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Swc4 positively regulates telomere length independently of its roles in NuA4 and SWR1 complexes

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ABSTRACT

Telomeres at the ends of eukaryotic chromosomes are essential for genome integrality and stability. In order to identify genes that sustain telomere maintenance independently of telomerase recruitment, we have exploited the phenotype of over-long telomeres in the cells that express Cdc13-Est2 fusion protein, and examined 195 strains, in which individual non-essential gene deletion causes telomere shortening. We have identified 24 genes whose deletion results in dramatic failure of Cdc13-Est2 function, including those encoding components of telomerase, Yku, KEOPS and NMD complexes, as well as quite a few whose functions are not obvious in telomerase activity regulation. We have characterized Swc4, a shared subunit of histone acetyltransferase NuA4 and chromatin remodeling SWR1 (SWR1-C) complexes, in telomere length regulation. Deletion of *SWC4*, but not other non-essential subunits of either NuA4 or SWR1-C, causes significant telomere shortening. Consistently, simultaneous disassembly of NuA4 and SWR1-C does not affect telomere length. Interestingly, inactivation of Swc4 in telomerase null cells accelerates both telomere shortening and senescence rates. Swc4 associates with telomeric DNA *in vivo*, suggesting a direct role of Swc4 at telomeres. Taken together, our work reveals a distinct role of Swc4 in telomere length regulation, separable from its canonical roles in both NuA4 and SWR1-C.

INTRODUCTION

Telomeres are essential for genome stability and integrity in eukaryotes (1,2). Telomeric DNA is usually elongated through telomerase, a specialized reverse transcriptase that uses its intrinsic RNA moiety as template. While in the absence of telomerase, homologous recombination can function as a back-up means to replicate telomeres (3–6). In budding yeast *Saccharomyces cerevisiae*, telomeric DNA is composed of 300 ± 75 bp double-stranded $TG_{1–3}/C_{1–3}A$ sequences and a 12–14 nt single-stranded $TG_{1–3}$ overhang (2,7,8). Sub-telomeric regions contain X and Y' elements (9,10). Telomerase contains two core subunits of Est2 (catalytic subunit) and Tlc1 (RNA subunit) (11,12), as well as other accessory subunits including Est1, Est3 (13), Yku70/Yku80 heterodimer (hereafter referred as Yku) (14), the Sm_7 heteroheptameric complex (15), and Pop1/Pop6/Pop7 complex of RNase P/MRP (16).

The ultimate execution of telomerase function at telomeres seems to involve quite a few processes, such as expression of telomerase components, assembly and nucleus import of telomerase core enzyme Est2/Tlc1, recruitment and physical loading of telomerase at telomeres, Tlc1 template–telomeric DNA pairing, telomerase elongation of telomeric G-strand and processive translocation. Molecular regulation of telomerase recruitment has been well documented. There are two pathways of telomerase recruitment. One is Cdc13–Est1 pathway. Est1 physically interacts with Cdc13, which specifically binds telomeric G-strand overhang, to recruit Est2/Tlc1 core enzyme (17–19). The other is Tlc1–Yku–Sir4 pathway. Both Sir4 and Yku bind to telomeres and recruit Est2/Tlc1 through specific Yku80–Tlc1 interaction (19–21). Cdc13-Est1 and Tlc1–Yku–Sir4 pathways appear to play dominant and accessory roles, respectively, in telomerase recruitment because *cdc13–2* allele that contains a E252K mutation results in ever-shorter-telomeres (est)

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phenotype (22), which can be suppressed by Est1 K444E mutation (18); while *yku80–135i* mutation, which abolishes Yku–Tlc1 interaction, only results in short telomeres, but not telomere-length dependent cellular senescence (14).

Physically tethering telomerase to telomeres, such as seen in the strains containing *CDC13–EST1* or *CDC13–EST2* fusion genes, results in telomere over-elongation (23). However, successful recruitment of telomerase core enzyme to telomeres seems not to be sufficient for efficient telomeric DNA elongation. Cdc13–Est2 fusion protein tethers telomerase core enzyme to telomeres, but could neither maintain minimum telomere length in the absence of Est3 (23), nor stabilize regular telomere length in the absence of Est1 (23). Consistently, overexpression of Est1 leads to telomere over-elongation (24), and Est1 has been proposed to play a role in telomerase activation (2,23,25,26).

Previously, several genome-wide screenings have identified >280 non-essential telomere-length-maintenance (TLM) genes that affect telomere length (27–30). Approximately 70% of TLM genes (TLM^+) positively regulate telomere length, and 30% of TLM genes (TLM^-) negatively regulate telomere length. However, a large portion of the genes have not been carefully characterized in the processes of telomerase recruitment and action. In order to identify the genes that affect telomerase function independently of telomerase recruitment, we performed a functional screening by examining telomere length change upon expression of Cdc13-Est2 fusion protein in 195 TLM^+ strains, which lack individual TLM^+ genes. We found that in 171 single gene-deletion strains, telomeres were efficiently elongated by Cdc13–Est2, but in 24 single gene-deletion strains, short telomeres could hardly be elongated. In this work, we characterized *SWC4* (also called *EAF2*), which encodes a subunit of both histone acetyltransferase NuA4 and chromatin remodeling SWR1 (SWR1-C) complexes. We showed that Swc4 regulates telomere length independently of its roles in either NuA4 or SWR1-C. Deletion of *SWC4* accelerates both telomere shortening and senescence rates of telomerase deficient cells. Swc4 associates with telomeres and plays a direct role in telomere length regulation.

MATERIALS AND METHODS

Yeast strains, plasmids and molecular manipulations

Yeast strains used in large scale screening were derived from systematic deletion strains (EUROSCARF), each deletion was replaced by a *KanMX* module. The other strains used in this study were listed in Supplementary Table S5. Gene deletion experiments were performed using standard genetic procedures and PCR-based gene deletion strategies as described previously (31,32). Myc tagging strain was constructed by a PCR-based gene modification strategy as described previously (32). *CEN* plasmid pRS316-*CDC13-EST2* was from (33), and pRS313-*SWC4* was from (34). The plasmids encoding Swc4 truncated mutants were generated by overlapping PCR.

Southern blotting assay

Yeast cells were collected from either liquid cultures or solid medium. Genomic DNA was extracted by phenol/chloroform method and digested by FastDigest XhoI (Thermo Scientific). DNA was separated on 1% agarose gel, and transferred to Amersham Hybond-N^+ membrane (GE Healthcare). A native telomeric $C_{1-3}A$ sequence (35) labeled by High Prime DNA Labeling Kit (Roche: 11585584001) in the presence of [α-^{32}P] dCTP was used to probe the membrane, telomere signals were detected by phosphor-image.

Native Southern blotting assay used to detect telomeric ssDNA was performed as previously described (36,37), with following modifications. Genomic DNA was in turn digested by RNaseA and XhoI, and separated on 0.7% agarose gel, the gel was treated by 0.5× SSC (8.5 mM trisodium citrate, 75 mM NaCl). The telomeric ssDNA was probed by a biotin-labeled $C_{1-3}A$ sequence (5′-CACCAC ACCCACACACCACACCCACA-3′).

Chromatin extraction, whole-cell extracts preparation and western blot

Yeast cells were cultured to mid-log phase, chromatin was extracted as described previously (38), whole-cell extracts were prepared by TCA methods. The proteins were separated by SDS-PAGE, and tranferred to Immun-Blot PVDF Membranes (BIO-RAD). 1× TBST (50 mM Tris pH 7.5, 150mM NaCl, 0.05% Tween 20) diluted primary and secondary antibodies were incubated overnight at 4°C and 1 h at room temperature respectively, the membranes were imaged by Amersham Imager 680 (GE Healthcare). Antibodies used of western blot: Anti-acetyl-Histone H4 Antibody (Millipore: 06-598), Histone H4 antibody (Active Motif: 61521), Anti-Histone H2A.Z antibody (Millipore: 07–718), Anti-α-Tubulin antibody (Sigma-Aldrich: T6199), anti-myc antibody is prepared by our laboratory.

Serial dilution assay

Cells grown to stationary phase in liquid cultures were diluted to OD_{600} of 0.5. Five-fold diluted cells were spotted on indicated plates and incubated for 72 h.

Cellular senescence assay

Cellular senescence assay was performed as previously described with few modifications (39). Briefly, a spore of indicated genotype was inoculated into 5 ml YPD medium and grown to stationary phase. The cell density was measured by spectrometry (OD_{600}) every 24 h (96 h of the strains contain *SWC4* deletion), and the cells were diluted to OD_{600} of 0.01 with fresh YPD medium. This procedure was repeated for 11 times.

Fluorescence-activated cell sorting (FACS) assay

Yeast cells (MATa) grown to mid-log phase were synchronized at G1 phase by adding 10 ug/ml α-factor (incubated at 30°C for 2 h, every 40 min replenished 5 ug/ml α-factor), and released into S and G2/M phases at 24°C. FACS assay was performed as previously decribed (40).

Chromatin immunoprecipitation (ChIP) assay and dot blot assay

ChIP assay was performed as previously described, with following modifications (40). Immunoprecipitation of cross-linked DNA was probed by monoclonal anti-c-Myc antibody (Sigma-Aldrich: M4439). For dot blot assay, 2-fold serial diluted input DNA and immunoprecipitated DNA were denatured and spotted on Amersham Hybond N^+ membrane (GE healthcare), the membrane was UV-crosslinked and telomeric DNA was probed by α-^{32}P-dCTP labeled $C_{1-3}A$ sequences. The grayscale of immunoprecipitated DNA by anti-myc antibody of WT and *SWC4*^{13myc} cells was measured by ImageJ software, and normalized to the grayscale of their corresponding input DNA. Error bars represent the standard deviation from three independent clones. *** represents $P < 0.001$ (Student's *t*-test).

For qPCR assay, a non-telomeric gene *ARO1* was used to normalize the enrichment of Swc4 at TEL6R. Primer pairs specific for TEL6R and *ARO1* were used for real-time PCR: TEL6R-F, 5′-GTAAATGGCAAGGGTAAAAACCA-3′; TEL6R-R, 5′-CCAGTCCTCATTTCCATCAATAGTAA-3′; *ARO1*-F, 5′- GGGTGGTATAAAGAATGCTCAAATTT-3′; *ARO1*-R, 5′-TCTTCTTAGAGCTTGGAACTCAGCTT-3′. The enrichment was measured by ΔΔCt-method, calculated as: $2^{[-((Ct_{IP\ TEL6R} - Ct_{Input\ TEL6R}) - (Ct_{IP\ ARO1} - Ct_{Input\ ARO1}))]}$. The fold of enrichment was calculated as: $IP_{myc}/IP_{non\text{-}tagged}$.

RESULTS

Functional screening for genes that are required for telomere over-elongation in the strain expressing Cdc13-Est2 fusion protein

Previous studies have shown that expression of Cdc13–Est2 fusion protein leads to telomere over-elongation (23), demonstrating that constitutively tethering telomerase to telomeres promotes telomere elongation. Interestingly, Cdc13–Est2 fusion protein could only maintain short telomeres in the absence of Est1 (23), suggesting that in addition to telomerase recruitment, Est1 is also essential for efficient telomerase activity. In order to screen genes that are required for telomere over-elongation in the strain expressing Cdc13–Est2 fusion protein, we introduced a *CDC13–EST2* fusion gene into 195 TLM^+ strains, in which each individual gene deletion results in shorter telomeres (compared to wild-type) (Supplementary Table S1). In this screening, we ignored TLM^- strains, i.e. the single-gene deletion strains that have longer telomeres than wild-type strain (Supplementary Table S2). The expression of *CDC13–EST2* fusion gene was driven by *CDC13* promoter (33). Then we examined telomere length by Southern blotting assay using telomeric specific $C_{1-3}A$ probe. The results showed that in 171 strains (out of 195), telomeres were efficiently elongated upon Cdc13–Est2 expression (Supplementary Figure S1A–L and Supplementary Table S1). In contrast, short telomeres in 24 strains could hardly be elongated (Table 1; Figure 1A), suggesting that fully execution of telomerase activity requires these genes. Among these genes, 18 have been well characterized in telomere length regulation: *TLC1*, *EST1* and *EST3* encode the subunits of telomerase; *YKU70*/*YKU80* are involved in telomerase recruitment, telomere protection, telomere silencing and telomere anchoring at nuclear periphery (41); *UPF3*, *NAM7* and *NMD2* regulate Stn1 and Ten1 expression, and function in nonsense mediated mRNA decay (NMD) (42); *KAE1*, *BUD32*, *CGI121* and *GON7* encode the subunits of KEOPS complex, which plays a role in telomere decapping (43). *SUA5* encodes a single-stranded telomeric DNA-binding protein that positively regulates telomere length (30). *RTF1* and *CDC73* encode the subunits of Paf1 complex, which is required for efficient expression of Tlc1 (44).

Figure 1. Identification of genes required for telomere over-elongation in the presence of Cdc13-Est2 fusion protein. The DNA markers are labeled on left. (**A**, **B**) Telomere Southern blotting assay. The isogenic strains (indicated on top of the panel) were transformed with pRS316 (−) or pRS316-*CDC13-EST2* (+), streaked on plates for at least 5 times (about 100–125 generations) and telomere length was examined by Southern blotting assay. (**C**) Deletion of *SWC4* results in telomere shortening. The *swc4*Δ haploid cells were restreaked on YPD plate for 8 times (indicated on top of the panel), and telomere length was examined by Southern blotting assay. (**D**) Complementation of *swc4*Δ by pRS313-*SWC4* plasmid. The *swc4*Δ mutants transformed with pRS313 or pRS313-*SWC4* were restreaked on histidine$^-$ plate for at least 5 times prior to Southern blotting assay.

Table 1. List of the TLM+ genes required for Cdc13-Est2 mediated telomere over-elongation

Gene	Function
TLC1, EST1, EST3	Telomerase components
YKU70, YKU80	Yku complex
UPF3, NAM7, NMD2	Components of the nonsense-mediated mRNA decay (NMD) pathway
KAE1, BUD32, CGI121, GON7	Subunits of KEOPS complex , required for telomere uncapping
SUA5	Telomeric DNA binding protein required for threonylcarbamoyl adenosine biosynthesis
RTF1, CDC73	Subunit of RNAPII-associated chromatin remodeling Paf1 complex
SWC4	A shared subunit of histone acetyltransferase NuA4 and chromatin remodeling SWR1 complexes
HFI1	Subunit of SAGA histone acetyltransferase complex
RNR1	The large subunit of ribonucleotide-diphosphate reductase (RNR) complex
MTR10	Nuclear import receptor, required for mRNA-nucleus export
SUM1	Transcriptional repressor that regulates middle-sporulation genes
MET7	Folylpolyglutamate synthetase, required for methionine synthesis
XRN1	5′-3′ exonuclease, component of cytoplasmic processing (P) bodies involved in mRNA decay
SAH1	*S*-adenosyl-L-homocysteine hydrolase
YDR396W	Dubious open reading frame, function unknown

Descriptions are referred to *Saccharomyces* Genome Database (SGD: https://www.yeastgenome.org/).

MTR10 is required for Tlc1 transportation between nucleus and cytoplasm (45). *RNR1* is required for the sustained telomere elongation by telomerase (46). *XRN1* promotes the association of Cdc13 to telomeres by downregulating the transcript encoding Rif1 (47). These results demonstrated the validity of our screening. Several genes, such as *SUM1*, *SAH1* and *MET7* are involved in the cellular processes that seem not to be directly correlated with chromatin or DNA metabolism (Table 1). *YDR396W* is a dubious open reading frame with unknown function (Table 1). Interestingly, *HFI1* and *SWC4* are the subunits of chromatin modification or remodeling complexes (Table 1) (48–50), and their functions in telomere regulation have not been characterized. Further deletion of the chromosomal copy *EST2* resulted in little change of telomere length in the strains of *yku70*Δ, *yku80*Δ, *nmd2*Δ, *met7*Δ, *cdc73*Δ, *rtf1*Δ and *hfi1*Δ (Supplementary Figure S2); but resulted in a modest elongation of the telomeres in the strains of *sum1*Δ, *xrn1*Δ, *cgi121*Δ, *sua5*Δ, *kae1*Δ, *mtr10*Δ, *sah1*Δ and *swc4*Δ, expressing Cdc13-Est2 fusion protein (Supplementary Figure S2).

Deletion of *SWC4/EAF2* causes telomere shortening

SWC4 caught our attention because Southern blotting results showed a severe failure of telomere over-elongation by Cdc13–Est2 fusion protein in *swc4*Δ mutant (Figure 1A and B), consistent with our previous result that *SWC4* deletion caused significant telomere shortening (30). *SWC4* has been considered as an essential gene (50,51) because *swc4*Δ cells display servere growth defect (34), and not much is known for its functions in NuA4 and SWR1-C. In order to validate the effect of Swc4 in telomere length regulation, we dissected *swc4*Δ spores from a *SWC4/swc4*Δ heterozygous diploid strain. Although the *swc4*Δ spores displayed low viability (Supplementary Figure S3), some of them remained viable successively on solid or liquid medium. We examined telomere length in the *swc4*Δ cells that were streaked on plates for 8 times, the result showed that deletion of *SWC4* caused gradual telomere shortening in early passages, and shortened telomeres remained stable in the late passages (Figure 1C). A *CEN*-plasmid bearing *SWC4* gene could complement the telomere length defect of *swc4*Δ cells (Figure 1D). These data confirmed that Swc4 plays a positive role in telomere length regulation (30).

Swc4 regulates telomere length independently of its roles in NuA4 and SWR1-C

Swc4 (Eaf2) is a shared subunit of NuA4 and SWR1-C (Figure 2A). NuA4 is a histone acetyltransferase. It is comprised of 13 subunits (encoded by essential genes *ACT1*, *ARP4*, *EPL1*, *ESA1*, and *TRA1*; non-essential genes *YAF9*, *EAF1*, *SWC4* (*EAF2*), *EAF3*, *YNG2* (*EAF4*), *EAF5*, *EAF6* and *EAF7*) (Figure 2A), and responsible for the acetylation of histone H4K5, 8, 12 and 16 (52,53). Esa1 possesses acetyltransferase activity (52), and is the catalytic subunit. SWR1-C is a chromatin remodeling complex. It is comprised of 14 subunits (encoded by essential genes *ACT1*, *ARP4*, *RVB1*, and *RVB2*; non-essential genes *YAF9*, *ARP6*, *BDF1*, *SWR1*, *VPS72* (*SWC2*), *SWC3*, *SWC4*, *SWC5*, *VPS71* (*SWC6*) and *SWC7*) (Figure 2A), and responsible for histone variant H2A.Z deposition (54–56). Swr1 possesses ATPase activity (56), and is indispensable for the chromatin remodeling activity of SWR1-C. NuA4 and SWR1-C share four common subunits Act1, Arp4, Swc4 (Eaf2) and Yaf9 (Figure 2A). *ACT1* and *ARP4* are essential genes, and deletion of either *ACT1* or *ARP4* leads to cell death, while *SWC4* and *YAF9* are not essential, but deletion of *SWC4* causes slow growth phenotype (34).

Because Swc4 is a shared subunit of NuA4 and SWR1-C, it remains possible that the effect of Swc4 on telomere length is attributed to its roles in either NuA4 or SWR1-C. To test this hypothesis, we first constructed $esa1^{E338Q}$ and *htz1*Δ mutants. The catalytic dead $esa1^{E338Q}$ mutant displayed a significant reduction of global histone H4 acetylation as expected (Figure 2B) (57–59), while the *htz1*Δ mutant lacked H2A.Z in chromatin (Figure 2C). Notably, both mutants had no defect in telomere length (Figure 2D), suggesting that telomere shortening in *swc4*Δ cells was not likely attributed to either the change of histone acetylation or the defect of histone variant H2A.Z deposition. We next examined telomere length in various mutants of both NuA4 and SWR1-C, in each of which a single non-essential gene was deleted. Interestingly, deletion of individual non-essential gene of either NuA4 (*YAF9*, *EAF1*, *EAF3*, *EAF5*, *EAF6, EAF7* and *YNG2*) or SWR1-C (*YAF9*, *VPS71*, *VPS72*,

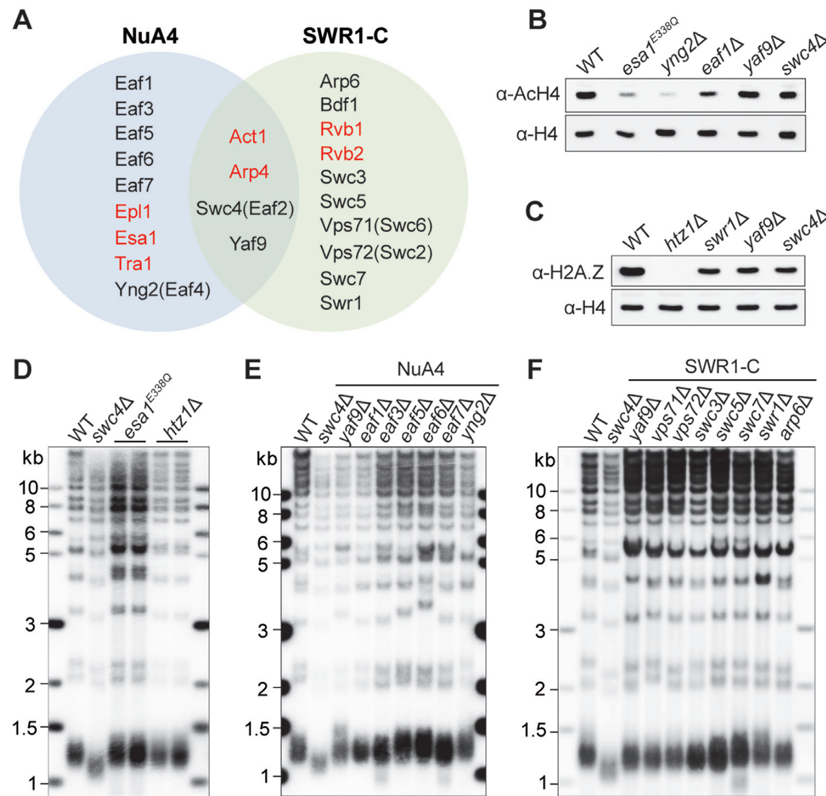

Figure 2. Swc4 affects telomere replication independently of its roles in NuA4 and SWR1 (SWR1-C) complexes. (**A**) Schematic diagram of yeast NuA4 and SWR1-C. The essential subunits of NuA4 and SWR1-C are highlighted in red. (**B**) Western blot examining acetyl-histone H4. Whole-cell protein extract from the isogenic strains (labeled on top) was subjected to western blot analysis with anti-acetyl-histone H4 antibody (Millipore: 06–598) and histone H4 antibody (Active Motif: 61521) (loading control). (**C**) Western blot examining chromatin associated histone H2A.Z. Chromatin from the isogenic strains (labeled on top) was extracted, and H2A.Z was detected with anti-histone H2A.Z antibody (Millipore: 07–718) and histone H4 antibody (loading control). (**D**) Southern blotting assay of telomere length in two independent clones of both *esa1*^{E338Q} and *htz1*Δ mutants. (**E**, **F**) Southern blotting assay of telomere length in the non-essential gene-deletion mutants of NuA4 (E) and SWR1-C (F). The genotypes of the strains are indicated on top of the panel.

SWC3, *SWC5*, *SWC7*, *SWR1*, *ARP6*) did not result in shorter telomeres seen in *swc4*Δ mutant (Figure 2E and F). Notably, deletion of *YAF9*, another shared subunit of NuA4 and SWR1-C, had little effect on telomere length (Figure 2E and F). Importantly, deletion of either *EAF1* or *SWR1* did not cause telomere shortening (Figure 2E and F), though *EAF1* and *SWR1* deletion resulted in collapse of NuA4 and SWR1-C, respectively (51,60). These results consistently indicated that Swc4 regulates telomere length independently of the integrity of either NuA4 or SWR1-C. To validate this notion further, we attempted to construct *eaf1*Δ *swr1*Δ double mutant, but *eaf1*Δ *swr1*Δ double mutant was inviable (Supplementary Figure S4) (54). Instead, we obtained *eaf3*Δ *swr1*Δ, *eaf5*Δ *swr1*Δ and *eaf7*Δ *swr1*Δ double mutants, and assumed that both NuA4 and SWR1-C were disassembled (60,61). A Southern blotting result showed little change of telomere length in these double mutants (Supplementary Figure S5). Thus, we concluded that Swc4 specifically regulates telomere length, and this function is independent of its roles in both NuA4 and SWR1-C.

Deletion of *SWC4* does not affect the expression of telomere associated genes

Previous studies have shown that Swc4 interacts with Yaf9 directly (62,63), and deletion of *YAF9* leads to changes of gene expression, histone H4 acetylation, and H2A.Z replacement near telomeres (64). It remains possible that the telomere length defect(s) in *swc4*Δ cells is resulted from the expression changes of telomerase components and/or telomere-associated proteins. To test this hypothesis, we analyzed the expression levels of TLM genes (Supplementary Table S3 and S4) in both *swc4*Δ and wild-type cells from our previous transcriptome array assay (34). The results showed these genes displayed similar transcriptional profile (Figure 3A and Supplementary Figure S6). Notably, *SWC4* deletion did not affect the transcription of genes, which encode telomerase catalytic subunit Est2, telomere capping factors (Cdc13, Stn1, Ten1, Rap1), telomerase recruitment factors (Yku70, Mre11, Rad50 and Tel1) and telomerase inhibitor Pif1 (Figure 3B). These results support the idea that Swc4 might directly regulate telomere length.

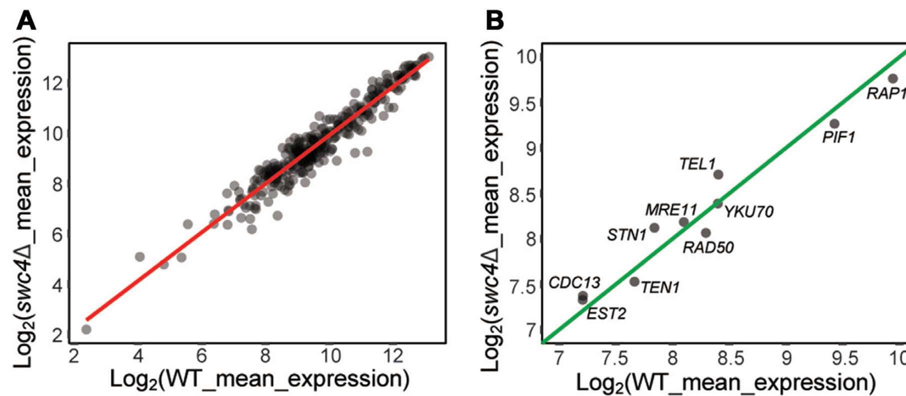

Figure 3. Deletion of *SWC4* does not alter the transcriptional profile of telomere associated genes. (**A**) Scatter plot showing the $\log_2$(mean_expression) value of two independent clones distribution of WT and *swc4*Δ cells. The fitted curve in red has a slope of 0.9659. *R*-square is 0.9424. (**B**) Scatter plot showing the $\log_2$(mean_expression) value of some representative telomere associated genes of WT and *swc4*Δ cells. The difference of the expression level of the genes indicated on plot between WT and *swc4*Δ cells is statistically insignificant ($P > 0.05$, Student's *t*-test). The green diagonal line represents correlations of +1.

The N-terminal 220 amino acids of Swc4 are required for its function at telomeres

Swc4 is a 55 kDa protein. Its N-terminal contains a SANT-domain, which is suggested to interact with histones (65); its C-terminal contains a YID (Yaf9 Interacting Domain) domain because it directly interacts with Yaf9 (62). Truncation of SANT domain of Swc4 in yeast cells nearly phenocopies the slow growth of *SWC4* deletion (34); while truncation of YID domain results in defects in H2A.Z deposition (34,50). In order to dissect further the role of Swc4 in telomere function, we constructed several deletion/truncation mutants of *swc4*, namely *swc4*-NΔ (lack of Ser2-Glu159), *swc4*-SANTΔ (lack of SANT domain, Ser160-Asn220), *swc4*-YIDΔ (lack of YID domain, Val406-Thr457), *swc4*-CΔ (lack of Pro221-Lys476) (Figure 4A). We performed growth curve and serial dilution assays, and found that *swc4*-SANTΔ mutant grew as poorly as *swc4*Δ mutant as previously reported (34) (Figure 4B and Supplementary Figure S7); *swc4*-NΔ mutant displayed a less severe growth defect than *swc4*Δ cells; while *swc4*-YIDΔ and *swc4*-CΔ mutants exhibited little growth defect (Figure 4B and Supplementary Figure S7). These results suggest that the SANT domain, but not the C-terminal part (including the YID domain), is important for the function of Swc4 in cell growth. Interestingly, the telomeres in both *swc4*-NΔ and *swc4*-SANTΔ mutants were as short as those in *swc4*Δ mutant (Figure 4C), indicating that the N-terminal part (Ser2 to Asn220, including the SANT domain) is required for the role of Swc4 in telomere length regulation. In contrast, the telomeres in both the *swc4*-YIDΔ and *swc4*-CΔ mutants were nearly as long as those in wild-type cells (Figure 4C), consistent with the result that deletion of *YAF9* did not affect telomere length (Figure 2E and F), and indicating that the C-terminal part (including the YID domain), is largely dispensable for telomere length control. A series of 40-amino-acids deletion in the N-terminal part of Swc4 confirmed that the N-terminal part except the very N-terminal 40 amino acids affects cell growth modestly (Figure 4D and E), but is indispensable in maintaining normal telomere length (Figure 4F). These results further support the conclusion that Swc4 functions alone, but not with other components of either NuA4 or SWR1-C, to regulate telomere length.

Epistatic analyses of Swc4 and other telomere length regulators

Cdc13-Est2 fusion protein mediated telomere over-elongation was not detected in *swc4*Δ cells (Figure 1A and B), suggesting that Swc4 is involved in a pathway other than Cdc13-Est1 telomerase recruitment. To clarify whether Swc4 affects Tlc1–Yku–Sir4 pathway of telomerase recruitment, we constructed the haploid strains of *swc4*Δ, *yku80–135i* and *swc4*Δ *yku80–135i* mutants, and performed Southern blotting assay to examine telomere length. The results showed that the short telomeres in *swc4*Δ and *yku80–135i* cells were further shortened in *swc4*Δ *yku80–135i* cells (Figure 5A). Consistently, *sir4*Δ *swc4*Δ double mutants displayed shorter telomeres than either *sir4*Δ or *swc4*Δ single mutant (Figure 5B), these data together suggested that Swc4 is not epistatic to Tlc1–Yku–Sir4 pathway in telomere length regulation. Additionally, the replacement of Yku80 with Yku80–135i could not grant the full function of Cdc13–Est2 fusion protein (Figure 5C). Moreover, deletion of *SWC4* in *est1*Δ *CDC13–EST2* resulted in telomere recombination (Supplementary Figure S8). This result was analogous to that observed in *yku80*Δ *est1*Δ *CDC13-EST2* cells (Supplementary Figure S9). Thus, we suggest Yku80 and Swc4 function independently in telomerase activity regulation.

We also analyzed the epistatic effect of *SWC4* on *RIF1* and *RIF2*, and found that telomeres in *rif1*Δ *swc4*Δ, *rif2*Δ *swc4*Δ and *rif1*Δ *rif2*Δ *swc4*Δ cells were shorter than that in *rif1*Δ, *rif2*Δ and *rif1*Δ *rif2*Δ cells, respectively, but were longer than that in *swc4*Δ cells (Figure 5D, E and Supplementary Figure S10), suggesting that over-elongation of the telomeres in *rif1*Δ and *rif2*Δ cells partially requires Swc4. Notably, *rif1*Δ *swc4*Δ cells grew as slow as *swc4*Δ cells (Supplementary Figure S11), indicating that the poor growth of *swc4*Δ cells was not directly attributed to the shorter telomeres.

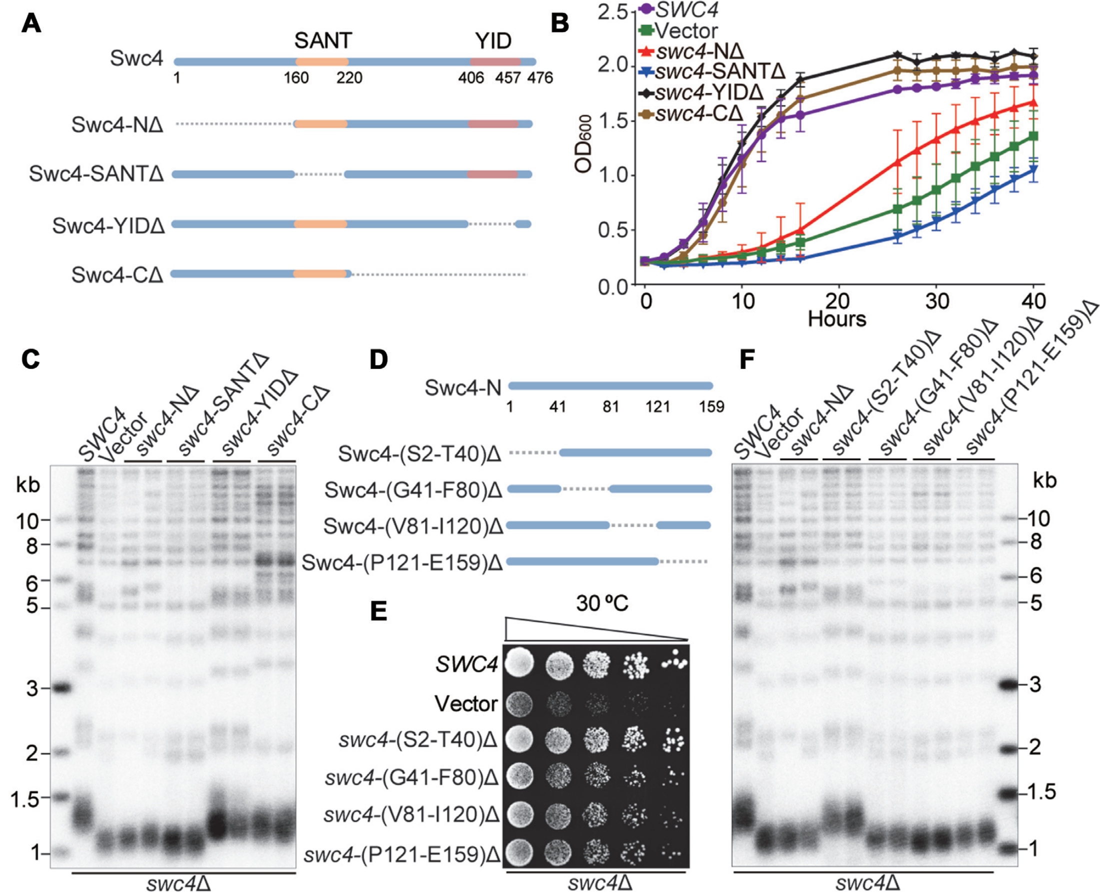

Figure 4. The N-terminal 220 amino acids of Swc4 are critical for telomere replication. (**A**) Schematic digram of structures of Swc4 and its mutants (labeled on left). The SANT domain (in canary) and Yaf9 Interacting Domain (YID, in pink) and deleted regions (dashed lines) are indicated. (**B**) Growth curves of Swc4 truncated mutants. Three individual colonies of indicated strains grown to stationary phase were diluted in 40 ml of fresh YC (His–) medium to the density at OD600 = 0.2, then the cell density (OD_{600}) was measured. Error bars represent standard deviation (s.d.), $n = 3$. (**C**) Southern blotting assay of telomere length in the isogenic strains labeled on top. Cells were restreaked on histidine$^-$ plate for at least 5 times prior to Southern blotting assay. (**D**) Schematic digram of structures of Swc4-N mutants, and the truncated amino acids are indicated at left of the panel. (**E**) Cell growth analysis. Five-fold serial dilutions of the mutants (labeled on left) were spotted onto leucine$^-$ plate and grown for ∼72 h at 30°C. (**F**) Southern blotting assay of telomere length in the isogenic strains labeled on top. Cells were restreaked on leucine$^-$ plate for at least 5 times prior to Southern blotting assay.

Deletion of *SWC4* accelerates senescence of telomerase null cells

If Swc4 functions independently of telomerase recruitment, it was intriguing to know whether Swc4 affects telomeres through telomerase pathway. We obtained haploid *tlc1*Δ, *swc4*Δ single and *tlc1*Δ *swc4*Δ double mutant spores derived from *TLC1*/*tlc1*Δ *SWC4*/*swc4*Δ heterozygous diploid strain, and performed cellular senescence assay. On solid media, *tlc1*Δ cells senesced at the third passage and reached crisis at the fourth passage, while *tlc1*Δ *swc4*Δ cells senesced at first passage and reached crisis at the second passage (Figure 6A). In liquid media, *swc4*Δ cells held regular growth rate during continuous 11 passages; *tlc1*Δ cells gradually lost viability, senesced at the sixth passage, and regained growth potential rapidly afterward (Figure 6B). In sharp contrast, *tlc1*Δ *swc4*Δ cells lost viability and senesced abruptly at the first passage, and recovered at the third passage (Figure 6B). These data together suggested that deletion of *SWC4* accelerated the senescence rate of *tlc1*Δ cells. Further telomere Southern blotting assay revealed that *tlc1*Δ *swc4*Δ cells displayed much faster telomere shortening and much sooner telomere recombination than *tlc1*Δ cells (Figure 6C). Consistently, telomere recombination took place at the third and second passage in *est1*Δ *swc4*Δ and *est2*Δ *swc4*Δ cells, respectively, much sooner than *est1*Δ and *est2*Δ cells (Supplementary Figure S12), supporting the notion that lack of *SWC4* accelerates the telomere shortening rate as well as the senescence rate of telomerase null cells.

To address whether *SWC4* deletion accelerated telomere shortening of telomerase null cells by affecting the resection of telomeric C-strand, we constructed *exo1*Δ, *exo1*Δ *swc4*Δ, *sgs1*Δ, *sgs1*Δ *swc4*Δ, *mre11*Δ and *mre11*Δ *swc4*Δ mutants, and detected the G-overhang by the native Southern blotting assay described previously (36,37). As positive and negative controls (8,66), *rif1*Δ *rif2*Δ and *mre11*Δ cells displayed significant increase and decrease of telomeric ssDNA respectively, compared with wild-type cells (Supplementary Figure S13A). Deletion of *SWC4* seemed to result in a notable decrease of the telomeric ssDNA in WT

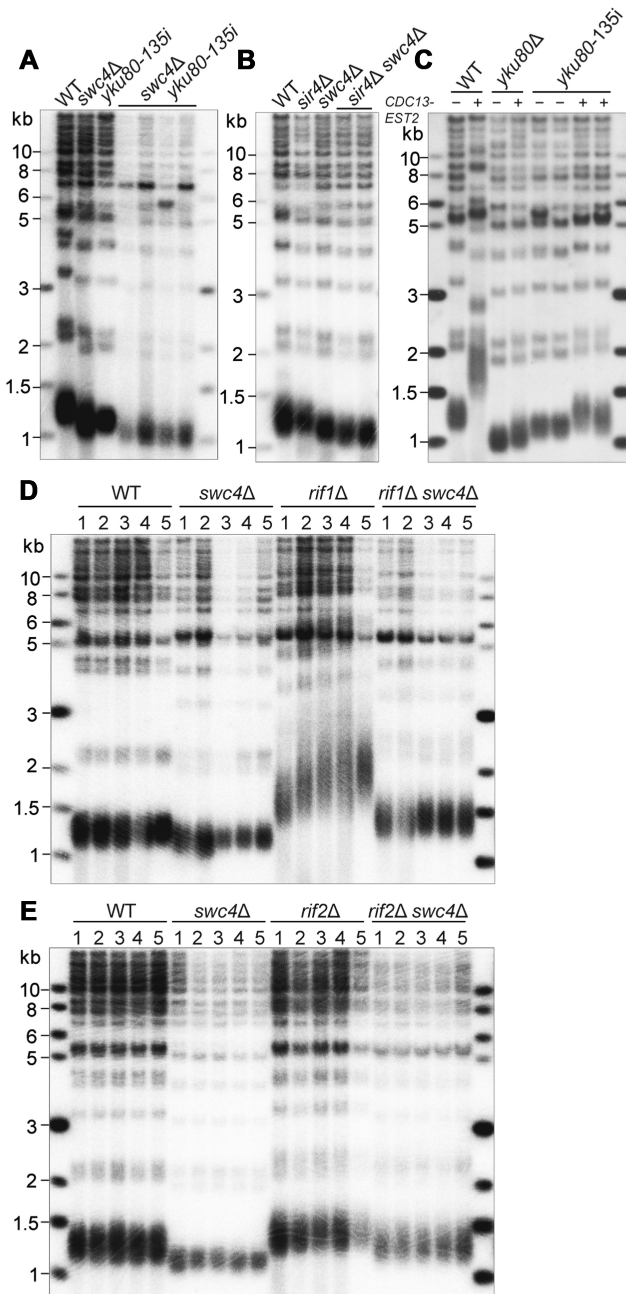

Figure 5. Epistatic analyses of Swc4 and other telomere length regulators. Southern blotting assay of telomere length in the isogenic strains labeled on the top of each panels. The DNA markers are labeled on left. (**A**) The *yku80*Δ and *swc4*Δ *yku80*Δ haploid strains contained episomal *yku80–135i* allele were restreaked at leucine⁻ plate for at least 5 times prior to Southern blotting assay. (**B**) *SWC4*/*swc4*Δ *SIR4*/*sir4*Δ diploid strain was sporulated, and the isogenic strains derived from the spores were restreaked at YPD plate for 5 times prior to Southern blotting assay. (**C**) The isogenic strains were transformed with pRS316 (−) or pRS316-*CDC13-EST2* (+), streaked on plates for at least 5 times and telomere length was examined. (**D**, **E**) *SWC4*/*swc4*Δ *RIF1*/*rif1*Δ (D) and *SWC4*/*swc4*Δ *RIF2*/*rif2*Δ (E) diploid strains were sporulated, and the spores dissected from tetrads were restreaked at YPD plate for 5 times prior to Southern blotting assay.

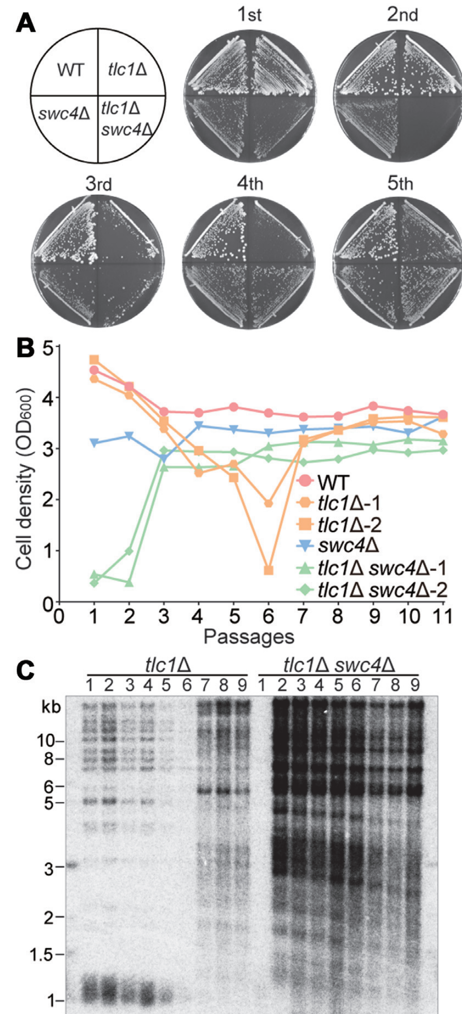

Figure 6. *SWC4* deletion accelerates telomere shortening and cellular senescence in *tlc1*Δ mutants. (**A**) Senescence assay on plate. WT, *swc4*Δ, *tlc1*Δ and *tlc1*Δ *swc4*Δ cells dissected from *SWC4*/*swc4*Δ *TLC1*/*tlc1*Δ diploid strain were continuously restreaked on YPD plate for 5 times, every plate was incubated at 30°C for ∼72 h before imaging. (**B**) Senescence assay in liquid medium. WT, *swc4*Δ, *tlc1*Δ and *tlc1*Δ *swc4*Δ cells dissected from *SWC4*/*swc4*Δ *TLC1*/*tlc1*Δ diploid strain were grown to staturation, and then diluted to $OD_{600} = 0.01$ in fresh medium for continuous passage. The passages were not the same time for WT/*tlc1*Δ cells and *swc4*Δ/*swc4*Δ *tlc1*Δ cells, WT and *tlc1*Δ cells were passaged once every 24 h, *swc4*Δ and *tlc1*Δ *swc4*Δ cells were passaged once every 96 h. (**C**) The telomeres of *tlc1*Δ and *tlc1*Δ *swc4*Δ cells collected from (**B**) were examined by Southern blotting assay. The numbers of passages indicated on top are corresponding to that in (**B**).

and *mre11*Δ cells, but not in *exo1*Δ or *sgs1*Δ cells (Supplementary Figure S13A). However, because deletion of *SWC4* causes cell cycle defects, i.e. most of the *swc4*Δ cells have prolonged G2/M phase (34,51), while telomeres acquire ssDNA late in S phase (67,68), it remains possible that the decrease of telomeric ssDNA seen in *swc4*Δ cells is resulted from the G2/M phase defect. To address this, we compared the telomeric ssDNA level of WT and *swc4*Δ cells arrested in G2/M phase, and the result showed that the amount of telomeric ssDNA of *swc4*Δ cells was comparable to that of WT cells (Supplementary Figure S13B), suggesting the de-

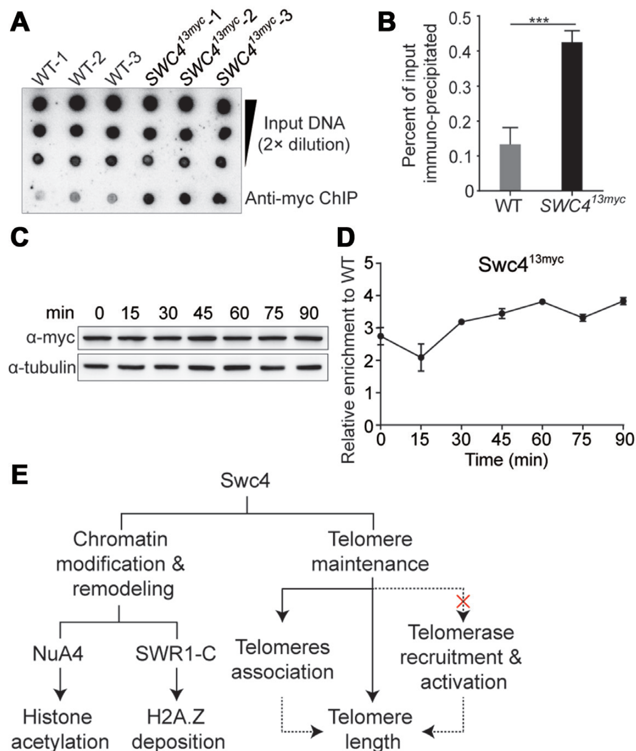

Figure 7. Swc4 associates with telomeres throughout the cell cycle. (**A**) Dot blot analysis of telomeric DNA precipitated by cross-linked Swc4^{13myc}. The anti-myc immunoprecipitated DNA and the 2-fold serial diluted input DNA were denatured and spotted on Amersham Hybond N^{+} membrane (GE healthcare), telomeric DNA was probed by $C_{1–3}A$ sequences. (**B**) Quantification results of (A). The grayscale of immunoprecipitated DNA by anti-myc antibody of WT and Swc4^{13myc} was measured by ImageJ software, and normalized to the grayscale of their corresponding input DNA. Error bars represent the standard deviation from three independent clones. *** represents $P < 0.001$ (Student's *t*-test). (**C**) Cells collected every 15 min during the cell cycle were subjected to western blot analysis. Tubulin serves as a loading control. (**D**) The myc tag–dependent fold enrichment of TEL6R over WT (non-tagged) was determined by ChIP-qPCR. Error bars represent the standard deviation. Results were observed in two independent clones. (**E**) A model for the independent functions of Swc4 on chromatin remodeling and telomere maintenance. Swc4 associates with telomeres and positively regulates telomere length, which is independent of telomerase recruitment and activation. The function of Swc4 on telomere maintenance is separable from its canonical roles in both NuA4 and SWR1-C.

creased telomeric ssDNA observed in *swc4*Δ cells is likely attributed to the prolonged G2/M phase.

Swc4 associates with telomeric DNA *in vivo*

In order to validate the direct role of Swc4 at telomeres, we performed chromatin immune-precipitation assay. The chromatin in the cells expressing Swc4^{13myc} and non-tagged cells was cross-linked and sonicated (40). Monoclonal anti-myc antibody was used to precipitate Swc4^{13myc}-associated chromatin, and precipitated DNA was purified and examined by dot-blotting with a telomeric $C_{1–3}A$ probe. The result showed that Swc4^{13myc}-associated telomeric DNA was significant enriched compared to that in non-tagged control cells (Figure 7A), quantification results showed about 0.4% of the input telomeric DNA was immunoprecipitated (Figure 7B), which was about 4-fold over non-tagged control, suggesting that Swc4 associates with telomeric chromatin *in vivo*, and the role of Swc4 at telomeres is direct. The association of Swc4 with telomeres in different stages of cell cycle was further analyzed. We arrested cells at G1 phase by alpha-factor and then released them into cell cycle, FACS analysis revealed *SWC4^{13myc}* strain displayed similar cell cycle progression to non-tagged strain (Supplementary Figure S14), and the protein level of Swc4^{13myc} remained unchanged during the cell cycle (Figure 7C). The telomere association of myc-tagged Swc4 were measured by ChIP assay followed by real time PCR quantitation, the result showed that Swc4 constantly associated with telomeres during the whole cell cycle (Figure 7D and Supplementary Figure S15). Consistently, the fold enrichment measured by ChIP-qPCR was comparable with the dot blot results (Figure 7B and D). Taken together, we concluded that Swc4 affects telomere length directly.

DISCUSSION

Although previous large scale screenings have identified >280 non-essential genes that affect telomere length in budding yeast (27–30), for most of the genes, their precise function and/or direct involvement in telomere length regulation are still largely unknown. In this study, we took advantage of the phenotype of over-long telomeres in the cells that express Cdc13–Est2 fusion protein, and performed a screening to search for TLM^{+} genes that sustain telomere maintenance independently of telomerase recruitment. On one hand, our screening seems to be very efficient. In most of the single-gene deletion mutants (171 out of 195), the expression of Cdc13–Est2 fusion protein results in over-long telomeres as seen in wild-type cells (Supplementary Figure S1A–L and Supplementary Table S1), suggesting that these TLM^{+} genes do not significantly affect telomerase activity once telomerase is tethered to telomeres. But on the other hand, our original idea, which uses Cdc13–Est2 fusion protein to screen genes that only function independently of telomerase recruitment, might be too simple (to be naïve). Instead, our screening likely helps to identify genes that affect several telomerase-associated events in addition to recruitment, including the genes affecting telomerase or telomere binding protein expression (such as Rtf1 and Cdc73 of Paf1 complex, NMD complex) (42,44), telomerase localization (Mtr10) (45), telomere deprotection (KEOPS complex and Xrn1) (43,47).

Chromatin associated activities such as histone modification, chromatin remodeling and chromatin cohesion have been reported to affect telomere replication (39,59,69–71). In our current screening, we also find out that *HFI1* (an adaptor protein required for structural integrity of the SAGA complex) and *SWC4* (a shared subunits of both NuA4 acetyltransferase and SWR1 chromatin remodeling complexes) are required for Cdc13–Est2 mediated telomere over-elongation (Table 1 and Figure 1A). *SWC4* has been thought essential for cell viability (*Saccharomyces* Genome Deletion Project), and thus it was not included in several genome-wide screenings that aimed for searching genes in telomere regulation (27–29,42). *SWC4* was found to be non-essential in a screening that we performed previously to

identify telomere maintenance genes (30). *swc4*Δ cells display low viability (Supplementary Figure S3), and a much slower growth rate than wild-type cells (Figure 4B and Supplementary Figure S7) (34), which explained why *SWC4* was considered as an essential gene.

Although Swc4 is a subunit shared by both NuA4 and SWR1-C that contain multiple subunits (Figure 2A), respectively, the telomere phenotypes of *swc4*Δ cells appear not to be resulted from the dysfunctions of NuA4 and/or SWR1-C activities. This conclusion is supported by several lines of evidence. (1) Deletion of any of the other non-essential genes in NuA4 or SWR1-C causes no significant change of telomere length (Figure 2E and F). (2) In previous genome-wide screenings carried out by independent groups, none of the non-essential genes (except *SWC4*) in NuA4 and/or SWR1-C were identified to affect telomere length (27–30). (3) Simultaneous disassembly of both NuA4 and SWR1-C does not affect telomere length (Supplementary Figure S5). Additionally, telomere shortening seen in *swc4*Δ is not likely an indirect cause of chromatin structure change. A few observations support this argument. (1) Deletion of *SWC4* has no apparent effect on the level of global histone H4 acetylation (Figure 2B) (34,51). (2) *swr1*Δ, *yaf9*Δ and *htz1*Δ cells have defects in depositing H2A.Z into chromatin (Figure 2C), but do not display telomere length defects (Figure 2D and F). (3) Truncation of YID domain of Swc4, which is required for H2A.Z deposition (34,50), does not cause telomere length defect (Figure 4C). Moreover, deletion of *SWC4* causes little change the expression of TLM genes (Figure 3A and Supplementary Figure S6), including those encoding telomerase and telomere binding proteins (Figure 3B). Therefore, we conclude that Swc4 plays a positive role in telomere length regulation independently of its canonical roles in NuA4 and/or SWR1-C (53,56).

Swc4 is identified in the current screening searching for genes that sustain telomere maintenance independently of telomerase recruitment (Figure 1A and B), but the molecular mechanism by which Swc4 regulates telomere length remains elusive. Swc4 seems not to function epistatically with Yku80, Sir4, Est1 or Rif1/Rif2 (Figure 5A, B, D and E and Supplementary Figures S8 and S10). Consistently and interestingly, deletion of *SWC4* in *tlc1*Δ, *est1*Δ or *est2*Δ cells accelerates telomere shortening and/or cellular senescence (Figure 6A-C and Supplementary Figure S12), suggesting that Swc4 might function in telomere protection, which is also supported by two other observations. (1) Telomere recombination seems to be induced in the absence of Swc4 (Figure 6C and Supplementary Figure S12). (2) In addition to telomere length defect, *swc4*-NΔ and *swc4*-SANTΔ mutants display slow growth (Figure 4B and C).

The SANT domain of Swc4, which is suggested to bind histone tails (65), appears to be important for its telomere function (Figure 4C). Given that Swc4 associates with telomeres (Figure 7A and D), but telomeric $TG_{1-3}/C_{1-3}A$ sequence is not likely to form nucleosomes (72). We thus favor the model that Swc4 interacts with subtelomeric chromatin to affect telomere length independently of telomerase recruitment and activation (Figure 7E), and the function of Swc4 on telomere maintenance is separable from its canonical roles in both NuA4 and SWR1-C (Figure 7E). Swc4 is evolutionarily conserved from yeast to higher eukaryotes (50). It will be intriguing to find out whether our findings on the telomere function of Swc4 is physiologically relevant in other organisms.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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