNA (Zhou *et al*, 2000; Boule *et al*, 2005). To further validate that Sua5p affects telomerase pathways, we examined the telomere lengths of *sua5Δpif1Δ* and *sua5Δrif1Δ* double-mutant cells when they were passaged about 350 and 275 generations (i.e. 14 and 11 times restreaking on plate), respectively. The long telomeres caused by *PIF1* or *RIF1* deletion were gradually shortened when Sua5p is absent (Figure 4D). Interestingly, Compared with telomeres of the *sua5Δrif1Δ* cells, which finally kept at wild-type length, telomeres of *sua5Δ pif1Δ* cells had similar telomere length to *sua5Δ* cells (Figure 4D). This result indicated that *SUA5* functions epistatic to *PIF1* in the telomerase pathway. To ask whether Sua5p has any direct influence on telomerase activity *in vitro*, we purified recombinant Sua5p (Figure 5C), and added it into the reaction mixture of telomerase activity assay (Liao *et al*, 2005). Neither the nucleotide addition activity nor the processivity of telomerase core enzyme was affected (Figure 4E).

Sua5p specifically interacts with telomeric DNA in vivo and in vitro

To find out whether Sua5p could specifically interact with telomere *in vivo*, a ChIP experiment was carried out as described earlier (Taggart *et al*, 2002). The chromosomal SUA5 gene was tagged with a Myc epitope, which did not affect telomere length (data not shown). The amount of Sua5p in cells remains unchanged along the cell cycle (Figure 5A), whereas the ChIP-qPCR result showed that Sua5p is associated with telomeric DNA, and the interaction peaked at late S phase (Figure 5B). These results indicated that the Sua5p interacts with telomeres *in vivo*, and its effect on telomere replication is direct.

The primary sequence analysis revealed that Sua5p is an evolutionarily conserved protein (Supplementary Figure 6A). Over-expression of either *Candida albicans* or *Schizosaccharomyces pombe* homolog of Sua5p in *sua5Δ S. cerevisiae* cells could rescue the telomere shortening phenotype (Supplementary Figure 6B), suggesting that the Sua5p's function in telomere maintenance might be conserved. Sua5p consists of N terminal yrdC and the C terminal SUA5 domains (Bateman *et al*, 2004). Both *Escherichia coli* yrdC and thermoacidophilic archaeon *Sulfolobus tokodaii* Sua5 have been proposed to bind nucleic acids (Teplova *et al*, 2000; Agari *et al*, 2008). However, their biochemical activity has not been formally shown. We over-expressed and purified the Sua5p, as well as the N terminal fragment (aa 1–250, named Sua5p-N), which contains yrdC domain, and the C terminal fragment (aa 251–426, named Sua5p-C), which contains SUA5 domain (Figure 5C). Interestingly, we found that the recombinant full length Sua5p, but not Sua5p-N or Sua5p-C, could bind single-stranded yeast telomeric DNA (Figure 5D), suggesting that the DNA binding of Sua5p requires both the yrdC and Sua5 domains. The amount of shifted DNA increased with higher concentrations of Sua5p (Figure 5D, asterisked) and a higher-shift band was detected when the GST–Sua5p was added in the reaction (Figure 5E, double asterisked), indicating that the shifted band is Sua5p–DNA complex. The ssTG DNA binding was completely competed out by the excess amount of unlabelled ssTG DNA, but not the dsTG/AC DNA or nontelomeric single-stranded DNA oligos (Figure 5F). Sua5p could not bind either ssCA or dsTG/AC DNA (Supplementary Figure 7). These results showed that Sua5p specifically binds ssTG DNA *in vitro*. To determine the consensus sequence for Sua5p binding, we made a series mutant oligos based on TG20 sequence (Table I), and carried out a systematical-affinity analysis. The binding affinity was significantly decreased when the core telomere sequence was mutated (Table I), which indicated that Sua5p binds to the core telomere sequence TGGGTGT.

The DNA-binding ability of Sua5p is crucial for its functions

The 3D structures of *E. coli* yrdC protein, *S. tokodaii* Sua5 protein (StSua5p) and the SUA5 domain of *Pyrococcus horikoshii* Sua5 protein (PhSua5p) have been solved (Teplova *et al*, 2000; Agari *et al*, 2008; Agari Y, unpublished data). The sequence alignment of *S. cerevisiae* Sua5p (ScSua5p) and StSua5p revealed 37% identity and 50% similarity (Supplementary Figure 8A). According to structure of StSua5p (PDB: 2EQA), we modelled the structure of ScSua5p (Figure 6A). The surface potential prediction had revealed a positive-charged region at the interface of these two domains (Figure 6B). As neither yrdC nor SUA5 domain alone could bind telomeric DNA (Figure 5D), we speculated that the DNA-binding region of Sua5p may be within the positive-charged region. To further examine the DNA-binding ability and validate the modelled Sua5p structure, we constructed 48-point mutants of *SUA5*. Most of the mutated residues are conserved among species or on the surface of Sua5p. In all, 19 of 48 alleles failed to restore telomere length (Supplementary Table III), and most of these lost-of-function mutations (15/19) located at the positive-charged region (Figure 6C; Supplementary Figure 8B).

To examine the *in vitro* activity of these mutants, six recombinant mutant proteins were purified as wild-type Sua5p (Figure 6D, lower panel). The mutants at the positive-charged region (K93, R95, D98, R248 and R414) (Figure 6C, upper panel) exhibited little DNA-binding activity (Figure 6D, upper panel), whereas the *sua5-1* mutant protein (S107F, outside the positive-charged region), which does not affect telomere length (Figure 6C), exhibited wild-type level of telomeric DNA-binding ability (Figure 6D). These results confirmed that the positive-charged region in Sua5p is responsible for its DNA binding, and the DNA-binding activity is essential for its positive regulatory function in telomere replication.

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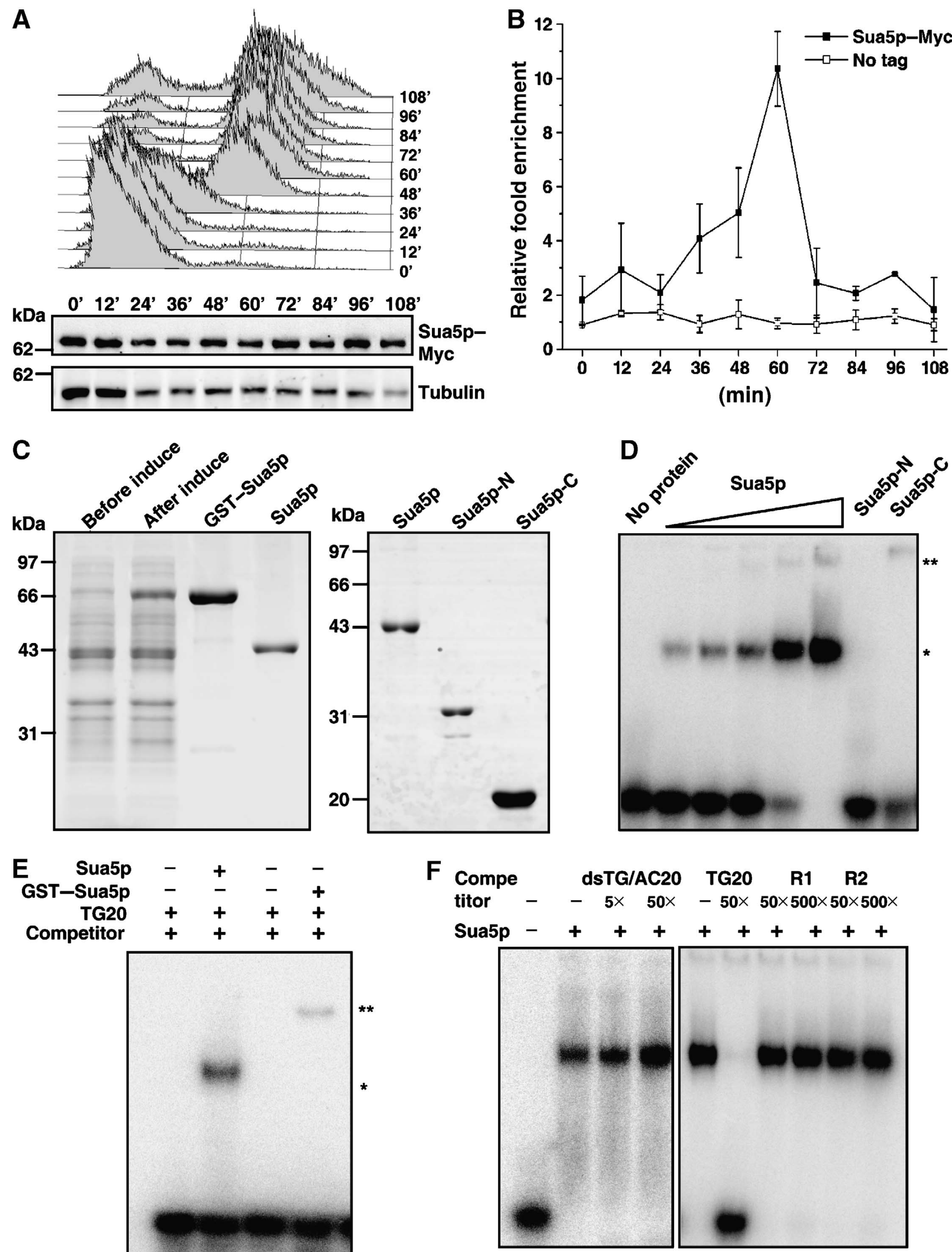

Figure 5 Sua5p interacts with telomeric DNA. (**A**) The expression of Sua5p remains unchanged during the cell cycle. The myc-tagged *SUA5* cells were arrested at G1 phase with α-factor, and allowed to progress into S and G2/M phases (upper panel). Sua5p was examined by western blot (middle panel). Tubulin serves as a loading control (lower panel). (**B**) Sua5p preferentially binds telomeres at late S phase of cell cycle. ChIP-qPCR was performed to analyse the association of Sua5p to telomeric DNA. (**C**) Purification of Sua5p proteins in *E. coli*. The GST–fusion proteins were over-expressed, and purified with the glutathione-affinity column. The GST-tag was cleaved by PreScission protease. The purified proteins were analysed by Coomassie blue stained SDS–PAGE. (**D**) Full-length Sua5p, but not Sua5p-N or Sua5p-C, binds ssTG DNA. Radioactively labelled oligo TG20 (2.4 nM) was incubated with increasing amounts of purified Sua5p (0.1, 0.27, 0.54, 1 and 2.1 μM, respectively), Sua5p-N (2 μM) or Sua5p-C (2 μM). The shifted Sua5p–DNA bands are indicated with a single asterisk, and super-shifted bands, which may represent higher-ordered protein–DNA complex, are indicated with a double asterisk. (**E**) GST–Sua5p–DNA complex (**) migrates slower than Sua5p–DNA complex (*). About 0.5 μM of GST–Sua5p and 1 μM of Sua5p were added in the reactions. (**F**) Sua5p specifically binds ssTG DNA. Competition assays were performed in the presence of the 5- or 50-fold excess of double-stranded telomeric DNA, 50-fold excess of unlabelled TG20 and 50- or 500-fold excess random-sequence oligos R1 and R2. About 2 μM Sua5p was used in each of the reactions.

Discussion

In this study, we have found that *sua5*Δ cells display progressively shortened telomeres during early passages in a large-scale screening. *SUA5* functions downstream the telomerase recruitment and may regulate the telomerase activity. Further analyses have revealed that Sua5p specifically interacts with ssTG DNA *in vitro* through a distinct ssTG-binding region. Sua5p directly binds telomeres *in vivo*. The DNA-binding ability of Sua5p is essential for its telomere function.

A large-scale screening of new telomere maintenance genes

In a genome-wide characterization of essential genes (total 1094 genes) in *S. cerevisiae*, we unexpectedly found out that

Table I Affinity changes for binding of the Sua5p to mutated TG20 oligos

Primer	$(TG)_{0-6}TGGGTGT(G)_{0-1}$	Relative K_d	ΔΔG (kcal/mol) ensemble
TG20	TGGTGTGTGTGGGTGTGGTG	1	
TG201	TaaTGTGTGTGGGTGTGGTG	3.7	0.79
TG202	TGGTaaGTGTGGGTGTGGTG	7.4	1.20
TG203	TGGTGTaTGTGGGTGTGGTG	1.2	0.14
TG204	TGGTGTGTaTGGGTGTGGTG	3.6	0.78
TG205	TGGTGTGTGaGGGTGTGGTG	1.1	0.034
TG214	TGGTGTGTGTaGGTGTGGTG	38	2.19
TG206	TGGTGTGTGTGaaTGTGGTG	175	3.11
TG215	TGGTGTGTGTGaGTGTGGTG	439	3.66
TG216	TGGTGTGTGTGGaTGTGGTG	36.8	2.16
TG207	TGGTGTGTGTGGGTaTGGTG	1.1	0.065
TG208	TGGTGTGTGTGGGTGTaaTG	0.78	−0.15

64 genes were 'mis-classified' as essential genes. The earlier genome-wide screenings of telomere maintenance genes in the nonessential gene deletion set have identified about 280 genes (Askree *et al*, 2004; Gatbonton *et al*, 2006), and our current study has added 29 more genes in this group. Some of the genes, for example, *SWC4*, *HRR25* and *PDS5*, function in the process of chromatin remodelling or DNA metabolism (Supplementary Table II), and it is unsurprising that they play roles in telomere length regulation. Our screening has recovered the *MTR10* and *KAE1*, which have been reported to regulate telomere length (Figure 1A). The *INO80*, which regulates telomere structure (Yu *et al*, 2007), has also been found to regulate telomere length (Supplementary Table II). Therefore, the new genes uncovered in our screening would provide new clues to understand telomere replication.

In the earlier screenings, nearly 5% of the yeast mutants were found to have abnormal telomeres (Askree *et al*, 2004; Gatbonton *et al*, 2006). Interestingly, in our current screening, 29 out of 64 nonessential genes (45%) appear to affect telomeres. A possible explanation is that most of the recovered mutants have severe growth defect, and they may affect telomeres indirectly. To find out the genes that are directly relevant to telomere replication, we examined the telomere changes during the early passages after sporulation. Sua5 particularly caught our attention because like *mtr10*Δ or *kae1*Δ cells, *sua5*Δ cells showed progressively shortened telomeres in their early passages (Figure 1), which is reminiscent of the EST phenotype seen in telomerase-deficient cells. *MTR10* and *KAE1* have been shown to regulate telomerase activity (Ferrezuelo *et al*, 2002; Downey *et al*, 2006), and the progressively shortening telomere phenotype of *sua5*Δ cells led us to suspect that *SUA5* may regulate telomerase activity.

Sua5 may positively regulate telomerase activity

The imbalance between telomere lengthening and shortening in *sua5*Δ cells suggested *SUA5* may function in telomere protection or telomerase activity regulation. Several lines of evidence argue against the telomere protection hypothesis: (1) *sua5*Δ mutant cell did not display temperature sensitivity, which is frequently observed in the telomere uncapping mutants (Supplementary Figure 4A); (2) the amount of single-stranded TG DNA in *sua5*Δ cells has not increased (Supplementary Figure 4B); (3) *sua5*Δ *exo1*Δ cells does not exhibit any growth advantage to *sua5*Δ cells, or telomere length restoration (Supplementary Figure 4C); (4) the telomere shortening rate of *est1*Δ*sua5*Δ cells is comparable to that of *est1*Δ cells (Figure 3A) and (5) the APD of the first-restreaked *sua5*Δ cell is comparable to that of the wild-type cells (Figure 2B). These observations encouraged us to postulate that Sua5 may participate in the regulation of telomerase activity.

Telomerase recruitment and activation are two important steps for telomerase activity. *SUA5* and Ku–Tlc1 interaction-deficient mutant function in different epistasis groups (Figure 4A). CDC13–EST2 and CDC13–EST1 fusion proteins cannot bypass the requirement of Sua5p for full telomerase activity (Figure 4B). In addition, the association of telomerase to telomeres is not changed in *sua5*Δ cells (Figure 4C). Therefore, Sua5p appears not to affect telomerase recruitment through Ku–Tlc1 at G1 phase, or through Cdc13–Est1 and RPA–Est1 at late S phase (Evans and Lundblad, 1999; Peterson *et al*, 2001; Taggart *et al*, 2002; Stellwagen *et al*, 2003; Fisher *et al*, 2004; Schramke *et al*, 2004).

In the absence of Sua5p, the elevated *de novo* telomere addition events in *pif1*Δ cells are dramatically decreased (Figure 3B), and the longer telomeres are less frequently elongated likely due to the limited telomerase activity (Figure 2). Apparently, the telomerase protein/RNA levels and assembling are not affected in *sua5*Δ cells (Figure 3). Sua5 functions epistatic to Pif1 in the telomerase pathway (Figure 4D), and Sua5p preferentially binds telomeres at late S phase (Figure 5B) as the telomerase does (Taggart *et al*, 2002). On the basis of these findings, we hypothesize that Sua5p may synergize the function of telomere-bound telomerase. *In vitro*, Sua5p seems not to affect telomerase activity (Figure 4E), raising the possibility that Sua5p may regulate telomerase activity through interaction with other telomere-binding proteins or telomeric DNA *in vivo* (Figures 5 and 6). It will be of great interest to investigate the genetic and physical interaction between Sua5 and the other telomere maintenance genes. The identification of Sua5p interacting proteins will help to explain its molecular mechanism.

Sua5p possesses a distinct ssTG DNA-binding structure

The OB-fold domain appears to be the fingerprint to many single-stranded nucleotide-binding proteins, including several ssTG DNA-binding proteins, that is, TEBPβ, Cdc13p, Stn1p, Pot1 and TPP1 (Horvath *et al*, 1998; Baumann and Cech, 2001; Lei *et al*, 2003; Mitton-Fry *et al*, 2004; Gao *et al*, 2007; Wang *et al*, 2007). Like these proteins, Sua5p could specifically bind ssTG DNA (Figure 5A). However, no OB-fold domain was founded in the Sua5p 3D structure (Figure 6A). Instead, both the yrdC and the SUA5 domains of Sua5p contributed to its interaction with single-stranded TG DNA

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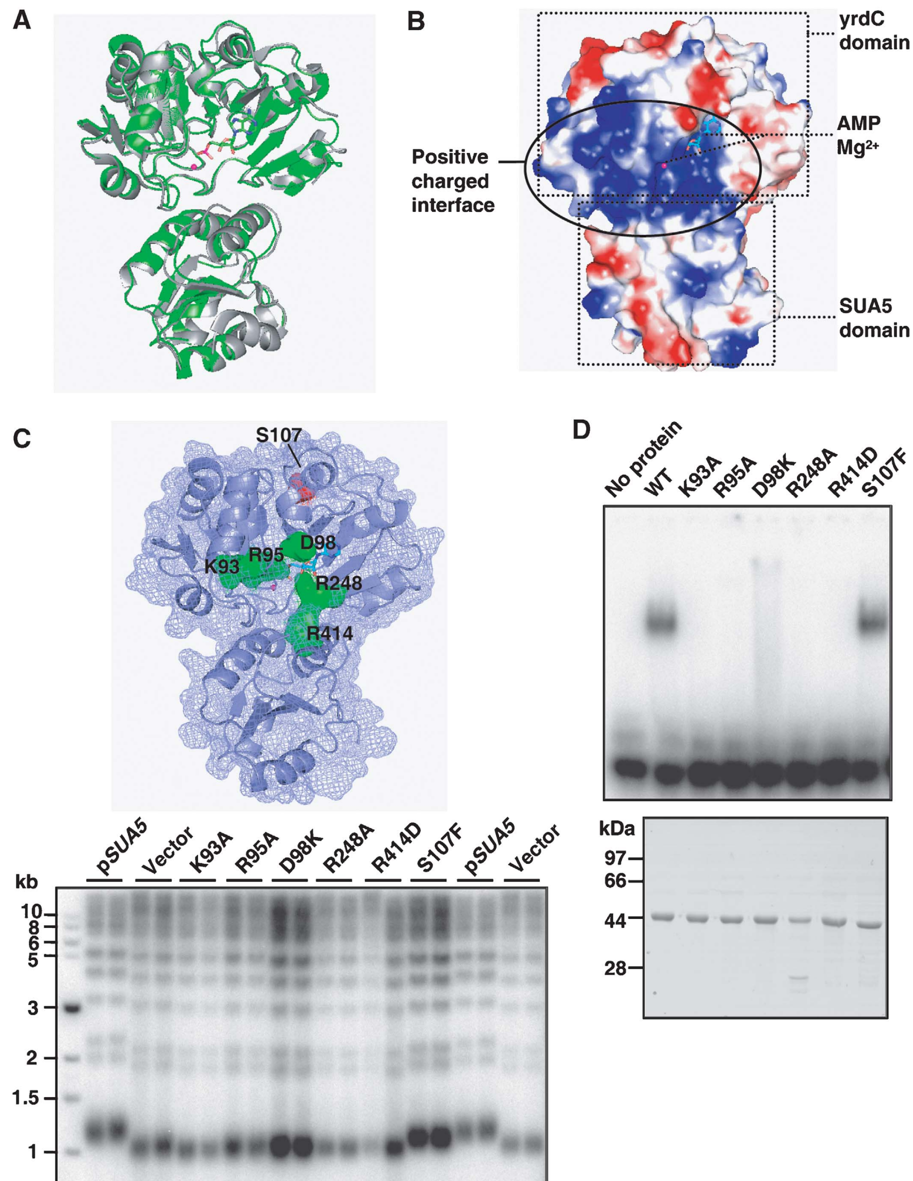

Figure 6 A positive-charged region of Sua5p is crucial for its function. (**A**) Modelled 3D structure of *S. cerevisiae* Sua5p. The modelled structure of *S. cerevisiae* Sua5p (green) is aligned with the template structure of *S. tokodaii* Sua5p (grey). (**B**) The relative protein surface potential is predicted and displayed with PyMOL software. A positive-charged region at the interface of yrdC and SUA5 domains is shown. (**C**) The point mutation in the positive-charged surface region of Sua5p affects its telomere maintenance function. Representative mutants, including K93A, R95A, D98K, R248A and R414D in the positive-charged region and S107F outside the positive-charged region, are pointed out in the upper panel. The mutant telomere lengths were examined (lower panel). (**D**) The point mutation in the positive-charged region of Sua5p abolishes its DNA-binding ability. 1 μM of wild-type or mutant Sua5 protein was used, and 50-fold excess of the random-sequence oligos were added into each reaction as competitor (upper panel). The purified wild-type and mutant Sua5 proteins are shown by Coomassie blue stained gel (lower panel).

(Figure 5D). Mutations in DNA-binding region at the interface abolished the interaction between Sua5p and ssTG DNA (Figure 6D), and caused the defects in telomere elongation (Figure 6C). The binding affinity of Sua5p ($K_d = 615$ nM) is relatively low compared with Cdc13p ($K_d = 28$ nM) under the same reaction condition. These data strongly suggested that the specific TG-rich DNA-binding mode of Sua5p represents a novel structure for ssTG-DNA recognition and/or binding.

One molecule of both AMP and Mg^{2+} ion were found in the crystal structure of StSua5p (Agari *et al*, 2008). The amino acids responsible for AMP and Mg^{2+} bindings are highly conserved in *S. tokodaii* and *S. cerevisiae* Sua5 proteins. Mutations of these amino acids cause loss of *SUA5* function (Supplementary Table III). It has been reported that StSua5p has ATPase activity (Agari *et al*, 2008), but our recombinant fungal Sua5p (Supplementary Figure 9A) did not show any ATPase (Supplementary Figure 9B) or GTPase (Supplementary Figure 9C) activity. This discrepancy makes the function of AMP and Mg^{2+} confusing. It remains elusive whether and how AMP and Mg^{2+} contribute to the Sua5p function.

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Evolutionary conservation of SUA proteins

The conserved yrdC domain by itself as well as conjugated with other motifs has been found in a broad-spectrum species (Teplova *et al*, 2000; Chen *et al*, 2003), and SUA5 domain exists in its self or at the C terminus of yrdC domain (Bateman *et al*, 2004). The homologs of Sua5p have been found in archaea, bacteria and eukaryotes. Bacterial yrdC protein is suggested to be involved in rRNA maturation (Kaczanowska and Ryden-Aulin, 2005). However, the rRNA maturation was not affected in *S. cerevisiae* when Sua5p was absent (Supplementary Figure 2D), and the shorter telomeres in *sua5*Δ mutant are not caused by an indirect translation defect.

In eukaryotes, most Sua5 homologs exist in Fungus Kingdom (Bateman *et al*, 2004). Therefore, it will be of great interest to investigate whether fungal Sua5p binds telomeric DNA and regulates telomeres in other yeast such as *C. albicans* and *S. pombe*. Unfortunately, Sua5 is essential for the growth of *S. pombe* and *C. albicans*, and we could not get the deletion mutant (data not shown). The over-expression of either *S. pombe* or *C. albicans* Sua5 protein in budding yeast can rescue the telomere shortening caused by the *S. cerevisiae SUA5* deletion (Supplementary Figure 6B). In addition, the purified recombinant SpSua5p and CaSua5p are able to specifically bind ssTG DNAs (data not shown), suggesting Sua5p's function in telomere maintenance may be conserved among fungi.

It will be intriguing to test whether the human yrdC protein (Chen *et al*, 2003; Jiang *et al*, 2005) also affect telomere length. However, the function of Sua5p at telomere seems to be difficult to spread to its archaeobacterial or bacterial homologs as there is not DNA end replication problem in these organisms. Therefore, it is hard to interpret why fungal Sua5p has adapted a specific single-strand telomeric DNA-binding activity. One possibility is that Sua5p and its homologs may have a higher affinity to T(U)G-rich nucleotides. Another possibility is that the fungal Sua5p have gained a telomere regulation function during the evolution.

In conclusion, the results presented here have identified Sua5 as a novel ssTG DNA-binding protein, which is important for telomere length homeostasis. Elucidating the molecular mechanism of Sua5p function will shed light on the understanding of telomere replication.

Materials and methods

Yeast strains and plasmids

Strains used in this work are derivatives of BY4743 or YPH501 as indicated in Supplementary Table I. Yeast strains were constructed using standard genetic procedures either by genetic cross or by homologous recombination. Systematic deletion strains are from EUROSCARF. All gene disruptions and taggings were verified by PCR or gene-specific phenotypes. *SUA5* CEN plasmids were constructed by cloning a 2.3 kb (from upstream 800 bp to downstream 200 bp of *SUA5* open reading frame) PCR-amplified fragment of *SUA5* in the *BamH*I-*Xho*I site of pRS316. The point mutations were introduced by site-directed mutagenesis.

Telomere blot

Genomic DNA was digested with *Xho*I or *Pst*I, separated on agarose gels, transferred to Hybond-N membrane (GE Healthcare), and hybridized to a telomere probe. The screening experiments for telomere-length changes were started with two asci; each spore was restreaked five times on YPD plates after sporulation. Genomic DNAs of the first- and fifth-restreaked cells were subjected to telomere blot. Telomere length was determined with Image Quant TL software. Linear log curve stimulation was used to calibrate the molecular weight and Student's t test was applied to state the significant changes.

Telomere PCR

Telomere PCR was performed as described (Forstemann *et al*, 2000; Lee *et al*, 2007). The telomere ends of chromosome I-L were amplified using 'o286S *Mlu*I' and 'G18 *BamH*I' primers (Lee *et al*, 2007). PCR products were separated on 2% agarose gel, and cloned into the pMD18-T simple vector (Takara). Individual clones were sequenced by Majorbio.

Primer extension assay for telomerase activity

Telomerase purification and primer extension were performed as described earlier (Liao *et al*, 2005). Purified Sua5p was pre-incubated with telomerase at 30°C for 20 mins before initiating the reaction by adding dNTPs. Reaction products were analysed by 18% polyacrylamide-urea gel electrophoresis.

Recombinant protein purification and gel shift assay

SUA5 ORF and truncated fragments were cloned into pGEX-6p-1 vector. The GST fusion proteins were over-expressed and purified according to the manufacturer's instructions (GE Healthcare). GST tag was cut by PreScission protease, and Sua5p was further purified with Q-sepharose and Superdex-200 columns (GE Healthcare). About 2.4 nM ^{32}P-labelled TG20 DNA (5′-TGGTGTGTGTGGGTGTGG TG-3′) was mixed with indicated amount of full length or fragments of Sua5p in 50 mM K-HEPES buffer (pH 7.5) containing 100 mM KCl, 5 mM $MgCl_2$. The reaction mixture was incubated at 30°C for 40 min, and analysed with a 6% nondenaturing polyacrylamide gel. Oligonucleotide CA20 (5′-CACCACACCCACACACACCA-3′) was annealed with TG20 to form double-strand TG20/CA20. Two random-sequence oligonucleotides R1 (5′-TCAATGTTATACTTGACTACTTG C-3′) and R2 (5′-AGTACTAGGATATATCAGACTATG-3′) were used as nonspecific binding competitors.

ChIP analysis

ChIP was performed according to Taggart *et al* (2002). Sua5p was tagged with Myc epitopes, and Sua5p–Myc strain exhibits wild-type telomere length (data not shown). No-tag or Sua5p–Myc strain, which harbour *bar1*Δ was arrested at G1 phase by α-factor, and then released to allow cell-cycle progression. Cells were harvested at indicated time points and subjected to FACS and ChIP analysis. Myc-tagged proteins were immunoprecipitated with monoclonal α-Myc antibody (Sigma). Quantitative PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems).

Structure modelling of Sua5p

Initial models of ScSua5p were obtained with the MODELLER program (Sanchez and Sali, 2000) by using the structure of StSua5p (PDB: 2EQA) as template. Structures of *E. coli* yrdC (PDB: 1HRU), PhSua5p (PDB: 2YV4) were also used as references. Buried side chains were manually adjusted to avoid steric conflict or have favourable interactions with neighbouring residues using the graphics program Coot (Emsley and Cowtan, 2004), consulting reference databases of known main chain and side chain conformations and preferred side chain rotamers. The regions of ScSua5 residues aa 1–17, 27–44, 120–131, 267–291, 304–313 and 338–355 have no equivalent in the known structures and were excluded from the model. The quality and stereochemistry of the model were evaluated using the program PROCHECK (Laskowski *et al*, 1993). The main chain conformations for 99.07% of amino-acid residues were within the favoured or allowed regions of the Ramachandran plot. Models of AMP molecule and Mg^{2+} were based on the template structure of StSua5p.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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