Regulation of Telomere Length by Fatty Acid Elongase 3 in Yeast

IN Volvement of Inositol PhosphatE Metabolism and Ku70/80 FunctioN

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In this study, we investigated the roles of very long-chain fatty acid (VLCFA) synthesis by fatty acid elongase 3 (ELO3) in the regulation of telomere length and life span in the yeast *Saccharomyces cerevisiae*. Loss of VLCFA synthesis via deletion of *ELO3* reduced telomere length, and reconstitution of the expression of wild type *ELO3*, and not by its mutant with decreased catalytic activity, rescued telomere attrition. Further experiments revealed that alterations of phytoceramide seem to be dispensable for telomere shortening in response to loss of *ELO3*. Interestingly, telomere shortening in *elo3Δ* cells was almost completely prevented by deletion of *IPK2* or *KCS1*, which are involved in the generation of inositol phosphates (IP4, IP5, and inositol pyrophosphates). Deletion of *IPK1*, which generates IP6, however, did not affect regulation of telomere length. Further data also suggested that *elo3Δ* cells exhibit accelerated chronologic aging, and reduced replicative life span compared with wild type cells, and deletion of *KCS1* helped recover these biological defects. Importantly, to determine downstream mechanisms, epistasis experiments were performed, and data indicated that *ELO3* and *YKU70/80* share a common pathway for the regulation of telomere length. More specifically, chromatin immunoprecipitation assays revealed that the telomere binding and protective function of *Yku80p* in vivo was reduced in *elo3Δ* cells, whereas its non-homologues end-joining function was not altered. Deletion of *KCS1* in *elo3Δ* cells recovered the telomere binding and protective function of Ku, consistent with the role of *KCS1* mutation in the rescue of telomere length attrition. Thus, these findings provide initial evidence of a possible link between Elo3-dependent VLCFA synthesis, and IP metabolism by *KCS1* and *IPK2* in the regulation of telomeres, which play important physiological roles in the control of senescence and aging, via a mechanism involving alterations of the telomere-binding/protection function of Ku.

Very long-chain fatty acids (VLCFAs), containing mainly 26 carbons in yeast, are synthesized from palmitoyl-CoA by microsomal fatty acid (FA) elongases (Elo1–3p) that catalyze the multistep chain elongation reactions (1–4). As summarized in Fig. 1A, the chain elongation, up to C24-FA, is catalyzed by both Elo2p and Elo3p, whereas elongation from C24- to C26-FA is exclusively catalyzed by Elo3p (2). Yeast sphingolipids contain predominantly C26-phytoceramide (5, 6) with hexacosanoic acid (C26-FA), which is a precursor for the synthesis of complex sphingolipids, such as inositol phosphorylceramide (IPC), mannosylinositol phosphorylceramide, and mannosylidinositol phosphorylceramide. Null mutations of *ELO3* (SUR4, YLR372W, *SRE1*, and *VBM1*), therefore, cause striking changes in the synthesis of sphingolipids and phosphatidylinositol/inositol phosphate metabolism (7–10), which are known to mediate diverse biological and signaling functions, including regulation of longevity, senescence, and telomere length (11–13).

Telomeres are multifunctional genetic elements that cap eukaryotic chromosome ends, and they play essential roles in genomic stability, oncogenic transformation, and cellular senescence/aging (14, 15). Telomeres are maintained by a specialized RNA-dependent DNA polymerase, telomerase, which adds species-specific, long-tandem nucleotide repeats (TG-repeats in yeast) to the ends of chromosomes (16). Telomeres are regulated by factors that either play a role in their protection, such as *Est1/2*, *Tel1*, *Yku70/80*, and *Rad50*, or in their rapid shortening, such as *Cdc13*, *Rif2*, and *Rap1* (14–19). The balance between these positive and negative factors determines the maintenance of telomeres in cells. Therefore, uncovering factors and signaling pathways involved in the regulation of telomeres is very important for senescence- and aging-related research.

Interestingly, in a recent study, in which the global analysis of the roles of all non-essential genes involved in the regulation of...
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TABLE 1

| Yeast strains | Genotype | Source |
|---------------|----------|--------|
| BY4742        | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Invitrogen |
| BY4742 elo1Δ  | BY4742 elo1::kanMX4 | Invitrogen |
| BY4742 elo2Δ  | BY4742 elo2::kanMX4 | Invitrogen |
| BY4742 elo3Δ  | BY4742 elo3::kanMX4 | Invitrogen |
| BY4742 elo2Δ::pGal-ELO3 (wt) | BY4742 elo2::kanMX4::pGal-ELO3(wt)::URA3 | This study |
| BY4742 elo2Δ::pGal-ELO3 (mut) | BY4742 elo2::kanMX4::pGal-ELO3(mut)::URA3 | This study |
| BY4742 elo3Δ::pGal-ELO3 | BY4742 elo3::kanMX4::pGal-ELO3(mut)::URA3 | This study |
| BY4742 elo3Δ::pGal-ELO3 | BY4742 elo3::kanMX4::pGal-ELO3(mut)::URA3 | This study |
| BY4742 pkp1Δ | BY4742 ipk2::kanMX4 | Invitrogen |
| BY4742 pkp2Δ | BY4742 ipk2::kanMX4 | Invitrogen |
| BY4742 kcs1Δ | BY4742 kcs1::kanMX4 | Invitrogen |
| BY4742 yku80Δelo3Δ | BY4742 yku80::kanMX4 elo3::URA3 | This study |
| BY4742 yku80Δelo3Δ | BY4742 yku80::kanMX4 elo3::URA3 | This study |
| BY4742 ipk1elo3Δ | BY4742 ipk1::kanMX4 elo3::URA3 | This study |
| BY4742 ipk2elo3Δ | BY4742 ipk2::kanMX4 elo3::URA3 | This study |
| BY4742 yku80-GFP | BY4742 yku80-GFP::kanMX4 | Invitrogen |
| BY4742 elo3Δ::yku80-GFP | BY4742 elo3::yku80-GFP::kanMX4 | This study |
| BY4742 kcs1Δelo3Δ::yku80-GFP | BY4742 kcs1::kanMX4 elo3::URA3 yku80-GFP::kanMX4 | This study |
| J9-3 eloΔ | J9-3 elo::URA3 | Guillas et al. (31) |
| J9-3 lag1Δ | J9-3 lag1::kanMX4 | Guillas et al. (31) |
| J9-3 eloΔ::ipk2Δ | J9-3 elo::kanMX4 elo2::URA3 | This study |
| J9-3 eloΔ::ipk2Δ | J9-3 elo::kanMX4 elo2::URA3 | This study |
| W303-1A | MATα can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 | Guillas et al. (31) |
| W303-1A lac1Δlag1Δ | W303-1A lac1::URA3 lag1::TRP1 | Guillas et al. (31) |

Telomere length in yeast were examined, the deletion of ELO3 was identified as one of the mutations that results in a significant telomere shortening (20). However, mechanisms by which Elo3p and VLCFA synthesis regulate telomeres are still unknown.

Therefore, in this study, roles and mechanisms of telomere length regulation by VLCFA synthesis via Elo3p in yeast were examined. Consistent with the previous report (20), our data also showed that deletion of ELO3, which resulted in the loss of VLCFA and C26-phytoceramide synthesis, mediated a rapid attrition of telomere length. Further experiments suggested a novel link between C26-FA synthesis by Elo3p, and inositol polyphosphate (IP) metabolism by Ipk2p and Kcs1p in the regulation of telomere length. Remarkably, the deletion of IPK2 or KCS1, which are involved in the synthesis of IP4, IP5, and PP-IP4, and not IPK1, which generates IP6, completely protected telomere attrition in elo3Δ cells. In addition, data also suggested that telomere length attrition in elo3Δ cells was concomitant with accelerated chronologic aging and reduced replicative life span, biological defects that were also recovered by the deletion of KCS1. In addition, epistasis experiments revealed that ELO3 and YKU70/80 share a common pathway for the regulation of telomere length. More specifically, deletion of ELO3 resulted in alterations of the binding and protection of telomeres by Ku70/80, whereas the non-homologue end-joining (NHEJ) function of Ku was not affected. Importantly, deletion of KCS1 in the elo3Δ strain also recovered the telomere binding and protective function of YKu70/80, consistent with the protection of telomere length shortening in these cells. Thus, these data provide initial evidence for a possible link between VLCFA synthesis by Elo3p, and IP metabolism by Ipk2p/Kcs1p in the regulation of telomeres, which are involved in the control of senescence and life span, via alterations of the telomere-binding/protective functions of Ku70/80.

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic Methods—Yeast strains used in this study are listed in Table 1. Yeast cells were grown at 30 °C in rich (YPD or YPG) or minimal media containing 2% glucose (D) or galactose (G). Theelo3::URA3 strains were generated by the one-step gene replacement method (21). The URA3 gene was amplified using 60- and 61-base oligonucleotides consisting of 40-base 5′ or 3′ flanking sequences of the ELO3 gene followed by 20- or 21-base URA3 sequences (5′-ATT CGG CTG TTT TCC GTT TGT TTA CGA AAC ATA AGT CAG CTT TTC AAT TAG TT TCT GTA AAC TCT CAG CTT TTC AT CA TCA AT T-3′, and 5′-TTT TCT TCT GTA TTC GTC GTC AAA TAT TCT CGC TCT TCA TGG GTA ATA ACT GAT ATA ATT-3′). Yeast cells were transformed with purified PCR products, and Ura+ cells were selected on minimal media lacking uracil. Desired deletion was confirmed by PCR of genomic DNA. The plasmid (designated as pGalElo3[wt] in this report) containing the ELO3 gene under the control of the GAL1 promoter, YCpGALELO3(U), was obtained from Dr. Charles E. Martin (Rutgers University, Piscataway, NJ). The pGal-Elo3(mut) containing a 24-bp deletion (corresponding to an 8-amino acid deletion at position 19–26) was generated using the QuikChange mutagenesis kit (Stratagene).

Analysis of Telomere Length—Telomere length was measured as described previously (20, 22) by Southern blotting using genomic DNA prepared from 1.5 ml of yeast cultures grown to saturation. After digestion with XhoI, DNA fragments were separated by electrophoresis on a 1% Tris/borate/EDTA/agarose gel, and transferred to a nylon membrane (Roche). Then, the membranes were probed with a digoxigenin-labeled C1–3A/mini. The average telomere length for each lane was estimated from digestion of the last Y element at chromosome termini.

FA Measurements—Total lipids were extracted from the logarithmic phase of yeast cultures, and FA levels were measured using gas chromatography/mass spectrometry (GC/MS) as...
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FIGURE 1. Roles of ELO1, ELO2, and ELO3 in the generation of FAs, and regulation of telomere length. A, the generation of VLCFAs by ELO1–3 in yeast. B, FAs were measured in WT versus ELO1, ELO2, and ELO3 mutants (BY4742 strain) as described under "Experimental Procedures." FA levels were normalized to the levels of P, C, telomere length was measured by Southern blotting using genomic DNA, isolated from ELO1, -2, and -3 mutant strains (lanes 2–4, respectively) as described under "Experimental Procedures." Lanes 1 and 5 contain molecular weight (Mr) markers. The vertical dashed lines indicate a gap between lanes M and 1 on the blot. FAs and telomere lengths were measured in duplicates at least in two independent trials. Error bars represent standard deviations.

Measurement of Phytoceramide Levels—The measurement of the levels of endogenous phytoceramides was performed using high performance liquid chromatography–tandem mass spectrometry, as described previously (24). Phytoceramide contents were normalized to total lipid phosphate (Pi) levels.

Measurement of Inositol Phosphates—The levels of IP molecules after labeling with [3H]inositol were analyzed using HPLC as described previously (25).

Determination of Chronological Aging—Chronologic aging of yeast population was determined as previously described (26). Yeast cultures were grown for 4–5 days until they reach a stationary phase in 10 ml of YPD media. Then, the cells were washed twice, and an equal number of cells were re-suspended in 10 ml of sterile distilled water, and their growth was monitored on YPD plates after serial dilutions.

Analysis of Replicative Life Span in Yeast—The replicative life span was analyzed as described previously (27). In summary, new buds (virgin cells) from overnight cultures were deposited in a row in isolated spots on an YPD plate. The number of buds removed by micro-dissection prior to cell death is defined as the life span of the cell in generations. The significance of the differences in the life spans of aging cohorts of each of the strains in these experiments was assessed using the Mann-Whitney test.

RESULTS

Deletion of ELO3, Which Results in the Loss of VLCFA Synthesis, Mediates Telomere Shortening—In addition to their structural roles in biological membranes, lipid molecules are known to transduce a myriad of signaling pathways involved in the regulation of a wide variety of biological processes, such as regulation of cell growth, proliferation, and/or senescence (11). Recently, global analysis of the roles of all non-essential genes in the regulation of telomeres in yeast revealed that loss of ELO3 results in a significant reduction in telomere length (20). However, mechanisms by which ELO3 and/or VLCFA synthesis regulate telomere length have not been determined previously. Given our interest in the roles of lipid/sphingolipid metabolism and signaling in the regulation of telomeres, we set out experiments to determine mechanisms involved in the regulation of telomeres by VLCFA synthesis, which is also an integral part of sphingolipid metabolism, via the function of Elo3 in yeast.

First, to further confirm the possible roles of Elo3-generated VLCFA containing 26 carbon units (Fig. 1A) in the regulation of telomeres, we examined the FA contents and telomere length in elo3Δ compared with WT, elo1Δ, and elo2Δ strains, using GC/MS and Southern blotting, respectively. Consistent with previous reports (2, 3, 7, 8), our data showed that deletion of ELO3 completely prevented the synthesis of C26-FA, and decreased C24-FA synthesis about 33% as compared with controls, with concomitant accumulation of C16–C22-FAs, both hydroxylated (Fig. 1B and Table 2) and unhydroxylated (data not shown) forms. A moderate decrease in the levels of C24- and C26-, and an increase in C16–22-FAs were observed in elo2Δ cells, and no significant changes were detected in VLCFA...
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TABLE 2
2-OH fatty acid levels (nmol/nmol of P_i)
The levels of 2-OH FAs in yeast strains were measured using GC/MS, and normalized to P_i levels as described under “Experimental Procedures.” C26 FA levels were undetectable (UD) in elo3Δ cells. The numbers presented represent standard deviations (n = 3).

| Mutant Strain | 2-OH C16 | 2-OH C18 | 2-OH C20 | 2-OH C22 | 2-OH C24 | 2-OH C26 |
|---------------|---------|---------|---------|---------|---------|---------|
| WT            | 32.24 ± 5.60 | 3.68 ± 0.29 | 0.4 ± 0.07 | 1.07 ± 0.18 | 8.86 ± 1.98 | 53.81 ± 5.67 |
| elo1Δ         | 29.67 ± 4.88 | 2.81 ± 0.41 | 1 ± 0.15 | 1.71 ± 0.21 | 5.19 ± 0.75 | 49.6 ± 3.1 |
| elo2Δ         | 65.37 ± 8.41 | 11.01 ± 1.87 | 12.3 ± 1.22 | 3.10 ± 0.28 | 3.6 ± 0.43 | 18.23 ± 2.11 |
| elo3Δ         | 90.52 ± 9.01 | 8.6 ± 1.54 | 14.7 ± 1.39 | 16.92 ± 1.72 | 5.92 ± 0.69 | UD |

More importantly, the data also showed that deletion of ELO3 (Fig. 1C, lane 4) decreased telomere length about 100 (+12) bp, whereas deletion of ELO1 or ELO2 (Fig. 1C, lanes 2 and 3, respectively) did not cause detectable effects when compared with the WT strain (Fig. 1C, lanes 1 and 5). Similar data were also observed in another strain (JK9–3d), in which the deletion of ELO3 decreased telomere length ~110 bp compared with the WT strain (shown in Fig. 4C). These data are consistent with the previous study (20), and suggest that alterations of the VLCFA synthesis by the deletion of ELO3 participate in a rapid reduction of telomere length.

Overexpression of WT ELO3 Reconstructs the Synthesis of VLCFA, Leading to the Prevention of Telomere Attrition in the elo3Δ Mutant Strain—Next, to examine whether the shortening of telomeres in elo3Δ cells can be reversed, VLCFA synthesis was reconstituted in these mutants using an expression vector containing WT ELO3 under the control of a galactose (GAL)-inducible promoter (2). Expression of ELO3 in the presence of GAL reconstituted the synthesis of C26-FA within 24, 48, or 72 h of induction (Fig. 2A and Table 3), and more importantly, induction of Elo3p expression markedly reversed the shortening of telomere length in elo3Δ/pGAL1-ELO3(wt) cells at these time points compared with that of uninduced or WT cells (Fig. 2B, lanes 2, 4, and 6, 3, 5, and 7, respectively). Collectively, these data suggest that deletion of ELO3 results in the reduction of telomere length, which can be recovered in response to reconstitution of WT ELO3 expression and the synthesis of VLCFA in elo3Δ cells.

Then, to determine the functional role of Elo3p activity in the regulation of telomere length at 72 h, a mutant form of ELO3, which is defective in generation of C26-FA, was expressed in elo3Δ mutants, and its effects on FA synthesis and telomere length were examined. Because the domains that are required for distinct catalytic functions of Elo1–3p are still unclear, we truncated a stretch of amino acids (LNSSSSCSF) at positions 19–26 near the N terminus of Elo3p, which is present only in yeast Elo3, but not present in Elo1p or Elo2p. The mutant form of Elo3p was then expressed in elo3Δ cells under control of the GAL promoter, and its effects on FA synthesis and on the recovery of telomere length attrition were determined. Interestingly, the data showed that expression of the mutant Elo3p, lacking amino acids 19–26, was unable to generate C26 FA as efficiently, whose activity was about 70% less than that of the WT Elo3 (Fig. 3A and Table 4). In addition, the results also showed that C22- and C24-FA levels were comparable in response to the expression of WT or mutant Elo3p (Fig. 3A and Table 4). Moreover, whereas the expression of WT-ELO3 recovered loss of telomeres of about 70%, expression of the mutated-Elo3p did not efficiently protect telomeres in elo3Δ cells (Fig. 3, B and C, lanes 3 and 4). Taken together, these data suggest that enzymatic activity of Elo3p for C26-FA synthesis, and not in the alterations of C22- or C24-FA synthesis, plays important roles in the regulation of telomere length in yeast.

Alterations of the Phytoceramide Generation Might Be Dispensable for the Shortening of Telomere Length in elo3Δ Cells—In yeast, hydroxy-C26-phytoceramide, containing the VLCFA chain, constitutes the majority of ceramides, therefore, the synthesis of VLClFA is directly involved in the regulation of phyto-

FIGURE 2. Reconstitution of WT ELO3 expression prevents telomere attrition in elo3Δ cells. A, the levels of FAs were measured as described under “Experimental Procedures.” B, the plasmid containing the full-length WT-ELO3 under a galactose (Gal)-sensitive promoter was transformed into elo3Δ cells. Then, telomere length was measured after cells were grown in the presence (“+”) or absence (“−”) of galactose for 24, 48, and 72 h (lanes 2, 4, 6, and 3, 5, 7, respectively). Lane 1 contains samples obtained from WT cells. Molecular weight markers are indicated on the left. Experiments were performed in at least two independent trials.
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TABLE 3
2-OH fatty acid levels (nmol/nmol of P_i)
The levels of 2-OH FAs in yeast strains were measured using GC/MS, and normalized to P_i levels as described under “Experimental Procedures.” C26 FA levels were undetectable (UD) in elo3Δ cells. The numbers presented represent standard deviations (n = 2).

|          | C22 | C24 | C26 | C22 | C24 | C26 | C22 | C24 | C26 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| WT       | 1.07 ± 0.2 | 8.86 ± 3.3 | 53.81 ± 11.2 | 1.08 ± 0.24 | 9.0 ± 3.45 | 50.1 ± 10.2 | 1.0 ± 0.25 | 8.9 ± 2.9 | 51.2 ± 10.9 |
| Uninduced | 18.10 | 32.09 | 7.6 | 11.2 | 3.4 | 53.8 | 7.6 | 23.19 | 8.3 |
| Induced  | 9.11 ± 2.3 | 32.09 ± 7.6 | 23.19 ± 5.2 | 7.15 ± 2.8 | 38.63 ± 8.3 | 41.85 ± 6.8 | 6.67 ± 1.8 | 19.27 ± 0.9 | 36.79 ± 13.1 |

Figure 3. Effects of the expression of WT- and mutant-elo3 on FA synthesis, and regulation of telomere length in elo3Δ cells. A, the roles of WT and mutant Elo3p in the generation of VLCFA were determined as described above. B, the reduction in telomere restriction fragment length of elo3Δ, elo3Δ/wt-elo3, and elo3Δ/mutant-elo3 were analyzed and represented in a graphical format. C, telomere length was measured by Southern blotting as described under “Experimental Procedures” using genomic DNA isolated from WT, elo3Δ, elo3Δ/wt-elo3, and elo3Δ/mutant-elo3 (lanes 1–4). Molecular weight markers are indicated on the left. Experiments were performed in at least three independent trials.

To determine whether the increase in phytoceramide with shorter FA chain lengths, especially C22-phytoceramide, which was elevated only in elo3Δ cells, is important in telomere shortening, effects of LAG1 or ISC1 gene deletion on telomere length were determined in Jk93d strain (Fig. 4C). The rationale behind these experiments was that if the higher levels of shorter chain ceramides were involved, then, deletion of LAG1 or ISC1 would play protectice roles against telomere shortening in elo3Δ cells. The data showed that a single deletion of LAG1, ISC1, or ISC1 did not have any detectable effects on telomere length (Fig. 4C, lanes 3, 5, and 7, respectively), and double deletions (elo3Δlag1Δ, elo3Δlac1Δ, or elo3Δisc1Δ) were not protective; they exhibited the same degree of telomere shortening as a single deletion of ELO3 (Fig. 4C, lanes 4, 6, and 8, respectively). Thus, these results indicated that the altered levels of phytoceramide by Lag1p or Isc1p might be dispensable in the attrition of telomeres in elo3Δ cells.

In addition, to determine whether the loss of C26-phytoceramide plays a role in telomere attrition, we also examined telomere length in lag1Δlac1Δ double mutant (in the W303-1A strain), in which C26-phytoceramide synthesis is reduced with concomitant accumulation of C26-FA (29). The data revealed no detectable shortening of telomeres in lag1Δlac1Δ cells compared with the WT strain (Fig. 4C, lanes 10 and 9, respectively), indicating that limited synthesis of C26-phytoceramide and/or an overabundance of C26-FA may not influence the shortening of telomere length in these cells.

Attrition of Telomeres in elo3Δ Cells Might Be Linked to the Alterations of IP Metabolism and/or Signaling—VLCFA and sphingolipid synthesis are known to influence phosphatidylinositol/IP metabolism (2, 4, 8, 9). Because ceramide metabolism by Lag1p/Lac1p/Isc1 did not seem to affect telomeres in elo3Δ cells, and IP signaling has been previously shown to regulate telomeres (33, 34), we examined whether alterations in IP metabolism and/or signaling are involved in telomere length shortening in response to the loss of ELO3. Telomere length was measured in ipk1Δ, ipk2Δ, or kcs1 single-deletion strains, or in mutant strains that contain their double deletions with ELO3. These genes encode inositol polyphosphate kinases involved in the synthesis of IP6, IP4/IP5, PP-IP4, or IP7 (PP-
TABLE 4
2-OH fatty acid levels (nmol/nmol of P\(_\text{i}\))
The levels of 2-OH FAs in yeast strains were measured using GC/MS, and normalized to P\(_i\) levels as described under “Experimental Procedures.” C26 FA levels were undetectable in elo3\(\Delta\) cells. The numbers presented represent standard deviations (n = 2).

|        | 2-OH C16 | 2-OH C18 | 2-OH C20 | 2-OH C22 | 2-OH C24 | 2-OH C26 |
|--------|----------|----------|----------|----------|----------|----------|
| elo3\(\Delta\) + pGalElo3\(\Delta\) (wt) | 38.75 ± 4.97 | 4.11 ± 0.82 | 1.8 ± 0.27 | 8.13 ± 1.91 | 2.1 ± 0.18 | 31.64 ± 4.7 |
| elo3\(\Delta\) + pGalelo3\(\Delta\) (mut)   | 51.44 ± 6.43 | 3.8 ± 0.79 | 4.2 ± 0.81 | 6.93 ± 1.33 | 3.96 ± 0.41 | 9.92 ± 1.43 |

FIGURE 4. Roles of ceramide metabolism in telomere shortening in response to the loss of Elo3. A, the involvement of VLCFA synthesis in the regulation of the generation of phytoceramide (PHC) via the acylation of phytosphingosine (PHS) is shown. Phytoceramide can be a precursor for the generation of complex sphingolipids, such as IPC, mannosylinositol phosphorylceramide (MIPC), and mannosylidinositol phosphorylceramide (MI[P]C). B, effects of the loss of ELO3 on the generation/accumulation of phytoceramides were determined using LC/MS/MS as described under “Experimental Procedures.” Error bars represent S.D. ± mean. C, telomere length of WT, elo3\(\Delta\), lag1\(\Delta\), elo3\(\Delta\) lag1\(\Delta\), lac1\(\Delta\), elo3\(\Delta\) lac1\(\Delta\), isc1\(\Delta\), elo3\(\Delta\) isc1\(\Delta\) cells (Jk9–3D strain) was measured by Southern blotting (lanes 1–8, respectively). The genomic DNA from WT and lag1\(\Delta\)/lac1\(\Delta\)/isc1\(\Delta\) mutants (W303-1A strain) were used in lanes 9 and 10 for telomere length measurements. Molecular weight markers are indicated on the left. Experiments were performed in at least three independent trials.
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TABLE 5
2-OH phytoceramide levels (pmol/nmol of P_i)
The levels of phytoceramides were measured using LC/MS, and normalized to P_i levels as described previously (16, 19). C26-phytoceramide levels in elo3Δ cells were undetectable. The numbers presented represent standard deviations (n = 3).

|     | OH PhyC16 | OH PhyC18 | OH PhyC20 | OH PhyC22 | OH PhyC24 | OH PhyC26 |
|-----|-----------|-----------|-----------|-----------|-----------|-----------|
| WT  | 1.9 ± 0.18| 1.8 ± 0.16| 4.5 ± 1.12| 1.5 ± 0.05| 1.6 ± 0.09| 7.9 ± 0.94|
| Elo1Δ | 3.3 ± 1.01| 3.7 ± 0.91| 3.5 ± 0.98| 1.9 ± 1.16| 1.3 ± 0.51| 7.6 ± 0.71|
| Elo2Δ | 16.4 ± 3.27| 11.3 ± 1.26| 17.8 ± 2.59| 2.6 ± 1.22| 8.5 ± 1.46| 3.8 ± 0.23|
| Elo3Δ | 3.3 ± 0.54| 3.2 ± 0.54| 3.9 ± 0.33| 4.9 ± 1.01| 11.1 ± 1.9 | UD         |
| Elo3Δ+pGalElo3 Δmut | 3.6 ± 0.77| 3.2 ± 0.88| 5.6 ± 1.00| 4.5 ± 0.81| 7.0 ± 1.11| 1.3 ± 0.09|

* UD, undetectable.

A significant decrease (p < 10^-5) was also observed when KCS1 was deleted, resulting in a mean life span of 13.3 generations and a maximum of 23. When both deletions were combined (elo3Δkcs1Δ), the life span was statistically indistinguishable (p = 0.37) from the WT, and the mean and maximum life spans were 26.4 and 43, respectively. Thus, these results suggest that loss of ELO3 and KCS1 cooperate for telomere-dependent and/or independent regulation of replicative life span.

Mechanisms of Telomere Shortening in elo3Δ Mutants
Involves Alterations of Telomere Binding and a Protective Function of Yku80Δ—To determine the downstream mechanisms involved in telomere shortening in elo3Δ cells, epistasis experiments were performed with double mutants containing deletions of genes, which result in shortening of telomeres, such as TEL1, EST1, RAD50, and YKU70Δ/80Δ (20) in the elo3Δ mutant strain. Telomeres were measured in single and double deletion mutants compared with WT cells. As shown in Fig. 8A, single deletions of TEL1, RAD50, and EST1 (Fig. 8A, lanes 5, 7, and 9, respectively, and Fig. 8B) resulted in about a 220-, 230-, or 232-bp decrease in telomere length when compared with the WT strain, respectively. Deletion of ELO3 in these mutants (tel1Δelo3Δ, rad50Δelo3Δ, and Δest1Δelo3Δ) further decreased telomere length (Fig. 8A, lanes 6, 8, and 10, respectively), suggesting that ELO3 and these telomere-associated proteins function in distinct pathways for the regulation of telomere length. In contrast, deletion of ELO3 in yku80Δ and yku70Δ strains (Fig. 8A, lanes 4 and 12, respectively) did not further decrease telomere length compared with yku80Δ and yku70Δ single deletions (Fig. 8A, lanes 3 and 11, respectively, and Fig. 8B), which caused ~220-bp reduction in telomere length. These data suggest that ELO3 and YKU70/80 might share a common pathway for the regulation of telomere length.

The KU70Δ/80Δ binds and protects telomeres (13, 35). To determine whether loss of ELO3 alters the telomerase-binding/protection function of YKu, endogenous Yku80Δ was fused to GFP at its C terminus in WT and elo3Δ cells, and its in vivo association with telomeric DNA was examined by ChiP analysis. The data showed that although there were no alterations in nuclear localization (data not shown) or expression (Fig. 8C, right panel, lanes 3 and 4), the telomere-binding function of Yku80Δ-GFP was reduced around 60% in elo3Δ compared with WT cells (Fig. 8C, left panel, lane 2). More importantly, deletion of KCS1 in elo3Δ mutants (elo3Δkcs1Δ), which prevented telomere attraction, also markedly recovered the telomere-binding function of Yku80Δ-GFP (Fig. 8C, left panel, lane 2).

It is known that KU70Δ/80Δ also plays a key role in NHEJ activity (36–38), which can be measured as the ability of cells to form viable colonies after transformation with a linearized plasmid that contains the IURA gene. To examine whether deletion of ELO3 has any effect on the NHEJ function of Ku, WT, yku80Δ, and elo3Δ cells were transformed with the linearized
pYES2 plasmid containing LIRA, and their growth in the absence of uracil was measured. Interestingly, the data showed that loss of ELO3 did not alter the NHEJ function of Ku70/80, and the number of surviving colonies after transformation using linearized plasmids in elo3Δ cells was similar to WT cells (Fig. 8D). As expected, in yku80Δ cells, the colony numbers were decreased ~70% when cells were transformed with linearized plasmids compared with controls (cells transformed using circular pYES2), indicating altered NHEJ in the absence of Ku80 (Fig. 8D). Thus, taken together, these data reveal that telomere attrition mediated by the loss of VLCFA in elo3Δ cells might be mechanistically linked to the altered telomere-binding/protection function of Yku70/80, and not its NHEJ activity.

DISCUSSION

Results presented here demonstrate that loss of VLCFA synthesis via deletion of ELO3 results in a significant attrition of telomere length, which is prevented by the deletion of IPK2 or KCS1, encoding inositol polyphosphate kinases, suggesting the involvement of IP metabolism and/or signaling in this process. Additional data showed that deletion of ELO3 accelerated chronologic aging/senescence, and reduced replicative lifespan, and these biological defects were corrected by the deletion of KCS1 in elo3Δ cells. Epistasis studies suggested that ELO3 and YKU share a common pathway for the regulation of telomeres. Importantly, mechanistic studies revealed that alterations of the telomere binding/protection function of YKu is involved in telomere shortening in response to loss of ELO3/VLCFA, which was also recovered by the deletion of KCS1. These studies then provide novel mechanistic information about the Ku-dependent regulation of telomeres via VLCFA synthesis by Elo3.

In Saccharomyces cerevisiae, VLCFA comprises 1–2% of the total FA composition, and plays critical roles in maintaining cellular integrity, regulation of vacuolar function (3), membrane trafficking (39), or raft association of Pma1p (plasma membrane ATPase) (40). In addition, our data suggested a novel role for VLCFA synthesis by Elo3p in the regulation of telomere length. These data are consistent with the previous study (20) that identified the deletion of ELO3 as one of the mutations that caused attrition of telomeres, after the global analysis of the roles of all non-essential genes in yeast. Importantly, FA measurements suggested that loss of C26-FA (or its decreased levels below 10 nmol/nmol of P, as detected after the
Strains were 28.1 (48), 23 (44), 13.3 (23), and 26.4 (43) generations, respectively.

**FIGURE 8.** Elo3 shares a common pathway with Ku70/80 for the regulation of telomere length.

Regulation of Telomere Length by Fatty Acid Elongase 3

Telomere length was measured in WT, whereas the difference between WT and life span in yeast was determined. Dilutions following growth in stationary phase in water for several days, and then grown at 30 °C for 24 h, the replicative life span characteristics of cells were analyzed as described under “Experimental Procedures.” The number of daughter cells produced by a single mother cell was counted using microdissection. In each life span, determination of 43 cells/strain were used, which were performed in three independent experiments. The statistical analysis of the data were performed using Mann-Whitney (rank test), and p < 0.05 was considered significant. The differences in life span between WT and elo3Δ, and kcs1Δ were significant (p = 0.0043, and p < 10⁻⁶, respectively), whereas the difference between WT and elo3Δkcs1Δ was not (p = 0.37). The mean (maximum) life spans for these strains were 28.1 (48), 23 (44), 13.3 (23), and 26.4 (43) generations, respectively.

**FIGURE 7.** Roles of FA elongation and inositol diphosphate signaling in chronologic aging and replicative life span in yeast. A, WT, elo3Δ, elo3Δipk2Δ, and elo3Δkcs1Δ (lanes 1–4) cells were spotted on YPD plates in different dilutions following growth in stationary phase in water for several days, and then grown at 30 °C for 24 h. The replicative life span characteristics of cells were analyzed as described under “Experimental Procedures.” The number of daughter cells produced by a single mother cell was counted using microdissection. In each life span, determination of 43 cells/strain were used, which were performed in three independent experiments. The statistical analysis of the data were performed using Mann-Whitney (rank test), and p < 0.05 was considered significant. The differences in life span between WT and elo3Δ, and kcs1Δ were significant (p = 0.0043, and p < 10⁻⁶, respectively), whereas the difference between WT and elo3Δkcs1Δ was not (p = 0.37). The mean (maximum) life spans for these strains were 28.1 (48), 23 (44), 13.3 (23), and 26.4 (43) generations, respectively.

**FIGURE 8.** Elo3 shares a common pathway with Ku70/80 for the regulation of telomere length. A, the telomere length was measured in WT, elo3Δ, yku80Δ, elo3Δyku80Δ, tel1Δ, elo3Δtel1Δ, rad50Δ, elo3Δrad50Δ, est1Δ, elo3Δest1Δ, yku70Δ, and elo3Δyku70Δ cells (lanes 1–12, respectively). The vertical dashed lines indicate a gap between lanes 1–10 and 11–12 on the blot. Molecular weight markers are indicated on the left. B, the average reductions in telomere length in mutants mentioned above were detected in at least three independent experiments, and the data are shown in a graphical format. C, effects of loss of ELO3 in telomere binding/protective function of endogenous Ku-GFP in WT versus elo3Δ cells (left panel, lane 2) were measured using ChIP analysis as described under “Experimental Procedures.” Lane 1 contains input DNA used as loading controls. The expression of Yku80p-GFP protein in WT and elo3Δ (C, right panel, lanes 3 and 4, respectively) cells was measured by immunoprecipitation using anti-GFP antibody-coupled microbeads, followed by Western blotting using a soluble antibody against GFP protein. **D, the role of ELO3 deletion in the regulation of the NHEJ function of Ku was examined as described under “Experimental Procedures.”** The linearized and circular pYES2 plasmids, harboring the URA gene, were transformed into WT, elo3Δ, and yku80Δ cells. Then, the percent of WT, elo3Δ, and yku80Δ colonies grown in URA-negative media were counted in at least two independent trials as duplicates. Error bars represent S.D. ± mean.

**TABLE 1.** Table containing data for telomere length in different strains.

**TABLE 2.** Table containing data for life span in different strains.

**TABLE 3.** Table containing data for replicative life span in different strains.

**TABLE 4.** Table containing data for telomere shortening in different strains.

Synthesis of the mutant Elo3, which did not effectively protect telomere shortening) in elo3Δ cells plays important roles in the attrition of telomeres.

VLCA synthesis is closely associated with sphingolipid metabolism (5–9), and loss of ELO3 results in almost complete loss of the generation of C26-phytoceramide. Because sphingolipids are known to be involved in the regulation of senescence, life span, and telomeres, we also investigated a possible role of a down-regulation of C26-phytoceramide synthesis in telomere attrition using the lag1Δlac1Δ double-mutant strain (in the W303-1A strain, which lacks C26-phytoceramide synthesis (29). Telomere length was not altered in lag1Δlac1Δ cells when compared with the WT strain. Similarly, deletions of LAG1 or ISC1, which alter ceramide generation (29, 31, 32), in WT or elo3Δ cells did not have any detectable effect on telomere length. These data suggest that alterations of phytoceramide synthesis by LAG1/ISC1 might be dispensable for attrition of telomeres mediated by the loss of ELO3.

Recently, IP metabolism and signaling have been reported to regulate telomere shortening in yeast (33, 34). Accumulation of inositol pyrophosphate (PP-IP4) has been shown to result in telomere shortening, which can be rescued by deletion of IPK2 and KCS1 (33, 34). Our data, which revealed that telomere attrition in elo3Δ cells was completely prevented by the deletion of KCS1 or IPK2, but not by the deletion of IPK1, support the previously reported results (33, 34). Interestingly, in our experiments, measurement of IP molecules showed that IP4 was increased in elo3Δipk1Δ cells, which contain shorter telomeres, compared with ipk1Δ cells, suggesting a role for IP4 generation in the regulation of telomeres. There were no detectable changes in the levels of other IPs in these cells. Because IPK2 is the principal enzyme responsible for IP4 synthesis in yeast, we can infer...
that normal telomere length in elo3Δ/pk2Δ cells may be due to the lowering of IP4 levels. The levels of IP4 in cells lacking KCS1 were too low to determine whether the reversal of telomere shortening in elo3Δ/kcs1Δ is due to loss of IP4, or whether inositol pyrophosphates (PP-IP4, (PP)2-IP3, or PP-IP5) are involved. Nevertheless, these data suggest that alterations of the synthesis and/or metabolism of IP4, and possibly its further metabolism for the synthesis of its pyrophosphate derivatives, might play a role in the regulation of telomere length in elo3Δ cells (IP4 levels were also very low to detect in these mutant cells, most likely due to its rapid metabolism). The distinct roles of IP molecules in various different nuclear functions, such as in the regulation of chromatin remodeling complexes, or the cyclin-CDK/CDK-inhibitor complex, have been reported previously (41–43). However, specific IP molecules involved in the regulation of C26-FA synthesis, telomere length, and/or telomere protective function of Yku70/80 in elo3Δ cells are still unknown. It is interesting that IP6 has been shown to directly bind to mammalian Ku, but not yeast Yku70/80, which regulates its DNA-PK-associated repair and NHEJ functions (45, 46). However, whether IP4 or its pyrophosphate derivatives bind Yku70/80 to regulate its telomere-binding/protection function has not been shown, and needs to be investigated.

In conclusion, the data presented in this study suggest that alterations of IP metabolism for the synthesis of its pyrophosphate derivatives, and possibly its further metabolism and/or signaling by IPK2/KCS1 are involved in the regulation of C26-FA synthesis, telomere length, and/or telomere protective function of Yku70/80 in elo3Δ cells are still unknown. It is interesting that IP6 has been shown to directly bind to mammalian Ku, but not yeast Yku70/80, which regulates its DNA-PK-associated repair and NHEJ functions (45, 46). However, whether IP4 or its pyrophosphate derivatives bind Yku70/80 to regulate its telomere-binding/protection function has not been shown, and needs to be investigated.

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