ROLE OF TELOMERASE IN REGULATION OF CHROMOSOME STABILITY IN CELLS LACKING THE KU70/KU80 HETERODIMER

THESIS

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CHAPTER I

INTRODUCTION

Genomic deoxyribonucleic acid (DNA) is compacted and organized into linear structures called chromosomes in eukaryotic cells. These chromosomes contain genetic information that is encoded by adjacent sequences of nucleotides called genes. In humans, approximately 1.5% of the genome is regions that code for genes (1). Chromosomes not only contain genes but also extensive noncoding regions of DNA that play numerous structural roles. For example, the ends of chromosomes are comprised of two distinct stretches of DNA repeat sequences that are referred to as the subtelomere and the telomere. The subtelomere in the budding yeast *Saccharomyces cerevisiae* is made up of three individual regions: the X region, a short TG repeat sequence, and Y' elements (Figure1). Telomeres are the ~400 base pair long, $TG_{(1\text{-}3)}$ repeat regions at the ends of the chromosomes (2).

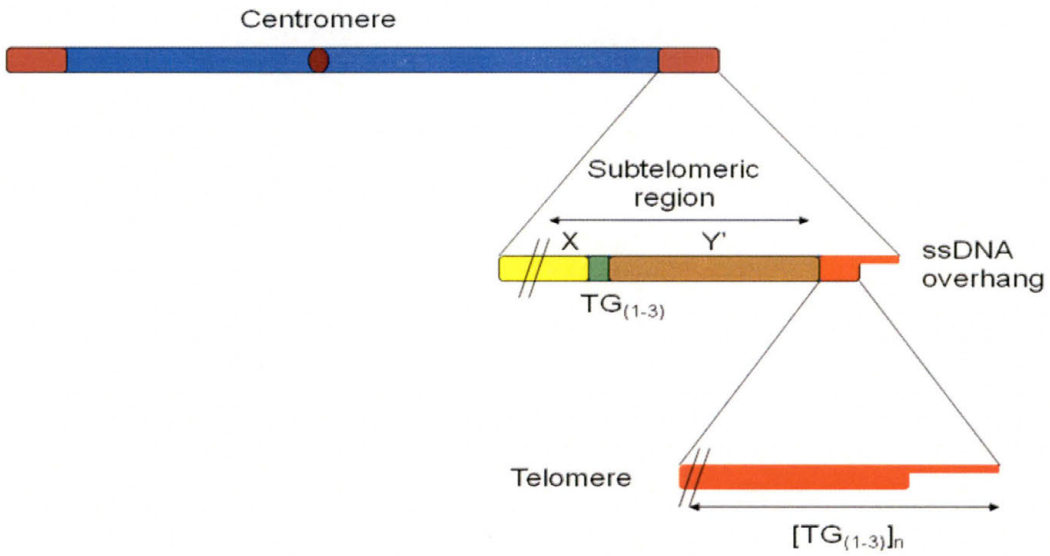


Figure 1. Diagram of a linear eukaryotic chromosome from the budding yeast *S. cerevisiae.*

Numerous proteins have affinity for subtelomeric and/or telomeric DNA. In addition, many proteins associate with those subtelomere or telomere binding proteins. Together, the subtelomeric and telomeric DNA along with their associated proteins make up a cap-like structure at the ends of linear chromosomes that helps mask the ends of the DNA from nucleases and cell cycle checkpoint responses (3). Loss of the telomeric cap structure can lead to attempted aberrant repair of DNA ends, though they are not actually breaks in the chromosome.

Some telomere binding proteins, such as Ku, Cdc13, Rif1, and Rif2 bind to telomeres throughout the cell cycle (4). Other proteins such as Tel1 and Pif1 associate transiently, depending on the stage of the cell cycle and/or the telomeric structure (5, 6, 7, 8). Figure 2 is a schematic displaying telomere-associated proteins and their interactions.

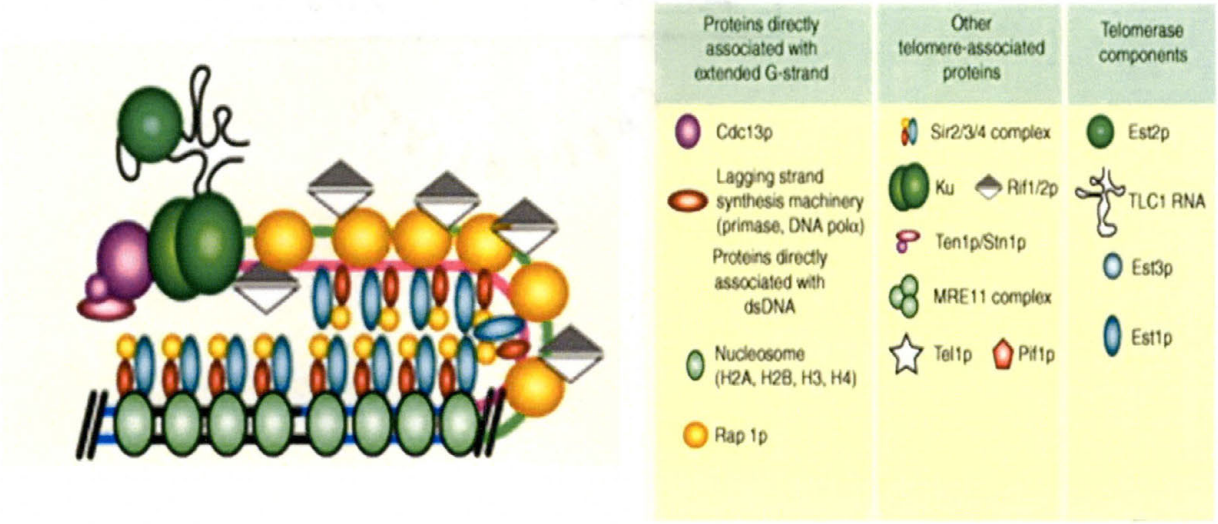


Figure 2. Illustration of telomere-binding proteins (9).

Telomeric length is maintained via a specialized reverse transcriptase enzyme, telomerase. Telomerase lengthens telomeres, adding bases to the 3' ends of the DNA, during S phase of the cell cycle. This is accomplished by utilizing an RNA subunit template complementary to the telomeric sequence. As seen in Figure 3, the *TLC1* RNA subunit anneals to a short telomeric sequence at the end of the chromosome. Then the polymerase subunit adds nucleotides to the 3' end of the DNA by utilizing a region of the *TLC1* RNA as a template.

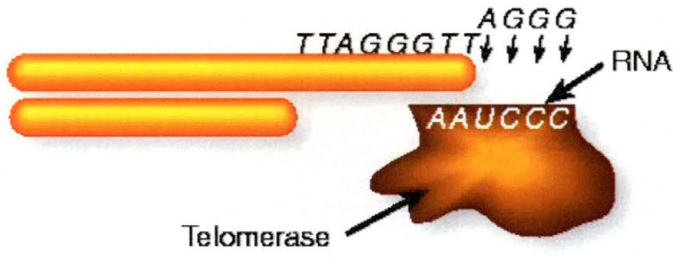


Figure 3. Representation of the mechanism of action of telomerase extension of telomeres. (Dorothy Crawford, The New Scientist Sept. 2008)

In humans, the enzyme is deactivated in most cells prior to birth. In most eukaryotic cells, the length of the telomeric nucleotide repeats is proportional to the lifespan of the cell (e.g., as cells age, the lengths of their telomeres decrease). In cell culture, aging human somatic cells age to the point where their telomeres eventually reach a critical length at which cells stop dividing and enter a quiescent state called senescence (10). This is due to cell cycle signals that are initiated by the exposed DNA ends which halt the cell cycle (11).

In single cell organisms such as the budding yeast *Saccharomyces cerevisiae*, as well as in most human cancer cells, telomerase actively maintains telomeric length and thus cells are in a sense immortal (12). Additionally, in both human stem cells and germ cells the telomerase enzyme is active and lengthening telomeres (13). Approximately 90% of human cancer cells in some way reactivate the telomerase enzyme, which stabilizes their telomeres, thus rendering the cells immortal (14).

Telomere length and stability are dependent on many genes and genetic pathways in the budding yeast *S. cerevisiae*. For example, Askree *et al.* analyzed ~5000 independent yeast mutants and found 173 genes whose mutation led to changes in telomere size (15). This study showed that telomere stability depends not only on obvious telomere-associated pathway genes such as those involved in DNA metabolism and chromatin structure, but also on seemingly unrelated cellular pathways. Genes associated with vesicular trafficking, cell polarity, ribosome translation, protein modification, and other pathways were also implicated in altered telomere structure (15).

This signifies the complexity of the cellular pathways involved in maintaining the telomeric structure.

Two genes known to be required for telomere stability are *YKU70* and *YKU80*, which encode the two subunits of the Ku complex (16). The Ku complex is a heterodimer found in all eukaryotes comprised of Ku70 and Ku80 which binds double-stranded DNA ends independent of DNA sequence. Ku binds DNA without interacting with bases and minimal interaction with the sugar phosphate backbone. The main interaction between Ku and DNA ends is likely contacts between the major and minor grooves of the double stranded DNA and the interior of the ring-like cavity formed between the Yku70 and Yku80 protein subunits (17).

Ku is a multifunctional protein complex that facilitates numerous biological pathways. The Ku complex is highly conserved among both lower and higher eukaryotes, suggesting its significance. Initially, Ku was isolated in humans as an autoantigen in patients with scleroderma-polymyositis (PM) overlap syndrome (18), and later associated with systemic lupus erythematusus (19), systemic autoimmune rheumomatic disease (SARD) (20), inflammation (21) and tumorgenesis (22).

Ku is also essential for site-specific recombination of V(D)J antibody and T cell receptor gene segments in humans and animals (23). V(D)J antibody and T cell receptor generation requires the induction of dsDNA breaks and subsequent repair of appropriate gene segments via NHEJ. Thus, since NHEJ is abolished if *KU70* or *KU80* is inactivated, the gene segments in V(D)J antibody and T cell receptor production cannot be accurately repaired. Ku-deficient individuals suffer from extremely low antibody and T cell receptor diversity. The intensively studied SCID (Severe Combined

Immunodeficiency) mice have mutations within the *KU80* gene. These mice are unable to create the immense antibody diversity needed to fight bacterial, viral, and fungal infections (24). Mutations in human *KU70* or *KU80* genes also result in defects in generating antibodies against bacterial, viral, and fungal infections. *ku* individuals are rendered immunodeficient and the resulting disease is commonly known as the "boy in the bubble" disease because patients must be kept in sterile environments (25).

The Ku complex is known to play a critical role in the non-homologous end-joining (NHEJ) pathway of repair of induced DNA double-strand breaks (Figure 4). This is critical in the repair of deleterious breaks, both from exogenous and endogenous DNA damaging sources. If one of the two Ku genes is inactivated, then the NHEJ pathway is abolished and the cell must rely on the homologous recombination pathway for repair (23, 26).

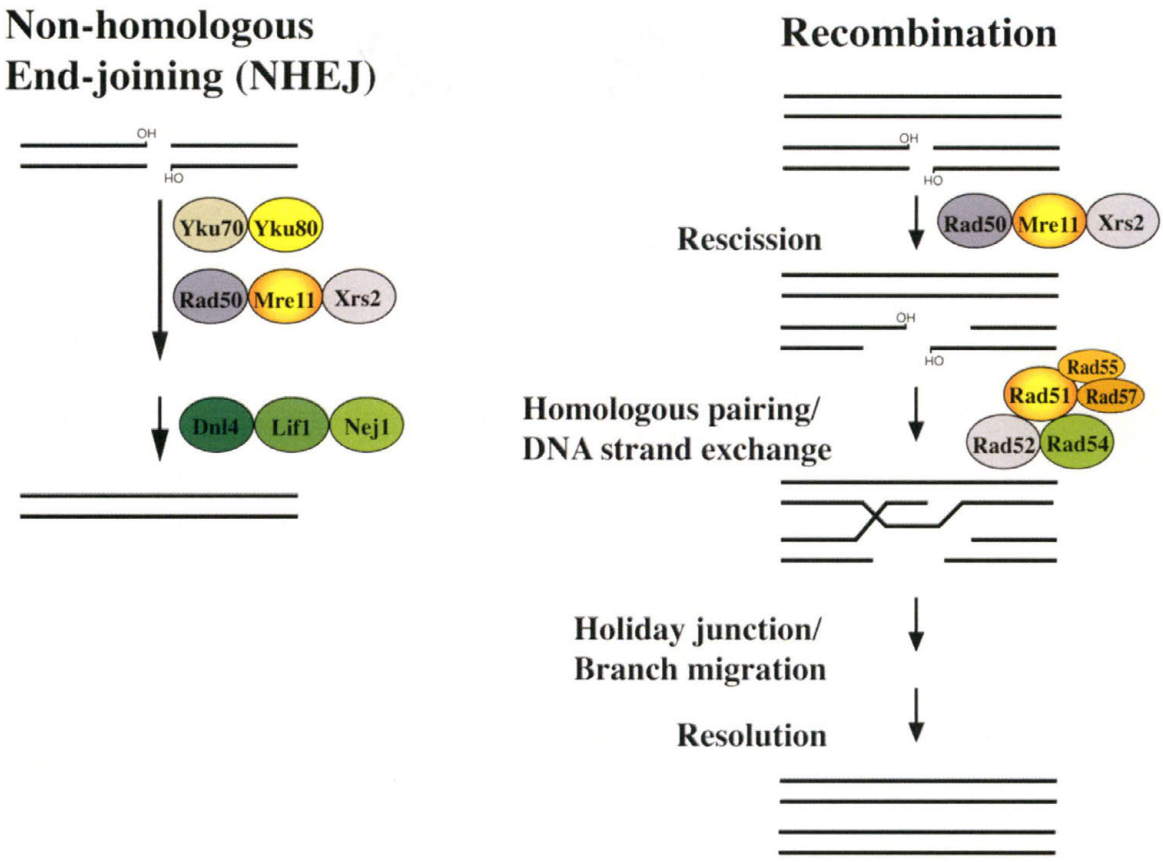


Figure 4. Schematic illustration of the two known major DNA repair pathways in eukaryotes.

The presence of Ku at the telomeres helps protect the telomeric DNA from deleterious events. Figure 5 shows some of the known roles of Ku at the yeast telomeres. Homologues of these yeast proteins exist in most eukaryotic organisms including humans.

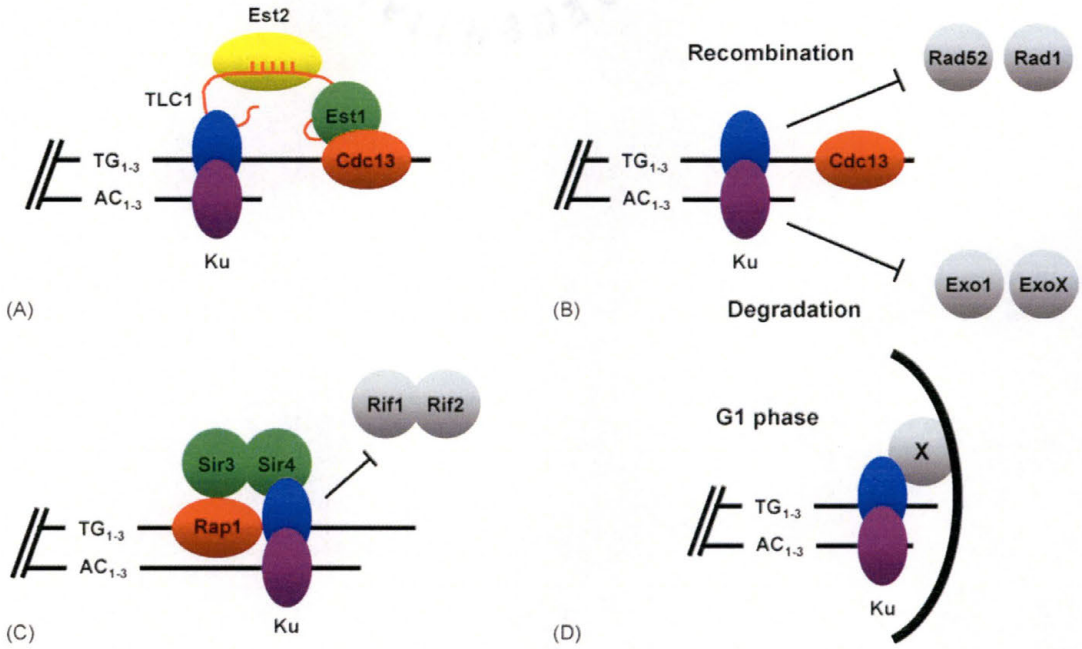


Figure 5. Illustration of the four known primary functions of the Ku complex at the ends of chromosomes in yeast cells. (Adapted from 27)

Figure 5A depicts how Ku stabilizes chromosome ends by recruiting telomerase subunits Est2 (catalytic subunit) of telomerase to the telomeres during the G_1 phase of the cell cycle. Ku also is thought to promote the binding of telomerase subunits Est1 and Est2 to the telomeres in late G_2/S phase (28). Specifically, recruitment of these telomerase proteins to the telomeres is thought to be due to an interaction between Ku and the telomerase RNA subunit, *TLC1* (28). The telomerase holoenzyme then adds nucleotides to the telomere sequences, thus extending the telomeres.

The presence of Ku at the ends of the chromosomes also protects the telomeres from chromosomal degradation by cellular nucleases (Figure 5B). Of the many nucleases within the nucleus, a few have been implicated in the degradation of uncapped telomeric DNA. Exo1 nuclease has become the focus of many studies in numerous biological

pathways (3, 29). The Exo1 protein has both 5'→3' exonuclease and a 5'-flap endonuclease activity which perform specific, and perhaps separate, functions during different cellular events (3). Specifically, Exo1 plays a backup role in meiotic crossing over and mitotic recombination (3, 30), and is critical for DNA mismatch repair (3, 31), and telomere integrity (3, 32). Generation of single-stranded, G-rich 3' DNA ends at compromised telomeres is not solely due to Exo1 degradation, but may also be an important function of the Mrx repair complex (3).

The length of telomeres is critical aspect of the chromosome and cell survival. Like short telomeres, long telomeres can lead to harmful cellular events. The length of telomeric repeats synthesized by telomerase is regulated by the Rif proteins, though the mechanism involved is not understood (Figure 5C).

Although the structure of telomeres is thought to be dynamic throughout the cell cycle, the telomeres are somewhat localized within the nucleus (33). Ku appears to play a role in the physical positioning or tethering of chromosome ends to the nuclear periphery (Figure 5D). The tethering of chromosomes is believed to be due to interactions between the Ku complex and protein subunits of the nuclear pores (34, 35). Specifically, Ku is thought to associate with myosin like proteins 1 and 2 (Mlp1 and Mlp2, respectively), forming specific contacts with Mlp2 (34, 35). Although the exact interactions that contribute to chromosomal tethering are not certain, it is believed that tethering of the telomeres to the nuclear membrane may help to protect telomeric DNA from degradation (33).

Inactivation of Ku genes in *S. cerevisiae* leads to numerous cellular phenotypes other than NHEJ abolition. A deletion of the *YKU70* or *YKU80* gene results in an altered

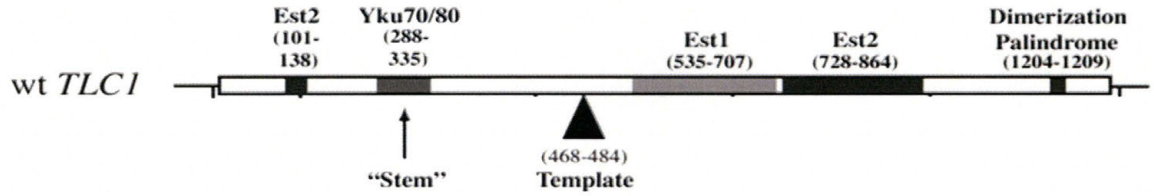
telomeric cap structure and disruption of silencing (36). Also, *ku* mutant telomeres become delocalized from the nuclear periphery i.e., they are no longer attached efficiently to the nuclear envelope (35). Lack of telomeric positioning could be deleterious to cells since this tethering to the nuclear envelope may contribute to protection from nuclease degradation.

When either *YKU70* or *YKU80* is altered, yeast telomeres become shorter. Telomere length is ~200 bp shorter at 30 °C (the normal growth temperature for yeast). Notably, yeast cells grown at higher temperatures (37 °C) not only have short telomeres, but also have an increased amount of single-stranded, TG-rich 3' overhang DNA, which has been created by exonuclease degradation (29). Such Ku-deficient cells, which proliferate at the permissive temperature (30 °C), exhibit a DNA damage checkpoint response and stop growing at elevated temperatures (e.g., 37 °C).

This telomere instability resulting from the lack of Ku at the telomeres can be alleviated by overexpression of specific subunits of the telomerase complex. The yeast telomerase holoenzyme consists of four protein subunits (Est1, Est2, Est3, and Cdc13) and an RNA subunit (*TLC1*) (37). As with most protein complexes, there are numerous interactions between the telomerase subunits, not only with other subunits, but with non-telomerase proteins. Est2 is the polymerase subunit that extends the 3' end of the telomeric DNA (38, 39). *TLC1* RNA serves as a template on which the reverse transcriptase Est2 protein polymerizes DNA (40). Est2 and *TLC1* RNA are considered to be the core of the enzyme. The *TLC1* RNA subunit also serves as a scaffold upon which the other subunits bind. Figure 6A contains a diagrammatic representation of the major freatures and protein binding sites of *TLC1* RNA. Figure 6B is a computer-generated

model of the *TLC1* RNA molecule (41). This schematic shows the 2-dimensional folding of the RNA as well as known telomerase subunit binding sites.

A.



B.

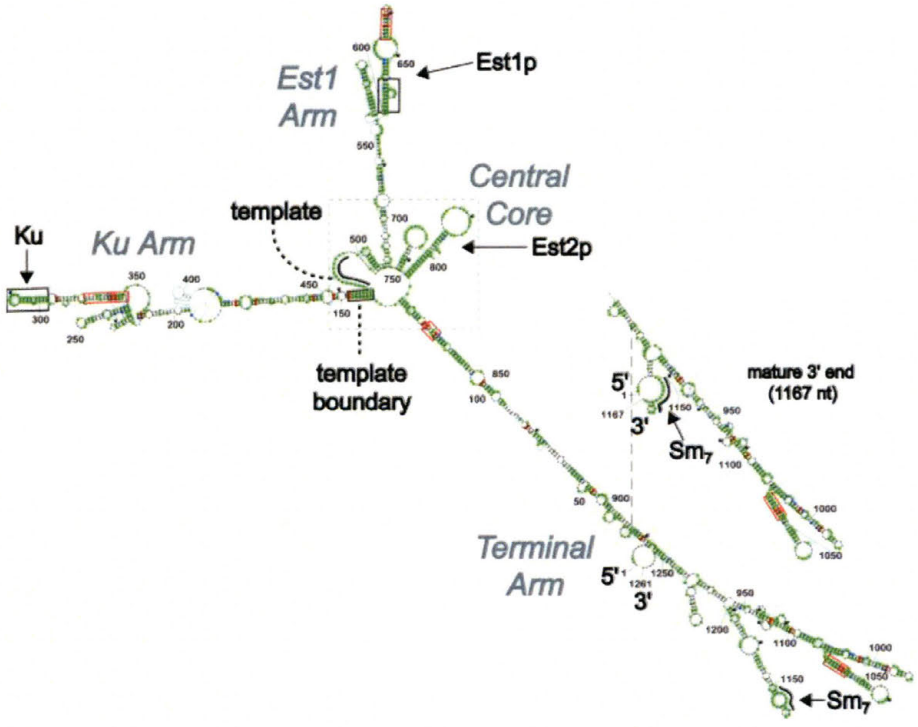


Figure 6. A. Schematic representation of *TLC1* depicting protein binding sites. B. Computer generated two dimensional representation of telomerase RNA subunit *TLC1* (41).

Est1 protein, in conjunction with Ku, is believed to recruit the *TLC1*/Est2 core enzyme to the ends of the telomeres for addition of new DNA during S phase (42). This recruitment seems to be a consequence of the Ku/Est1 association with *TLC1* RNA. Cdc13 is a single-stranded telomeric DNA binding protein that is also believed to help initiate telomere elongation (43).

Past studies have shown that overexpression of either Est2, the telomerase polymerase subunit or the RNA subunit *TLC1* can rescue the death of Ku mutants at 37 °C (4, 44, 45). Overexpression of *TLC1* RNA has been shown to affect several metabolic processes. Raising the levels of cellular *TLC1* RNA disrupts silencing that is usually observed at the telomeres (46). Also, *TLC1* overexpression alters the concentration of Est2 bound to the PinX protein in the nucleolus, which may be a regulator of Est2 function (47). High *TLC1* RNA levels can also alleviate the DNA repair defects of *mre11, rad50 and xrs2* mutants, which draw new connections between the telomerase complex and DNA repair (48).

The precise mechanism by which *TLC1* RNA or Est2 overexpression rescue the telomere instability of Ku mutants at elevated temperatures is unknown. Experiments described in this thesis were designed to increase our understanding of telomere instability caused by lack of the Yku70/Yku80 heterodimer and to investigate the mechanism of suppression by telomerase.

CHAPTER II

MATERIALS AND METHODS

I. MATERIALS

General Reagents

Agarose and ethidium bromide were purchased from Shelton Scientific, Inc. (Shelton, CT). Tris base, lithium acetate dehydrate, 99% glycerol, and polyethylene glycol were from Invitrogen Life Technologies (Carlsbad, CA). Dimethyl sulfoxide (DMSO), methyl methanesulfonate (MMS), and $MgCl_2$ were purchased from Sigma Chemical Co. (St. Louis, MO). Ethylenediaminetetraacetic acid (EDTA), hygromycin B (HygB), sodium citrate dihydrate, sodium hydroxide, and boric acid were obtained from EM (Darmstadt, Germany). G418 sulfate was purchased from Cellgro (Hardon, VA). 5-fluoroorotic acid (5-FOA) was purchased from Zymo Research (Orange, CA).

Enzymes and PCR reagents

Restriction enzymes, Klenow fragment DNA polymerase, and Taq DNA polymerase were purchased from New England Biolabs (Beverly, MA). Shrimp alkaline phosphatase (SAP) was obtained from USB (Cleveland, OH). ExTaq DNA polymerase was purchased from Takara (Madison, WI).

Bacteriological and yeast media

All amino acids, ampicillin (Amp), and galactose were purchased from Sigma. Difco bacto peptone, bacto agar, bacto yeast extract, bacto tryptone and yeast nitrogen base dropout media were purchased from Becton Dickinson Microbiological Systems (Sparks, MD). D-(+)-glucose was from Mallinckrodt Baker, Inc. (Paris, Kentucky).

Cell culture solutions and media

Non-selective YPDA yeast plate growth media contained 1% bacto yeast extract, 2% bacto peptone, 2% glucose, 2% bacto agar, and 0.001% adenine. YPDA liquid media was prepared as YPDA, but without agar. In order to assay mitochondrial integrity, cells were grown on YPG (1% bacto yeast extract, 2% bacto peptone, 2% bacto agar, 3% glycerol). Plasmid selection was achieved by growing yeast cells on synthetic media with drop-out mix (0.17% yeast nitrogen base without amino acids or ammonium sulfate, 0.5% ammonium sulfate, 2% glucose or 2-3% galactose, 2% bacto agar, plus all essential amino acids minus the amino acids used for selection). Synthetic media containing galactose was used to induce plasmid-encoded genes regulated by galactose promoters. For selection of resistant strains, G418 sulfate and hygromycin B were added to media at concentrations of 200 μg/ml and 250 μg/ml, respectively. In order to assess methyl methanesulfonate (MMS) sensitivity, cells were grown on YPDA or synthetic plates containing MMS.

E. coli cells were grown in LB + Amp broth (1% bacto tryptone, 0.5% yeast extract, 0.5% NaCl, 0.01% Amp) or on LB + Amp plates (as broth, with 1.5% agar).

Table 1 Strains

Strain	Genotype	Source
BY4742	*MATα his3Δ1 leu2Δ0 leu2Δ0 lys2Δ0 ura3Δ0*	49
VL6α	*MATα ura3-52 trp1(Δ63) lys2-801 his3-Δ200 met14 ade2-101*	50
VL648	VL6α *Δleu2. G418^r*	50
YLKL499	VL6α *Δrad50· hisG*	Lab strain
YLKL649	BY4742, *Δrad50 G418^r*	Lab strain
YLKL593	VL648, *Δyku70 HIS3*	Lab strain
YLKL594	VL648, *Δyku80 HIS3*	Lab strain
YLKL623	BY4742, *Δrif1 HygB^r*	Lab strain
YLKL626	BY4742, *Δrif2:.HygB^r*	Lab strain
YLKL649	BY4742, *Δrad50..G418^r*	Lab strain
YLKL652	BY4742, *Δyku70 HIS3*	Lab strain
YLKL653	BY4742, *Δsir2 HygB^r*	Lab strain
YLKL654	BY4742, *Δmlp2 HygB^r*	Lab strain
YLKL701	*MATa HIS3 LEU2 LYS2 URA3 ade5*	Lab strain
YLKL803	BY4742, *Δest2 HygB^r* + pLKL82y	Lab strain
YLKL842	BY4742, *Δ trp1 hisG*	Lab strain
YLKL843	BY4742, *Δyku70 HIS3 trp1 hisG*	Lab strain
YLKL851	BY4742, *Δmus81 G418^r*	Lab strain
YLKL856	BY4742, *Δsir2 G418^r*	Lab strain
YLKL857	BY4742, *Δyku70. G418^r*	Lab strain
YLKL866	BY4742, *Δyku70 URA3*	Lab strain
YLKL867	BY4742, *Δmlp2 (1-5040) Δyku70 .URA3*	Lab strain
YLKL868	BY4742, *Δsir2 ·HYgB^r yku70 URA3*	This study
YLKL869	BY4742, *Δrif1 ·HygB^r Δyku70 URA3*	This study
YLKL870	BY4742, *Δrif2 HygB^r Δyku70 URA3*	This study
YLKL871	BY4742, *Δsir4 G418^r Δyku70 URA3*	This study
YLKL923	BY4742, *Δyku80 ·G418^r*	Lab strain
YLKL948	BY4742, *Δmms4 ·G418^r*	Lab strain
YLKL956	BY4742, *Δyku80 G418^r yku70 URA3*	This study
YLKL957	BY4742, *Δyku70 HIS3 + pCDNA KU ts- supp #13*	This study
YLKL958	BY4742, *Δyku70 HIS3 + pCDNA KU ts- supp #22*	This study
YLKL959	BY4742, *Δyku70··HIS3 + pCDNA KU ts- supp #23*	This study
YLKL960	BY4742, *Δyku70. HIS3 + pCDNA KU ts- supp #29*	This study
YLKL961	BY4742, *Δest2. HygB^r + pVL715*	Lab strain
YLKL981	BY4742, *Δest2 HygB^r yku70 ·G418^r* + pVL715	This study
YLKL982	BY4742, *exo1Δ G418^r yku70Δ URA3*	This study
YLKL983	BY4742, *mms4Δ G418^r yku70Δ URA3*	This study

YLKL984	BY4742, *rad1Δ G418^r yku70Δ··URA3*	This study
YLKL985	BY4742, *rad10Δ G418^r yku70Δ. URA3*	This study
YLKL986	BY4742, *mus81Δ G418^r yku70Δ.·URA3*	This study
YLKL987	BY4742, *rad17Δ G418^r yku70Δ URA3*	This study
YLKL988	BY4742, *rad24Δ:·G418^r yku70Δ :URA3*	This study
YLKL989	BY4742, *mlp1Δ HygB^r yku70Δ. URA3*	This study
YLKL990	BY4742, *mlp1Δ HygB^r mlp2Δ0 yku70Δ URA3*	This study
YLKL991	BY4742, *tlc1Δ.:HygB^r yku70 HIS3* + pLKL83Y	This study
YLKL992	BY4742, *sir3Δ· G418^r yku70Δ ·URA3*	This study

Table 2 Plasmids

Plasmid	Description	Source
pRS314	*CEN/ARS TRP1*	51
pRS315	*CEN/ARS LEU2*	51
pRS316	*CEN/ARS URA3*	51
pRS424	*2μ TRP1*	52
pRS425	*2μ LEU2*	52
pVL715	*2μ URA3 ADH1p EST2*	53
pVL784	*2μ LEU2 ADH1p EST1*	53
pVL999	*2μ LEU2 ADH1p EST2*	53
pLKL74y	*2μ TRP1 GAL1p TLC1*	53
pLKL76y	*pLKL74Y, Δ(Ecl136II-HpaI)*	54
pLKL77y	*pLKL74Y, Δ(Ecl136II-NcoI)*	54
pLKL78y	*pLKL74Y, Δ(BamHI-StuI)*	54
pLKL79y	*pLKL74Y, Δ(BamHI-NcoI)*	54
pLKL83y	*2μADH1p..TLC1 URA3*	54
pCDNA50.1	*CEN/ARS URA3 GAL1p EXO1*	55
pGEM4Z S-H/URA	*yku70Δ URA3*	56
pLKL64y	*2μ LEU2 ADH1p TLC1*	54
YCpGal::RI_b	*Gal1p EcoRI URA3*	57
pAG32	*$HygB^r$*	58
pFA6MX4	*$G418^r$*	58
pTCG	*2μ TRP1 GAL1p*	59
pTRP61	*2μ TRP1 GAL1p TLC1*	60
pKu-S1	*yku70* 37 °C sensitive suppressor: *ANB1*	This study
pKu-S2	" " *HYP2*	This study
pKu-S3	" " *URA5*	This study
pKu-S5	" " *REP1*	This study
pKu-S6	" " *HYP2*	This study
pKu-S7	" " *CPR1*	This study
pKu-S8	" " *HYP2*	This study
pKu-S10	" " *RTF1*	This study
pKu-S11	" " *RRP14*	This study
pKu-S12	" " *UBP6*	This study
pKu-S14	" " *CPR1*	This study
pKu-S15	" " *UBP6*	This study
pKu-S16	" " *TLC1*	This study
pKu-S17	" " *YLR003C*	This study
pKu-S18	" " *BUD21*	This study

pKu-S19	"	" *HYP2*	This study
pKu-S20	"	" *HYP2*	This study
pKu-S21	"	" *HYP2*	This study
pKu-S24	"	" *RPL9A*	This study
pKu-S25	"	" *CPR1*	This study
pKu-S26	"	" *HYP2*	This study
pKu-S27	"	" *NUG1*	This study
pKu-S28	"	" *HYP2*	This study
pKu-S30	"	" *MPP6*	This study
pKu-S31	"	" *URA5*	This study

II. METHODS

Gel electrophoresis

Gel electrophoresis was performed in a Life Technologies Horizon 11-14 gel rig using 0.8-1.2% agarose gels in TBE (90 mM tris-borate, 2 mM EDTA) running buffer. Ethidium bromide was used to stain agarose gels and images were captured using a Kodak IS440 CF imaging system with Kodak 1D imaging software.

DNA transformations

The high efficiency lithium acetate method described by Gietz *et al.* was used for DNA transformations for gene disruption (61). A rapid lithium acetate/DMSO transformation method described by Soni *et al.* was used to transform plasmids into yeast strains (62). The Miller and Chung *E. coli* transformation method was used for most *E. coli* transformations (63). For recovery of *pKu-S* library plasmids, XL1 Blue Supercompetent *E. coli* cells from Stratagene were used.

Chromosomal and plasmid DNA purification

Yeast DNA was purified using the MasterPure^{TM} Purification Kit and provided protocol by Epicentre Technologies (Madison, WI). The boiling lysis method was used to purify plasmid DNA (Sambrook and Russell, Molecular Cloning Manual).

Polymerase Chain Reaction

PCR cycles were performed using 94 °C for 30 seconds, annealing temperatures from 42-50 °C for 30-40 seconds, and an extension temperature of 72 °C for 90-120

seconds. PCR was conducted in an Applied Biosystems 2720 Thermal Cycler for 32 cycles.

Dilution pronging cell survival assays

Cells were harvested into sterile deionized H_2O, diluted 1/40, sonicated for 6 seconds at 2-3 watts using a Sonics Vibracell Ultrasonic Processor (Newtown, CT). Following sonication, the cells were quantitated on a Reichert (Buffalo, NY) hemocytometer using a Comcon (Russia) LOMO phase contrast microscope. Yeast cells were added to a microtiter dish at a concentration of $1 \times 10^7 - 4 \times 10^7$ cells per 220 µl. These cells were serially diluted 5-fold, 6 times across the length of the dish. The cells were then pronged onto either selective synthetic or YPDA plates depending on the assay. In order to induce *EXO1* and *Eco*RI in *yku70* mutants, cells were pronged to both synthetic plates containing glucose (control) and plates with galactose. Pronged cells were incubated at 30 °C as positive cell viability controls and also incubated at 37 °C, 38 °C, or 40 °C depending upon the assay being performed. Cells were allowed to grow for 2-4 days and images were taken of the plates using a Canon Powershot G3 digital camera and saved as JPEG files.

Yeast strain construction

yku70Δ::URA3 double mutant constructs

Yeast strains YLKL869 (*rif1*::*HygB*^{r} *yku70*::*URA3*), YLKL870 (*rif2*::*HygB*^{r} *yku70*::*URA3*), YLKL868 (*sir2*::*HygB*^{r} *yku70*::*URA3*), YLKL867 (*mlp2*::Δ0 *yku70*::*URA3*), YLKL992 (*sir3*::*G418*^{r} *yku70*::*URA3*), YLKL871 (*sir4*::*G418*^{r}

yku70::*URA3*), YLKL982 (*exo1*::*G418*^{r} *yku70*::*URA3*), YLKL984 (*rad1*::*G418*^{r} *yku70*::*URA3*), YLKL985 (*rad10*::*G418*^{r} *yku70*::*URA3*), YLKL983 (*mms4*::*G418*^{r} *yku70*::*URA3*), YLKL986 (*mus81*::*G418*^{r} *yku70*::*URA3*), YLKL956 (*yku80*::*G418*^{r} *yku70*::*URA3*), YLKL987 (*rad17*::*G418*^{r} *yku70*::*URA3*), and YLKL988 (*rad24*::*G418*^{r} *yku70*::*URA3*) were constructed by transforming single mutant cells with *Hind*III + *Eco*RI-digested *YKU70* deletion plasmid pGEM4Z S-H/URA (see Table 1). Cells were spread to glucose minus uracil plates and resulting colonies were then patched to fresh glucose minus uracil plates. Two to three isolates were then patched to a new glucose minus uracil plate and grown at 37 °C for two days, after which the cells were replica-plated to glucose minus uracil plate and grown at 37 °C for two days. Cells that exhibited the temperature sensitivity phenotype were then used for further analysis. The *yku70* deletion in YLKL956 (*yku80Δ*::*G418*^{r} *yku70Δ*::*URA3*) was PCR confirmed using 5'YKU70 and 3'YKU70 because the *yku80* initial strain already exhibited a temperature sensitive phenotype. Several other double mutants were also confirmed by PCR.

Antibiotic resistance double mutant constructs

YLKL981 was constructed from YLKL803 (*est2Δ*::*HygB*^{r} pLKL82Y [*Gal1p*::*EST2*, *URA3*]). YLKL803 was spread to a synthetic plate plus 5-FOA to select for plasmidless cells. Resulting *ura3*$^-$ cells were then patched to a fresh 5-FOA plate and grown for 2 days. The cells were then transformed with pVL715 (*ADH1*p::*EST2 URA3*) and then spread to glucose minus uracil plates. Colonies were then patched to a fresh glucose minus uracil plate. To delete *YKU70,* the *G418*^{r} gene was amplified from the plasmid pAG32 using gyku70 primers. These cells were then transformed with

yku70Δ::*G418*^{r} PCR product and spread to YPDA plates, grown 1 day 30 °C and replica-plated to YPDA plus G418. G418^r colonies were then patched to a new YPDA plus G418 plates to confirm resistance. These cells were then streaked to glucose minus uracil plates and grown for 2 days at 37 °C, replica-plated to a new glucose minus uracil plate, and grown another 2 days. The resulting temperature-sensitive isolates were used for additional assays.

YLKL991 was created from YLKL652 (*yku70*::*HIS3*). YLKL652 was transformed with pLKL83Y (*ADH1*p::*TLC1 URA3*) and spread to glucose minus uracil plates. After 2 days, colonies were then patched to a fresh glucose minus uracil plate. The pAG32 (*HygB*^{r}) plasmid was PCR amplified using 5'gtlc1AA (CTACGTTTGAGTTTTCCATCATGCAGGCCTCAGAAATTTGGTAGGCACTCGA TGGATGTGACTGTCGCCCGTACATT) and 3' gtlc1BB (GCTGTGAATACAACACCAAGATTCATAAAATGAACACACGGTTCCTTCCGCT TGGGACAAGTTCTTGAAAACAAGAATC) primers. Three isolates were then transformed with the *tlc1*::*HygB*^{r} PCR fragment and spread to YPDA plates and grown 1 day at 30 °C, then replica-plated to YPDA plus HygB plates. The *tlc1*::*HygB*^{r} disruptions in these isolates were confirmed by PCR using 5'TLC1 and 3'TLC1 test primers. Three out of thirty-four HygB^r colonies were found to have the correct gene disruption.

YLKL989 and YLKL990 were created by disrupting *MLP1* with *HygB*^{r}. pAG32 was PCR amplified using 5'gmlp1 and 3'gmlp1. The resulting *mlp1*::*HygB*^{r} PCR product was transformed into YLKL866 (*yku70Δ*::*URA3*) and YLKL867 (*yku70Δ*::*URA3 mlp2Δ0*), respectively. After transformation, the cells were spread to YPDA plates and grown 1 day at 30 °C, then replica plated to YPDA plates plus HygB. Resulting HygB

resistant colonies were patched to fresh YPDA + HygB plates as well as YPG. The *mlp1Δ*::*HygB*^{r} disruption was verified via PCR amplification of the *MLP1* locus using 5'MLP1 and 3'MLP1 primers.

***TLC1 RNA and Est2 polymerase rescue of yku70 with Eco*RI overexpression**

The rescue of *yku70* sensitivity to *Eco*RI expression by *TLC1* RNA and Est2 polymerase overexpression was assayed. *Eco*RI expression plasmid pYCpGAL::RI_b (*GAL1*p::*Eco*RI *CEN/ARS URA*) was transformed into YLKL568 (wildtype) and YLKL857 (*yku70Δ··G418*^{r}) and spread to glucose minus uracil plates and grown for 2 days at 30 °C. Resulting colonies contained pYCGAL::Ri_b and were patched to a new glucose minus uracil plate. Two YLKL568 + pYCGAL::Ri_b and two YLKL857 + pYCGAL::Ri_b were then separately transformed with pLKL64Y and pVL999 (*TLC1* and *EST2*, respectively) and spread to glucose minus uracil and leucine. Both *yku70* and wildtype contained an *Eco*RI overexpression plasmid plus pLKL64Y or pVL999. Cells were grown at 30 °C for 2 days and were subsequently patched to a fresh glucose minus uracil and leucine plate. After 2 days of growth, cells were then harvested, counted, and pronged to assess the rescue of *Eco*RI sensitivity in the presence of supracellular levels of *TLC1* or Est2. Cells were grown for 3 days at 30 °C before analyzing.

cDNA temperature-sensitivity suppressor screen

Novel genes that alleviate the temperature sensitivity of *yku70* mutants at 37 °C were discovered by screening a yeast cDNA library. A *GAL1*-regulated yeast cDNA expression library, constructed by Liu *et al.*, was transformed into *yku70* strain YLKL843

(61). The initial screen revealed 29 isolates, called pKU-S1 to pKU-S31, that grew on galactose at 37 °C, but not on glucose media at 37 °C. The plasmid DNA was then extracted from the *yku70* yeast cells using the Epicentre Yeast DNA extraction kit with the provided Epicentre protocol. In order to propagate the plasmid DNAs, the precipitated plasmid DNAs from these isolates were transformed into Stratagene's XL1-Blue Supercompetent Cells and spread to LB + Amp plates. Colonies could be generated for only 25 of the 29 pcDNAs, despite repeated efforts. The boiling lysis method was used to extract plasmid DNAs from at least 2 colonies for each isolate. A small aliquot of each of the 25 isolates was electrophoresed on a 0.6% agarose gel. Subsequent analysis included restriction digestion by which each of the 25 isolated cDNA plasmids were digested with restriction enzymes. In order to test whether any *YKU70* genes were present, *Pst*I and *Xba*I, which should release an internal 530 bp *YKU70* gene fragment were used. To test whether any isolates were the *TLC1* gene, *Nco*I and *Afl*II were used to test release of a 467 bp fragment. To reveal if any plasmids contained *EST2*, *Eco*RI and *Nhe*I were used and the presence of a 351 bp fragment analyzed. *EST1* was assayed the same way, using *Bse*RI and *Nde*I, which release a 901 bp fragment. The sequence of part of the plasmid-encoded gene of each of the 25 cDNA isolates was then determined by shipping purified DNAs to Retrogen, Inc (San Diego, CA). The partial sequences were determined using the *T7* primer and then compared to the gene sequence stored in the Saccharomyces Genome Database (yeastgenome.org).

yku70 cell division rate assay

YLKL843 cells containing either pRS316 or pCDNA50.1 (*GAL1p::TLC1 URA3*) were patched to 2% glucose minus uracil plates and grown for 2 days at 30 °C. The cells were then harvested in 500 µl of deionized H_2O, diluted 1/40, sonicated, and counted. Three cultures were prepared and grown at 30 °C and 3 cultures were also prepared and placed at 37 °C for 12 hours. Similarly, 6 samples were prepared as described above and 3 of them incubated at 30 °C and 3 at 37 °C and grown for 22 hours. After the allotted grow-out time, the cells were harvested and counted to assay the final cell titers. The number of generations of growth was calculated from comparison of the initial and final cell titers.

G_2/M arrest assay

The G_2/M cell cycle arrest of *yku70* cell cultures due to elevated levels of Exo1 was assayed by first patching and growing YLKL843 cells plus either pRS316 or pCDNA50.1 on 2% glucose minus uracil plates at 30 °C for 2 days. Cells were then taken from these plates and streaked to 2% glucose minus uracil, 5% glucose minus uracil, and 2% galactose minus uracil plates. Two of each type of plates was placed at 23 °C, 30 °C, 37 °C, and 39 °C. After two days of growth, the cells were then harvested into 500 µl deionized H_2O and sonicated. Afterwards the cells were counted according to their characteristic morphological shape. Unbudded (G_1 phase) and small-budded cells (S phase) were counted separately than those cells with a dumbbell shape (G_2/M).

CHAPTER III

RESULTS AND DISCUSSION

Deletion of the Ku complex results in numerous deleterious phenotypes in eukaryotic cells. In humans, deleting Ku leads to compromised immune systems due to abolishment of the NHEJ DNA repair pathway critical for immunoglobulin and T cell receptor production (64). Removing NHEJ in mammalian cells leads to chromosome instability and risk of cancer. In yeast, many cellular phenotypes are observed in *yku70* and *yku80* mutants, including disruption of silencing of genes in close proximity to the telomeres and delocalization of the telomeres at nuclear porin complexes. Also observed in *ku* cells are shortened, but stable telomeres at the normal growth temperature of 30 °C. When these cells are grown at elevated temperatures (37 °C), their telomeres develop unstable caps and long single-stranded 3' overhangs caused by increased nuclease degradation. Increasing intracellular levels of telomerase subunits *TLC1* and Est2 can alleviate the death of *ku* mutants at elevated temperatures. The mechanism by which telomerase subunits rescue *ku* cells is still unknown. The goal of this project was to investigate the death of *ku* mutants at high temperatures and its rescue by increasing telomerase levels in the cell.

Traditionally, phenotypic studies on Ku have been performed using *yku70* mutants and less frequently *yku80* mutants. Although the complex is disrupted by deleting either one of the two *KU* genes, the remaining protein product of the gene that was not disrupted still remains present in the cell. Ku is a heterodimeric complex that associates with numerous proteins. It is possible that the remaining subunit may still be present at the telomeres and could possibly be associating with Ku binding proteins. In order to see whether the remaining subunit of the Ku complex affects telomeric stability, a *yku70 yku80* double mutant was constructed. The pGEM4Z *YKU70* gene deletion plasmid was used to disrupt the *YKU70* gene in wildtype cells and in *yku80* cells. This plasmid contains the *URA3* marker gene, which is flanked on both sides with DNA sequences homologous to the ends of the *YKU70* gene. The *URA3* region plus flanks was removed from the plasmid via restriction digestion with *Eco*RI and *Hind*III and was subsequently transformed into cells. Once transformed, the fragment associated with the homologous sequences in the chromosomal *YKU70* gene and a recombination event occurred in a small fraction of transformed cells (Figure 7). This resulted in replacing the *YKU70* gene with the *URA3* gene and allowed these cells to grow on plates lacking uracil.

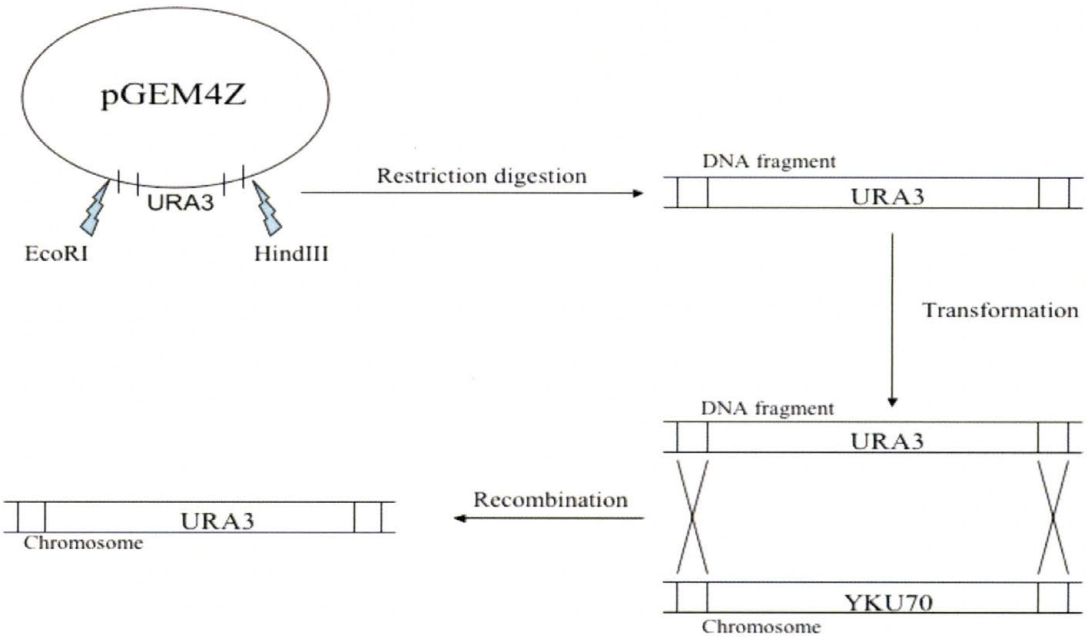


Figure 7. Schematic representation of the *yku70Δ*::*URA3* gene disruption procedure.

The *yku70Δ*::*URA3* deletion fragment was also transformed into a *yku80Δ*::*G418*^{r} single mutant. Since the *yku80Δ*::*G418*^{r} strain used to construct this double mutant was already temperature sensitive, the *yku70Δ*::*URA3* disruption was PCR confirmed with primers 5'BamKu and 3'BamKu (Figure 8). The wildtype *YKU70* gene PCR product including the 5' and 3' flanking regions is 2223 bp and the *URA3* gene including flanking base pairs is 2758 bp. The appearance of a new larger band at ~3 kb and the disappearance of the wildtype band at ~2.1 kb in the gel indicated that isolates 1 and 2 had *YKU70* replaced by the *URA3* gene (Figure 8). The 1500 bp band seen in lanes 2 and 3 (wt cell DNA) and lanes 4 and 5 (mutant isolates 1 and 2) is a result of non-specific priming during the PCR.

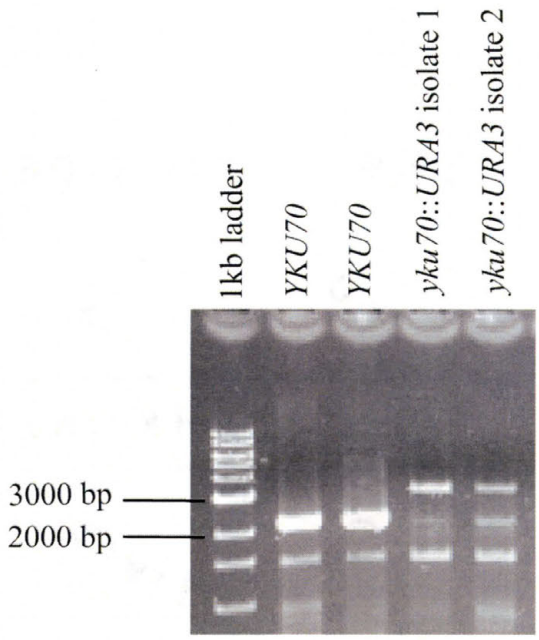


Figure 8. A 1.0% agarose gel assessing *yku70Δ*::*URA3* disruption in a *yku80Δ*::*G418*^{r} mutant strain.

pLKL64Y and pVL999 (plasmids that overexpress *TLC1* and *EST2*, respectively) were transformed into the *yku70*, *yku80*, and *yku70 yku80* strains in order to see whether Est2 polymerase or *TLC1* RNA rescues cells that are lacking both *YKU70* and *YKU80*. *TLC1* was able to rescue the death of *yku70* and *yku80* cells as well as *yku70 yku80* mutants (Figure 9). For this experiment, cells were grown at normal temperatures, harvested into water and counted in a microscope, and then diluted serially 5-fold into a 96-well microtiter dish. Cell suspensions were then spotted onto Petri dishes and incubated for 3 days at either 30 °C or 37 °C. Both cells containing a control plasmid vector (no *TLC1* gene) and cells containing *TLC1* overexpression plasmid pLKL64Y (*ADH1p*::*TLC1*) were tested. This data shows that the mechanism of rescue of *ku* mutants by *TLC1* RNA is independent of which subunit is deleted. This also illustrates that the presence of the remaining subunit of Ku in a single mutant is not required for the

TLC1 rescue of temperature sensitivity. Due to the many roles of Ku in yeast cells, numerous protein:protein interactions must be a consequence of these protein relationships. Therefore it was possible that the remaining subunit in a *ku* mutant could have been interacting with Ku binding proteins and destabilizing the protein cap. This data is critical due to seemingly contradictory studies in mice indicating that *ku80* $^{-/-}$ mutants exhibit aging phenotypes without the onset of cancer (65, 66) and *ku70* $^{-/-}$ mutants have high incidence of thymic lymphoma (67). In comparison, a recent study by Hasty *et al.* showed that the different phenotypes seen in *ku70* and *ku80* mutants may be due to differences in strain background in mice and not in the different roles of the Ku70 and Ku80 subunits (68).

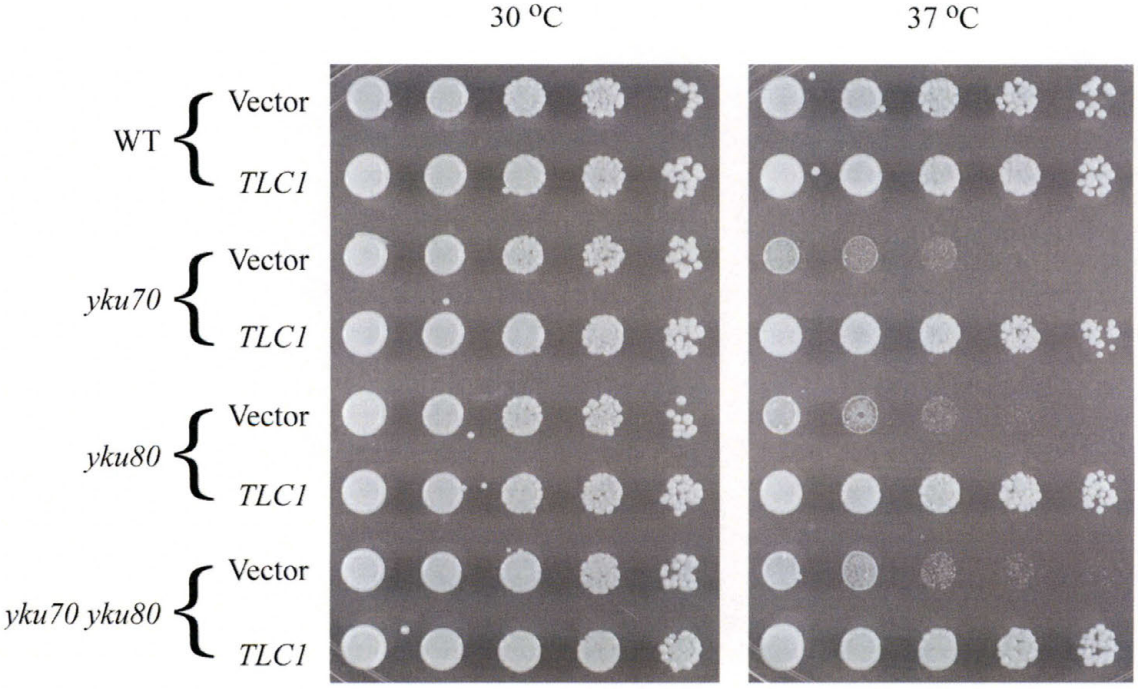


Figure 9. Rescue of *yku70*, *yku80*, and *yku70 yku80* mutants at 37 °C by overexpression of *TLC1* RNA. Vector, pRS315 control plasmid; *TLC1*, plasmid pLKL64Y (*ADH1p*::*TLC1*).

Est2 polymerase was also able to rescue the death of *yku70*, *yku80* and *yku70 yku80* cells (Figure 10). Like the *TLC1* experiment, Est2 rescues *ku* mutants independent of which subunit is deleted. The rescue of *yku70 yku80* mutants by Est2 shows that the remaining Yku80 subunit in a *yku70* single mutant is not associating with Est2 in order to stabilize telomeres.

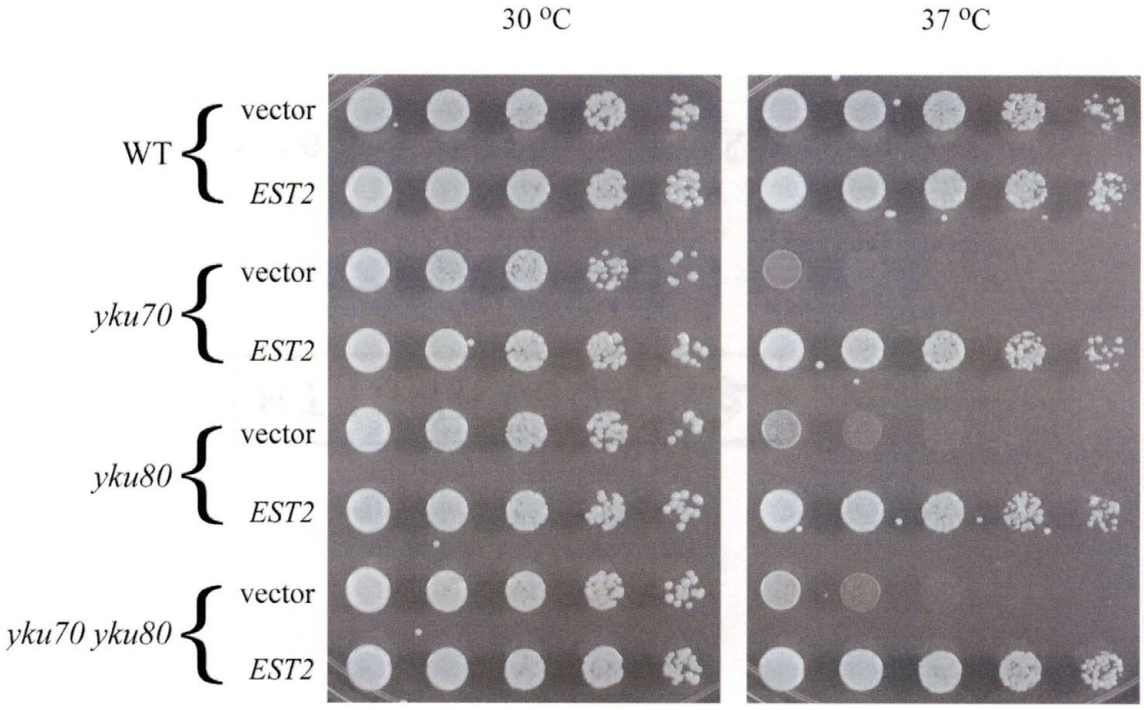


Figure 10. Est2 polymerase overexpression rescues the temperature sensitivities of *yku70*, *yku80*, and *yku70 yku80* mutants at 37 °C.

When shifted to 37 °C, *ku* mutants do not die immediately. The cells slow their growth and exhibit a cell cycle response in G_2 phase that is similar to what happens when cells are exposed to DNA damaging agents. In order to assess the number of generations that *yku70* cells undergo at 37 °C, cells were grown in YPDA liquid broth while shaking at 30 °C or 37 °C for 12 hours and 22 hours. Cells were counted before and after each incubation time and final titers and initial titers were used to calculate generations. The

number of generations of *yku70* cells at 30 °C and 37 °C after 12 hours and after 22 hours is compared in Figure 11. During 12 hours of growth, *yku70* mutants went through 3.4 generations at 30 °C but only 1.2 generations at 37 °C. After 22 hours of growth, *yku70* mutants went through 9 generations at 30 °C but just 5 generations at 37 °C. These results suggest that cells grown at 37 °C lose the ability to complete cell division after approximately 5 generations. Microscopic examination of the cultures demonstrated that most cells were arrested in G_2 phase.

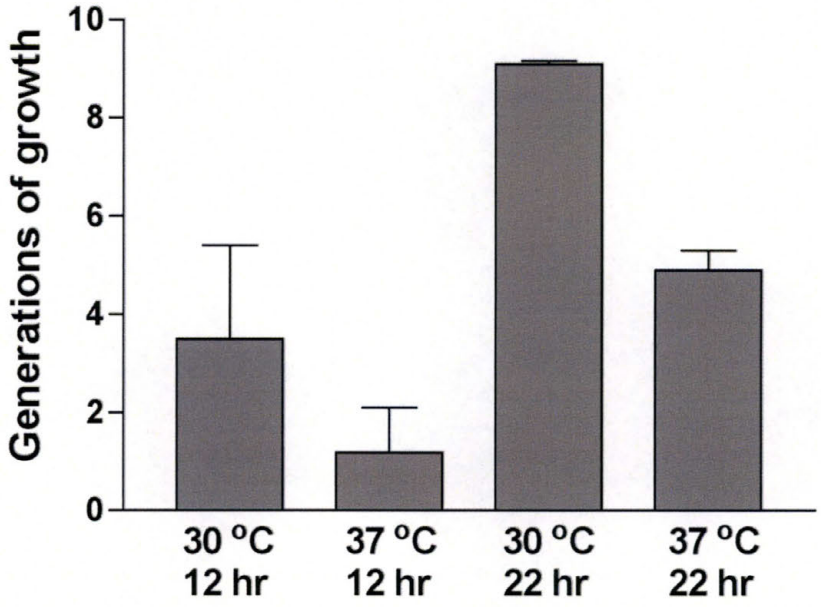


Figure 11. Graph showing the number of generations that *yku70* cells underwent in a 12 hour period and a 22 hour period at either 30 °C or 37 °C.

Work by the Lydall group demonstrated increased survival of *yku70 exo1* double mutants at 37 °C suggesting that the degradation of *ku* mutant telomeres is caused, at least in part, by the exonuclease activity of Exo1 (69). Exo1 normally functions in DNA mismatch repair and also plays a backup role in Okazaki fragment processing and in resection of DNA ends at double-stranded breaks to initiate repair (70). Wildtype telomeres are

protected by a complex of proteins including Yku70, Yku80, Rif1, Rif2, Rap1, Sir2, Sir3, Sir4, and other proteins. Once this telomeric cap is compromised, it is thought that Exo1 as well as other enzymes can gain access to the 5' end of the DNA. This exposed end of the DNA is degraded in a 5'→3' direction (Figure 12) creating a long single-stranded DNA region in the other strand that becomes susceptible to further degradation. Cells experiencing this telomeric shortening eventually stop dividing due to a cell cycle checkpoint response. Checkpoint response proteins recognize that the integrity of their chromosomes is compromised and will pause in G_2 phase of the cell cycle. When observed under magnification these cells resemble a dumbbell shape.

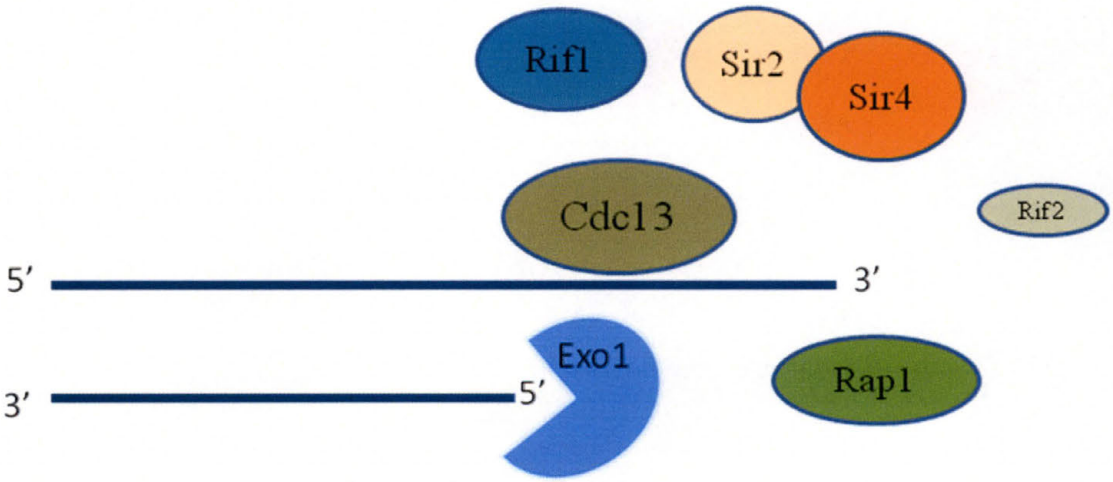


Figure 12. Schematic representation of Exo1 telomeric degradation due to a compromised telomeric cap in *ku* mutants grown at high temperatures. Exo1 (and possible other nucleases) degrades telomeric DNA in a 5'→3' direction.

The role of Exo1 in destabilization of telomeres in *ku* mutants was investigated further using plasmids that overexpress Exo1. First, we wished to determine if increased cellular levels of Exo1 would affect telomere stability in *ku* mutants grown at 30 °C or 37

°C. The G_2 arrest response of *yku70* mutants in response to Exo1 degradation was assayed by creating a *ku* strain containing plasmid pCDNA50.1. The *EXO1* gene on this plasmid is under control of the *GAL1* promoter and is only expressed when the cells are grown on galactose. To observe the effects of *EXO1* overexpression, *yku70* cells containing pCDNA50.1 were grown on solid media containing galactose as well as media containing glucose. Exo1 is not expressed in cells grown on glucose media due to transcriptional repression of the *GAL1* promoter. Cells were allowed to grow on either glucose or galactose at 23 °C, 30 °C, or 37 °C. After three days, the number of arrested cells was counted with respect to cells not arrested in the G_2 phase of the cell cycle. This assay showed that at permissive temperatures, the cells grown on either galactose or glucose plates did not arrest at either 23 °C or 30 °C, indicating resistance to Exo1 overexpression and stable protective protein caps. Approximately 10% of the cells grown at the lower temperatures were observed to be in G_2/M phase (Figure 13). In the procedure used here G_2 and M phase cells couldn't be distinguished in the light microscope, but most dumbbell-shaped cells are in G_2. This was expected because statistically, about 10% of cells in a culture will be going through the G_2 phase and will exhibit the dumbbell shape. At 37 °C, ~ 90% of the cells grown on either glucose or galactose were paused in G_2 (Figure 13). Overexpressing the *EXO1* gene did not alter the G_2 arrest of *ku* mutants at 37 °C. An explanation may be that the basal level of *EXO1* expression is adequate to trigger a cell cycle checkpoint response when the telomere caps become unstable at 37 °C.

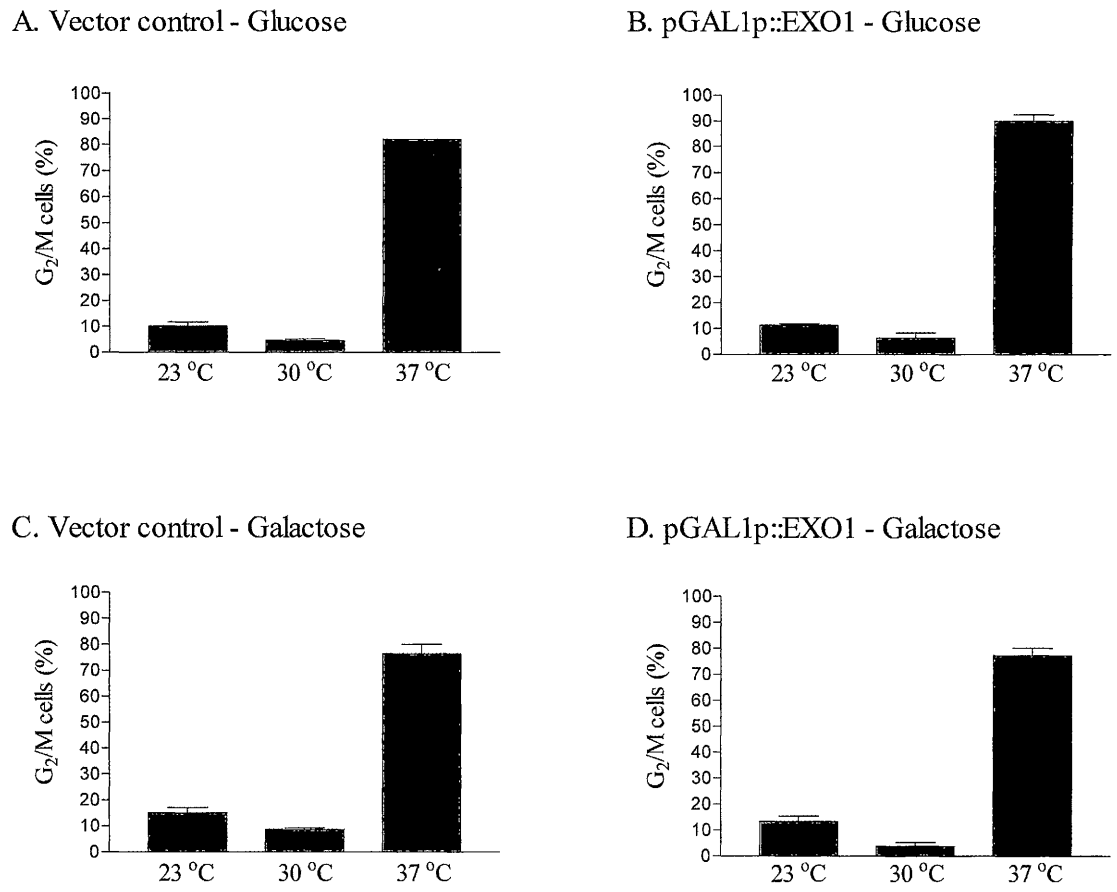


Figure 13. Graphs depicting G_2 arrest in *yku70* cells at 23 °C, 30 °C, and 37 °C. G_2 arrest of *yku70* cells containing A. vector plasmid grown on glucose; B. pGAL1::EXO1 grown on glucose; C. vector plasmid grown on galactose; D. pGAL1::EXO1 grown on galactose.

The fraction of non-growing cells in *yku70* cell cultures incubated at 37 °C in the presence of increased levels of *EXO1* was assayed by comparing survival of *yku70* cells and *yku70* cells containing pCDNA50.1. The cells were grown at 30 °C and 37 °C on glucose as a control as well as on galactose to induce *EXO1* overexpression. A larger number of cells was used for this experiment than for the ones in Figures 9 and 10 (4 x 10^7 cells in the first well versus only 1 x 10^7 cells in the earlier experiment). At 30 °C both *yku70* cells and *yku70* cells containing plasmid pCDNA50.1 (*GAL1p::EXO1*) grew

normally, but at 37 °C the *yku70* cells with overexpressed *EXO1* showed greater killing (Figure 14). This data supports evidence presented by Lydall *et al.* that Exo1 plays an important role in telomere degradation in *yku70* cells (69).

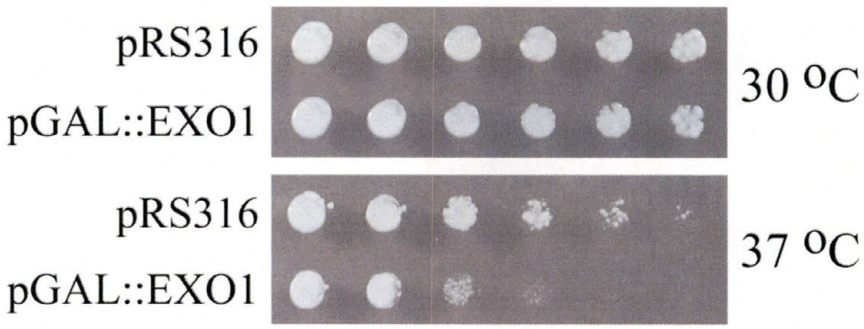


Figure 14. Overexpression of *EXO1* leads to accelerated death of *yku70* mutants at 37 °C, but not at 30 °C.

The death of *yku70* mutants at 37 °C can be alleviated by overexpressing telomerase subunits *TLC1* or Est2. In order to investigate whether *TLC1* and Est2 specifically antagonize the Exo1-accelerated death of *yku70* cells at 37 °C, plasmids containing *TLC1* and EST2 (pLKL64Y and pVL999, respectively) were transformed separately into *yku70* cells containing pCNA50.1 (*GAL1p*::*EXO1*). The pronging method of evaluating cell survival was used to assay the *TLC1* and Est2 rescue of Exo1 degradation in the mutants. When the cells were grown on glucose (Figure 15, top two panels) Exo1 nuclease was not produced from the *GAL1p::EXO1* plasmid. *yku70* cells pronged to glucose displayed strong killing at 37 °C (top two rows in upper right panel), and this lethality was suppressed in cells overexpressing *TLC1* or *EST2* (middle and bottom two rows in upper right panel). On galactose media all cells overexpressed *EXO1* from the *GAL1p::EXO1* plasmid pCDNA50.1. The death of *yku70* mutants at 37 °C was still rescued by *TLC1* and *EST2* overexpression when the cells were pronged to galactose

at 37 °C, even though Exo1 nuclease levels were higher. On both glucose and galactose plates *EST2* polymerase overexpression rescued cell killing more effectively than *TLC1* RNA.

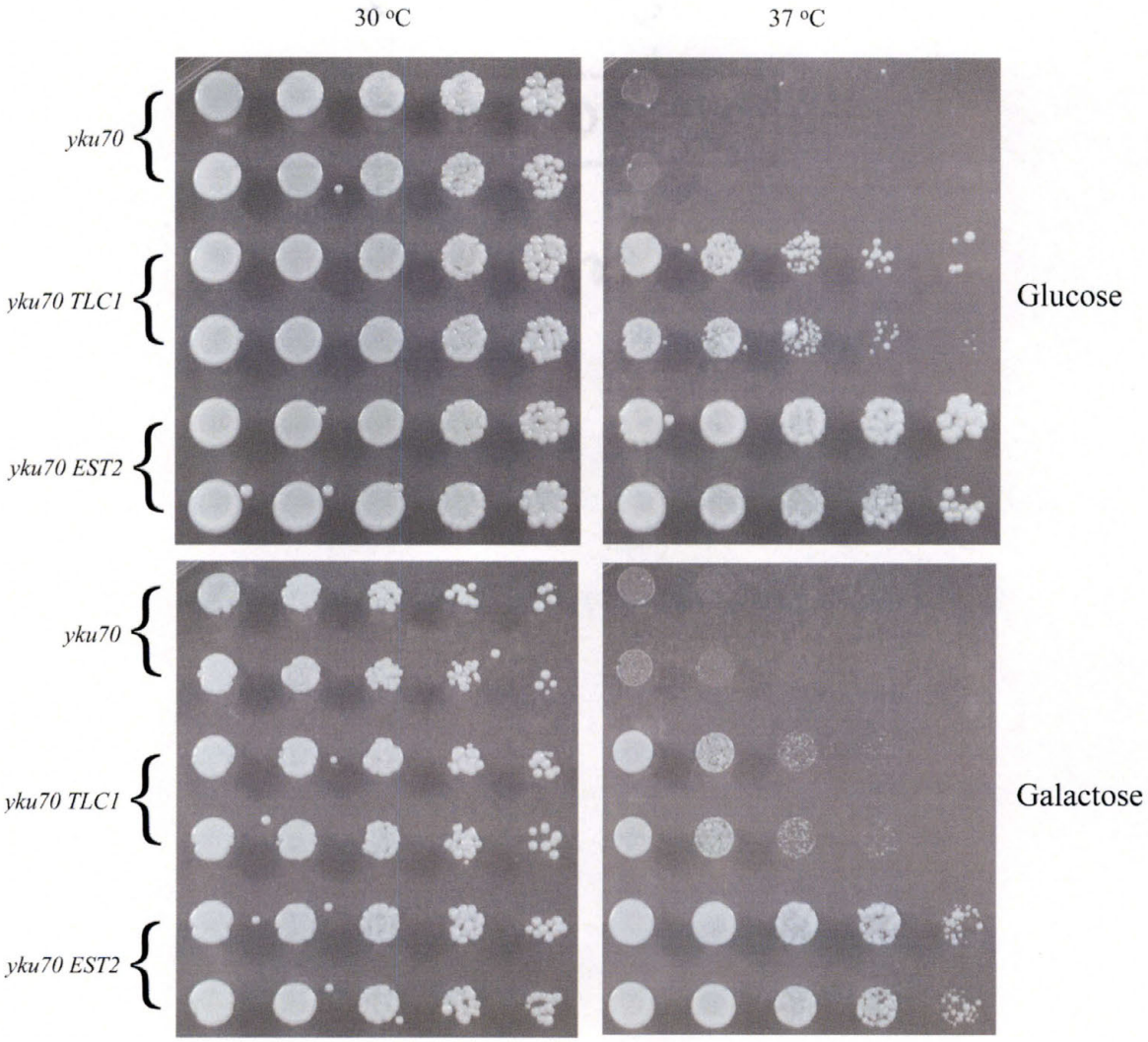


Figure 15. *TLC1* and *EST2* rescue of Exo1 accelerated death in *yku70* mutants. All cells contained the *EXO1* overexpression plasmid pCDNA50.1 (*GAL1p*::*EXO1*) which produces Exo1 in galactose media but not glucose.

Degradation of nucleic acids is an event that is critical in many cellular processes. Eukaryotic cells have evolved to produce numerous nucleases in order to perform specific functions within the nucleus of the cell, especially for DNA repair pathways.

Although Exo1 is considered to be the major contributor of 5' leading strand degradation in *ku* mutants, other nucleases may also play roles in telomere degradation. To investigate the importance of other nucleases, the impact of inactivating *RAD1*, *RAD10*, *MMS4*, or *MUS81* on *yku70* mutant death at 37 °C was assessed. *exo1 yku70* double mutants were included as a positive control since it is known that when *EXO1* is disrupted, *yku70* cells exhibit less killing at elevated temperatures. *rad1Δ::G418*^{r}, *rad10Δ::G418*^{r}, *mms4Δ::G418*^{r}, *mus81Δ::G418*^{r}, and *exo1Δ::G418*^{r} strains were obtained from a yeast deletion strain library (see Materials) and the *YKU70* gene was replaced with *URA3* in each strain as described earlier. The effect on survival of the deletions was assayed by pronging. Deletion of *RAD1*, *RAD10*, *MMS4*, or *MUS81* did not alter the ability of the *yku70* cells to grow at 37 °C (Figure 16). In contrast, *EXO1* gene disruption allowed cells to grow at elevated temperatures (Figure 16). These results show that although there may be additional nuclease degradation at compromised telomeres other than Exo1 degradation, the excess degradation is not due to the activity of the Rad1, Rad10, Mms4, or Mus81 nuclease proteins.

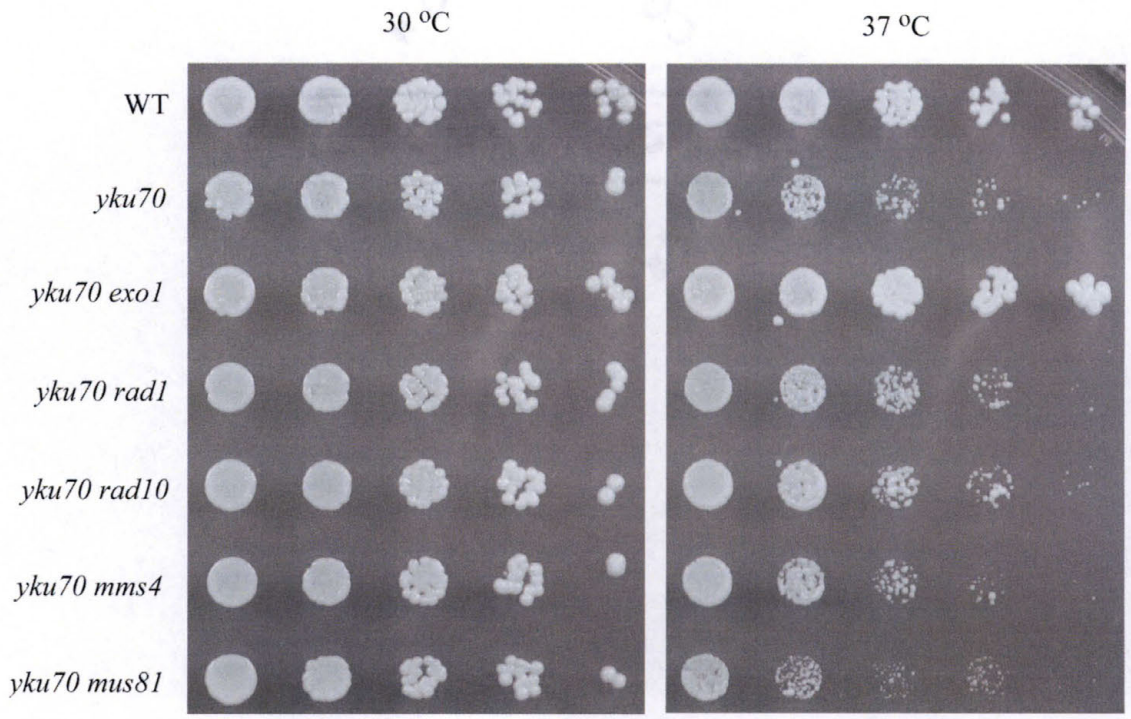


Figure 16. Deletion of the *EXO1*, *RAD1*, *RAD10*, *MMS4*, or *MUS81* nuclease genes does not rescue death of *yku70* mutants at 37 °C.

Recent work has indicated that the checkpoint protein Rad24 have an impact on compromised telomeres in *cdc13-1* mutants (32). *cdc13-1* cells make a mutant telomerase Cdc13 protein and show a temperature sensitivity phenotype similar to that of a *yku70* mutant. Since a *RAD24* deletion partially alleviated the death of *cdc13-1* mutants at 37 °C, it was possible that deleting this gene in *yku70* mutants could impact the death of *ku* cells at 37 °C. Associated with Rad24 is a potential nuclease called Rad17, which could also degrade telomeric DNA. In order to test the roles of either Rad24 or Rad17, *YKU70* was disrupted with *URA3* as before in *rad17Δ*::*G418*^{r} and *rad24Δ*::*G418*^{r} single mutants from the yeast mutant strain library. Growth of *yku70 rad17* and *yku70 rad24* double mutants was assayed at both 30 °C and 37 °C. The disruption of *RAD17* and *RAD24* in *yku70* mutants showed no effect on the death of

yku70 mutants at 37 °C (Figure 17). This suggests that Rad17 and Rad24 play a different role in telomere degradation in *cdc13-1* than in *yku70* mutants.

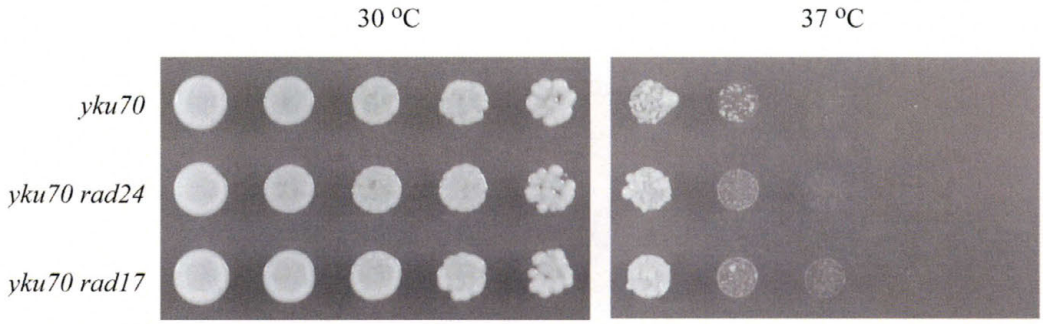


Figure 17. Disruption of *RAD24* or *RAD17* does not alter survival of *yku70* mutants at elevated temperatures.

Wildtype telomeres are composed of telomeric DNA as well as the many different proteins that form the protective cap structure. Some of these proteins associate with the subtelomeres as well as the telomeres. Other proteins have an affinity for the single-stranded DNA located at the telomere termini. Additional proteins associate with other proteins bound to telomeres. Figure 18 is a representation of some of the known protein interactions at the telomeres in yeast cells.

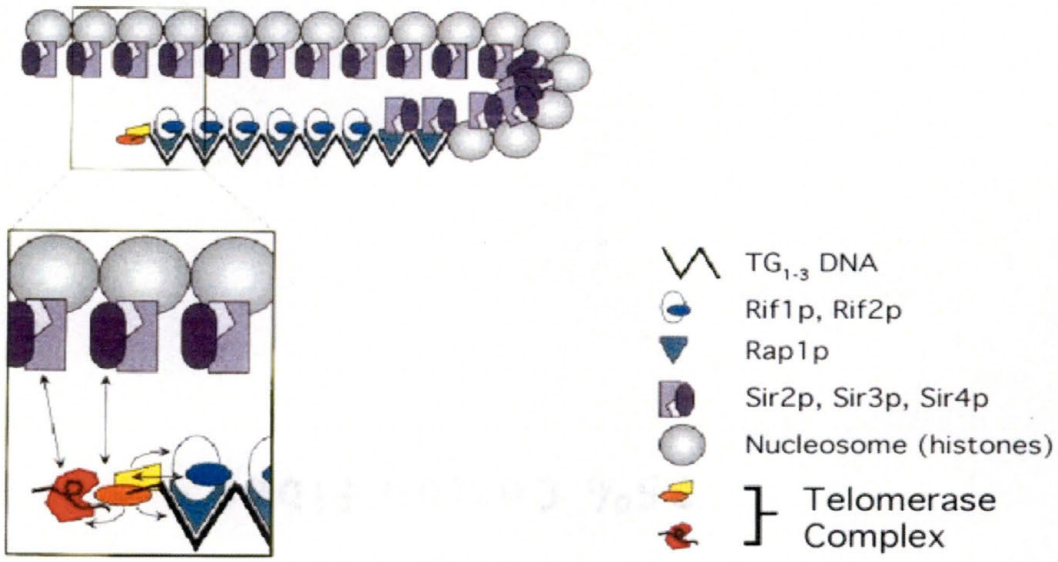


Figure 18. Representation of telomeric chromatin proteins and their associations (from 71).

When *yku70* cells are grown at elevated temperatures, it is believed that the protein telomeric cap becomes compromised. This is likely due to disrupted protein:protein interactions resulting from the absence of the Ku complex. Rif1 and Rif2 (Rap1-interacting factors 1 and 2) are telomere length regulating proteins that are recruited to telomeres by telomeric DNA binding protein Rap1 (72). Rif1 and Rif2 are thought to be part of the telomeric chromatin and may also negatively regulate the extent of DNA synthesis performed by telomerase during each S phase. A previous study demonstrated that inactivation of *RIF1*, which causes slight elongation of telomeres, helped rescue the death of *yku70* mutants at high temperatures (36). *rif2* mutants also have longer than normal telomeres, but the impact of *RIF2* inactivation on *yku70* strains has not been tested. To further investigate the roles of Rif proteins in *ku* mutant survival and rescue by telomerase, *yku70 rif1* and *yku70 rif2* double mutants were constructed.

These cells were grown at both 30 °C and 37 °C. As expected, *rif1 yku70* cells grew better at 37 °C than *yku70* single mutants, though there was reduction of growth at 39 °C (Figure 19). In contrast, *rif2 yku70* mutant growth was similar to *yku70* cells. Thus, removing *RIF2* from the remaining protein cap in *yku70* mutants does not affect extent of killing. When either of the telomerase negative regulators *RIF1* or *RIF2* is disrupted, cells have longer than normal telomeres. Interestingly though, only disruption of *RIF1* can alleviate the loss of viability of *ku* cells at high temperatures.

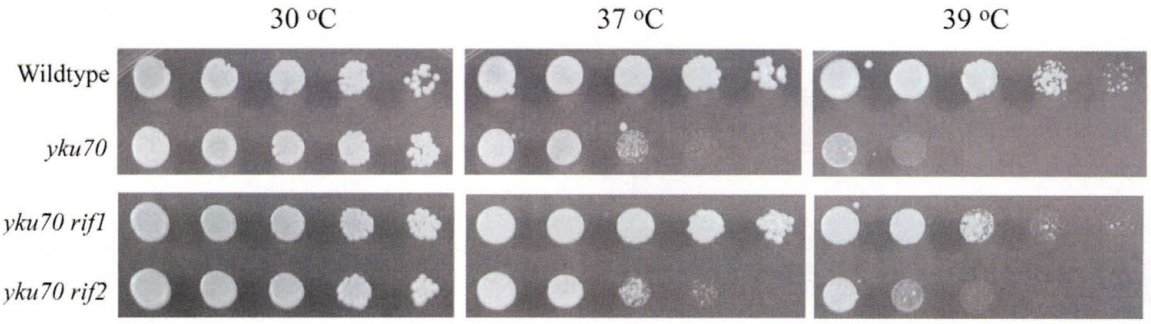


Figure 19. Effect of deleting *RIF1* or *RIF2* on the temperature sensitivity of a *yku70* mutant.

The mechanism of rescue of *yku70* mutants by *TLC1* RNA or Est2 polymerase overexpression may be a result of creation of a more stable structure that physically protects the telomeres. This complex may exist due to *TLC1* and Est2 binding to other proteins at the telomeres and stabilizing them or, alternatively, because the subunits bind to and titrate away a destabilizing protein. In order to assess dependence on possible interactions with Rif1 or Rif2 for the rescue of *yku70* mutants, *TLC1* RNA and Est2 polymerase were overexpressed separately in *yku70 rif1* and *yku70 rif2* cells. This experiment showed that deleting either *RIF1* or *RIF2* had no effect on the rescue of *yku70*

cells (Figure 20). In this experiment, a high temperature of 39 °C was used because *rif1 yku70* cells only display sensitivity at this higher temperature.

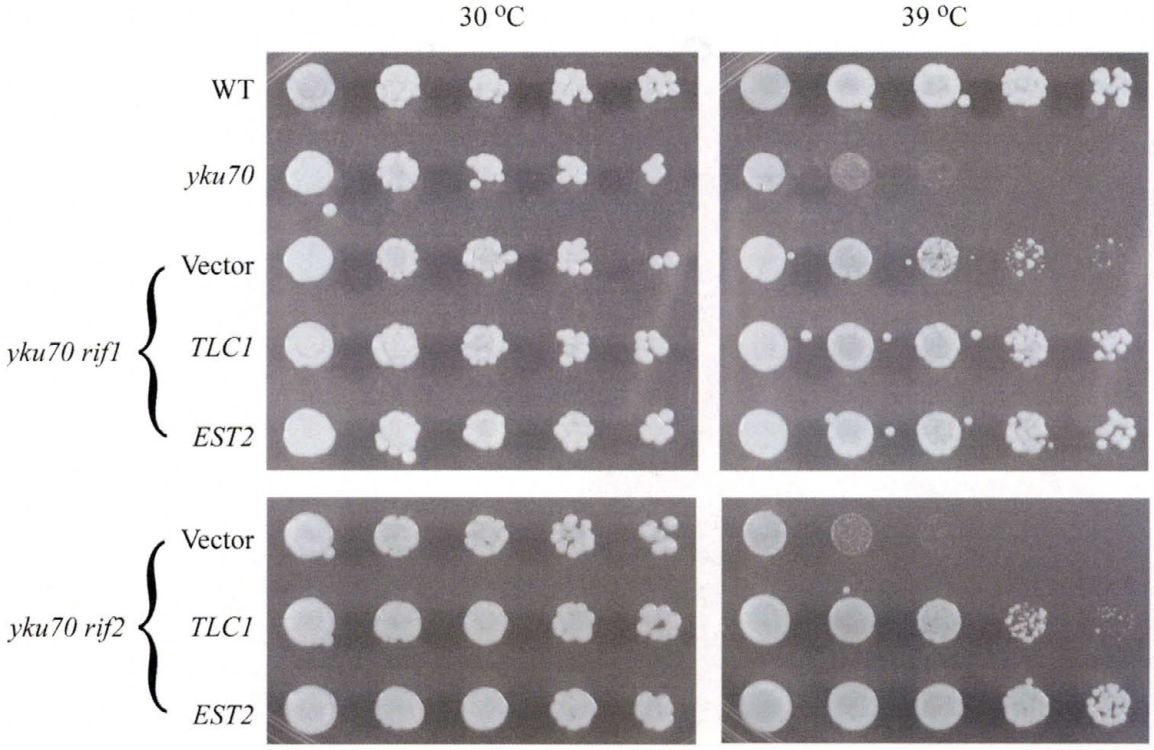


Figure 20. Overexpression *TLC1* and *EST2* rescues the death of *yku70* mutants independent of Rif1 or Rif2.

The Sir (Silent Information Regulator) proteins associate with telomeric histone tails and help to repress the transcription of genes in proximity to the telomeres in an event called telomeric silencing. Sir3 has been shown to bind the telomeric binding protein Rap1 and Sir4 can associate with Yku70 *in vivo* (73). Since the telomeric cap is unstable in *yku70* mutants at high temperatures, a mutation in a *SIR* gene within a *yku70* cell could lead to enhanced instability at the telomeres. *yku70Δ::URA3 sir2Δ::G418*^{r}, *yku70Δ::URA3 sir3Δ::G418*^{r}, and *yku70Δ::URA3 sir4Δ::G418*^{r} double mutant strains were created to see whether inactivation of *SIR* genes resulted in a phenotypic change in temperature sensitivity in *yku70* mutants. The *yku70 sir2*, *yku70 sir3*, and *yku70 sir4*

strains showed strong temperature sensitivity at 37 °C, but exhibited moderately better growth than the *yku70* single mutants, especially the *yku70 sir3* strains (Figure 21). The increased growth was approximately one column wide, or about 5 fold greater survival. This result indicates that telomeres are slightly more stable when both Ku and Sir proteins are absent compared to cells lacking only Ku.

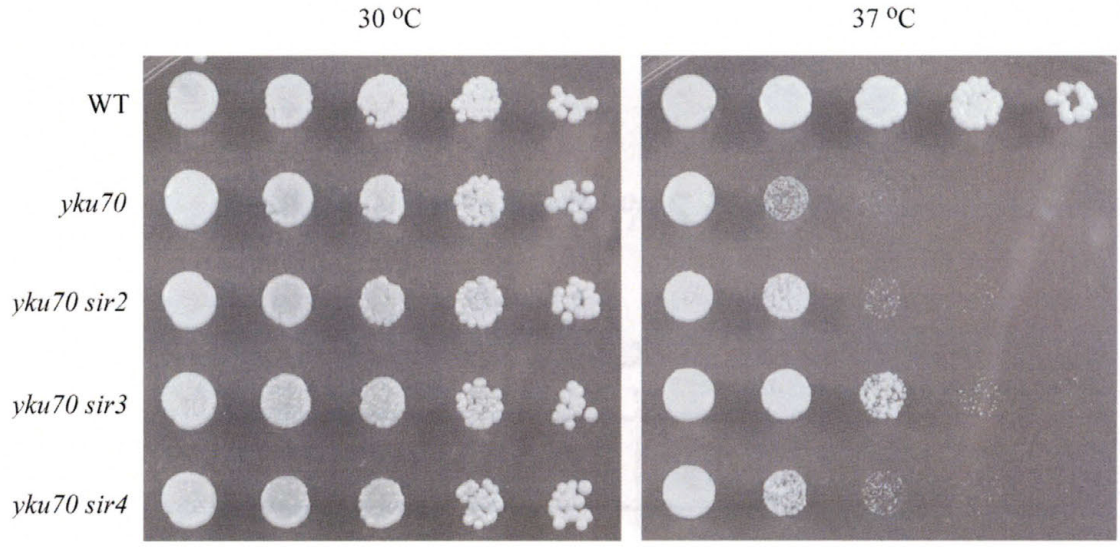


Figure 21. Deletion of Sir genes in *yku70* mutants resulted in slightly less killing than in *yku70* mutants at 37 °C.

In order to investigate whether the Sir proteins were critical for the rescue of *yku70* mutants by *TLC1* RNA or Est2 polymerase overexpression, pLKL64Y (*TLC1*) and pVL999 (*EST2*) were transformed into the *yku70 sir2*, *yku70 sir3*, and *yku70 sir4* double mutant strains. The cells were pronged to glucose minus uracil and minus leucine to select for the *yku70*::*URA3* mutation as well as to select for the plasmids. Interestingly, rescue by *TLC1* RNA was abolished when *SIR2* was inactivated and was reduced substantially by deletion of *SIR4* (Figure 22). In contrast, *yku70 sir3* cells were rescued even more efficiently than *yku70* single mutants. These results indicate that the rescue of

yku70 mutants by *TLC1* is dependent on the presence of Sir2 and Sir4 and is independent of Sir3.

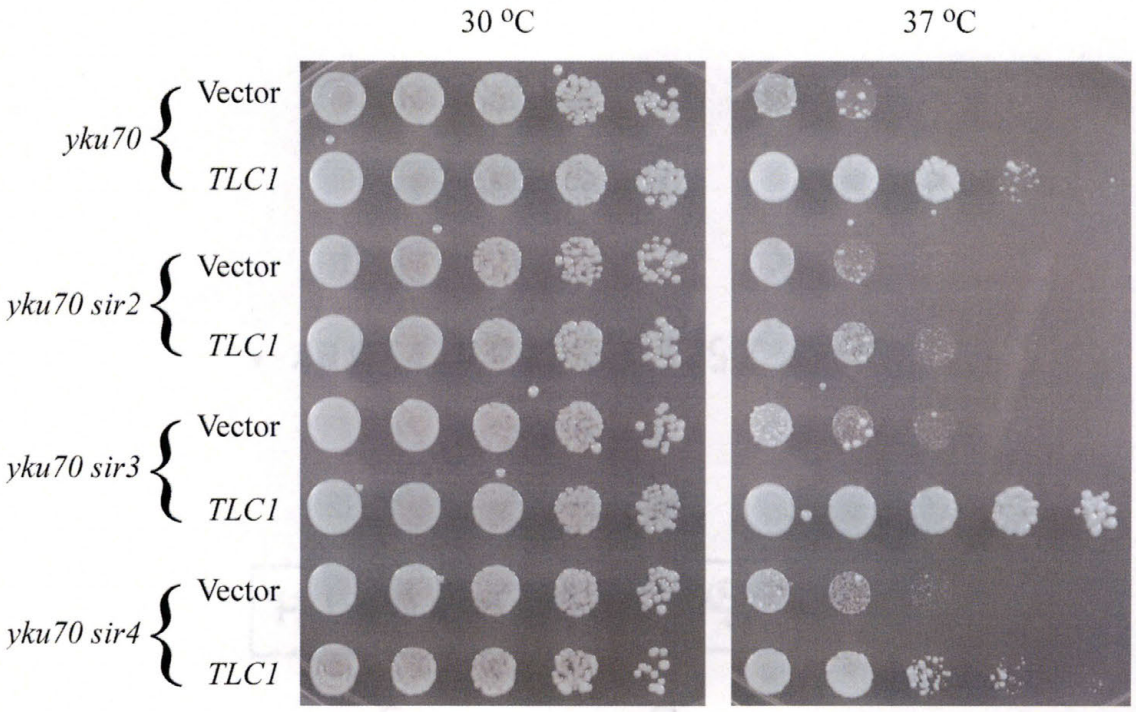


Figure 22. Inactivation of *SIR2* or *SIR4*, but not the *SIR3* gene, in *yku70* mutants abolishes rescue of temperature sensitivity by *TLC1* RNA.

To assess the effects of deleting the *SIR* genes on Est2 polymerase rescue of *yku70* mutants, the *yku70 sir2*, *yku70 sir3*, and *yku70 sir4* strains were transformed with pVL999 and a vector control, pronged, and grown at 30 °C or 37 °C. Est2 was capable of rescuing *yku70 sir2* and *yku70 sir3* strains as well as *yku70* single mutants. However, growth of *yku70 sir4* mutants was not rescued significantly by *EST2* polymerase overexpression (Figure 23). These results indicate that the rescue of *yku70* mutants at 37 °C by Est2 is independent of Sir2 and Sir3. It also suggests that Est2 requires Sir4 in order to rescue the death of *yku70* mutants at elevated temperatures.

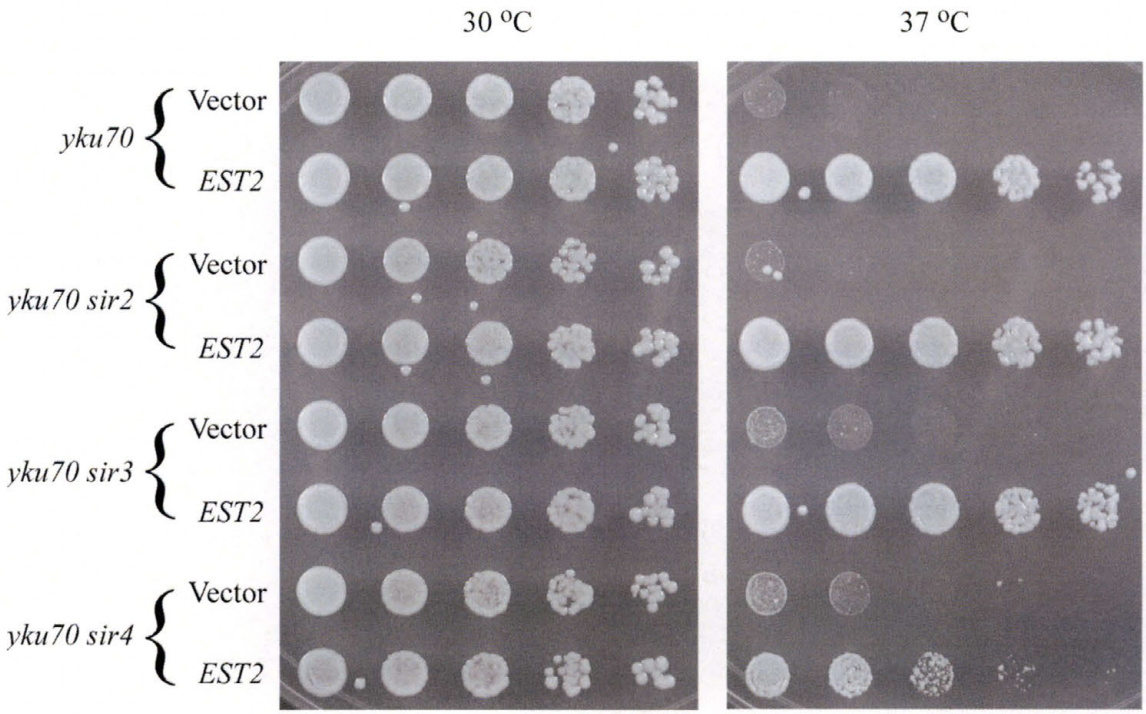


Figure 23. Rescue of temperature sensitivity of *yku70 sir* double mutants by overexpression of *EST2* polymerase at 37 °C.

The difference between the *TLC1* and Est2 rescue of *yku70 sir2*, *yku70 sir3* and *yku70 sir4* strains may be due to separate mechanisms of stabilization of *yku70* telomeres at 37 °C. Alternatively, it is possible that *TLC1* and Est2 rescue temperature sensitivity via similar mechanisms, since *TLC1* and Est2 associate to form the core of the telomerase holoenzyme, but Est2 is more effective at suppression. The dependence on the presence of a functional *SIR4* gene for rescue by both *TLC1* and Est2 suggests a common mechanism by both subunits, though it is also important to note the difference in ability to rescue *yku70 sir2* cells. *TLC1* RNA overexpression not only requires Sir4 to rescue the death of *yku70* mutants, but also requires Sir2 (Figure 22), but Est2 did not require *SIR2*.

Telomeres are anchored to the nuclear envelope via interactions between telomeric proteins and nuclear porin proteins. There is evidence that the tethering of telomeres to the nuclear porin complexes protects the telomeres from deleterious cellular events. The Mlp1 and Mlp2 proteins are required for efficient anchoring of telomere ends to the nuclear membrane and co-precipitation studies have shown that Mlp2 physically interacts with Yku70 (34). Since Mlp1 and Mlp2 tethering has been shown to protect the telomeres, it is possible they might affect chromosome stability and temperature sensitivity in *ku* cells. In order to test the impact of Mlp1 and Mlp2 on the survival of *yku70* mutants at elevated temperatures, a series of *yku70 mlp1*, *yku70 mlp2*, and *yku70 mlp1 mlp2* mutant strains were constructed. The viabilities of these mutant strains were compared to those of *yku70*Δ::*URA3* single mutants at 37 °C (Figure 24). Inactivating both *MLP1* and *MLP2* partially alleviated the temperature sensitivity of the *yku70* cells, resulting in approximately 25-fold higher survival at 37 °C. Deleting either *MLP* gene alone modestly rescued growth at higher temperatures, but not to the extent seen in *yku70 mlp1 mlp2* cells.

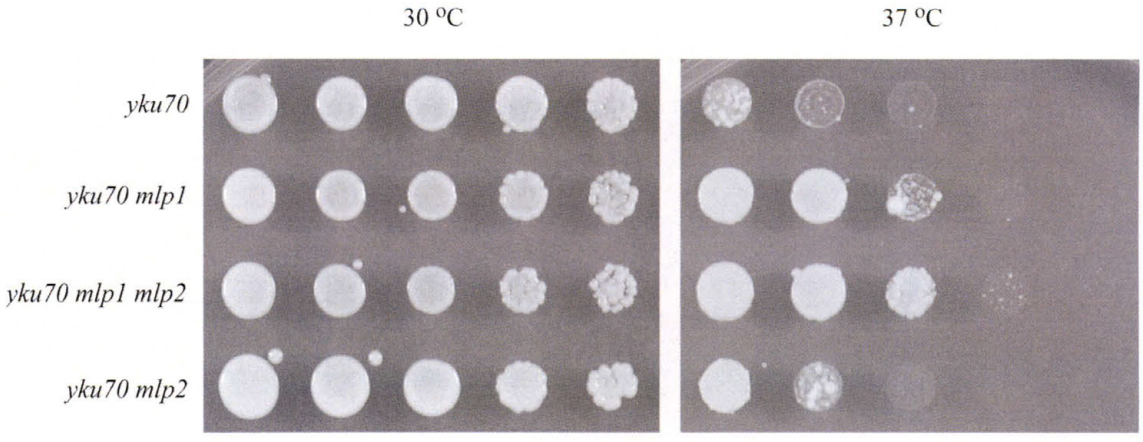


Figure 24. Effect of *MLP* deletions on the temperature sensitivity of *yku70* mutants.

Cells with disrupted *MLP* genes exhibit both decreased nuclear import of some molecules as well as extended telomeres. Since Mlp1 and Mlp2 interact with the telomeres and help anchor them to the nuclear periphery, the results presented here (Figure 24) suggest that reduction of tethering actually increases *ku* cell survival at 37 °C. Since DNA degradation by Exo1 is a primary cause of *yku70* cell death at 37 °C, the data suggests that this degradation is reduced under conditions where ends are not anchored efficiently. It is not clear why this might be true, but it is conceivable that less anchoring allows for increased telomere:telomere recombination events, which is known to affect the rate of killing of *yku70* cells at 37 °C.

It is possible that the rescue by *TLC1* or *EST2* of *yku70* mutants is due to a secondary stabilizing interaction between *TLC1* and Est2 with the Mlp proteins. This was tested by separately overexpressing *TLC1* and *EST2* in *yku70*, *yku70 mlp1*, *yku70 mlp2*, and *yku70 mlp1 mlp2* cells. Overexpression of *TLC1* RNA in all 4 mutant strains showed similar rescue at 37 °C (Figure 25). Additionally, *EST2* overexpression in *yku70 mlp1 mlp2* and *yku70 mlp2* cells rescued 37 °C temperature sensitivity (Figure 26).

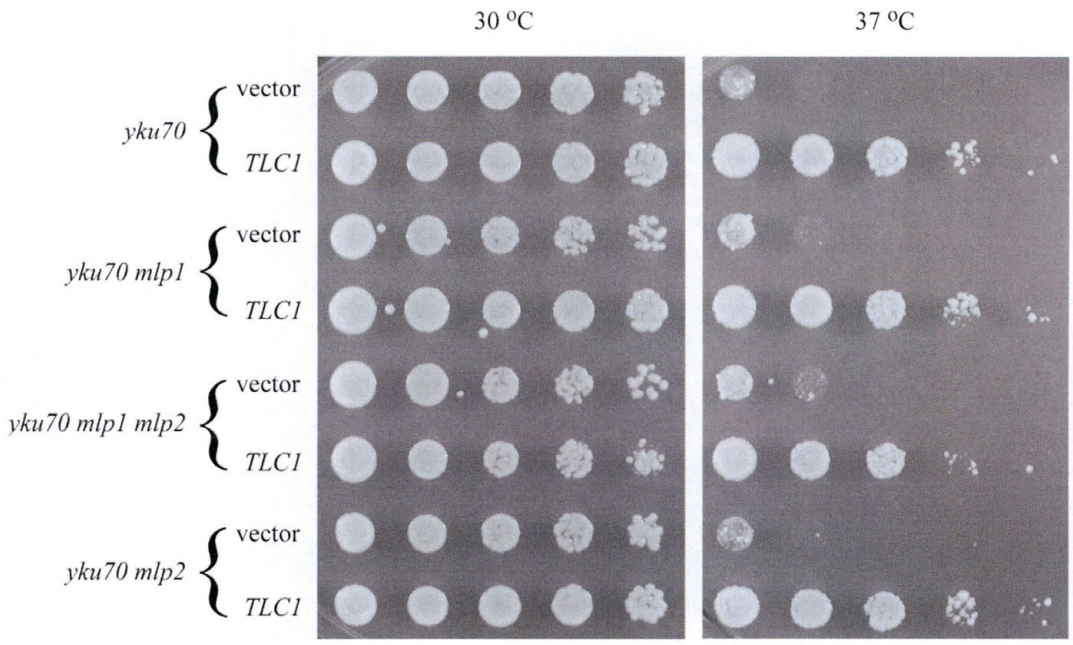


Figure 25. *TLC1* rescues the temperature sensitivity of *yku70*, *yku70 mlp1*, *yku70 mlp1 mlp2*, and *yku70 mlp2* cells at 37 °C.

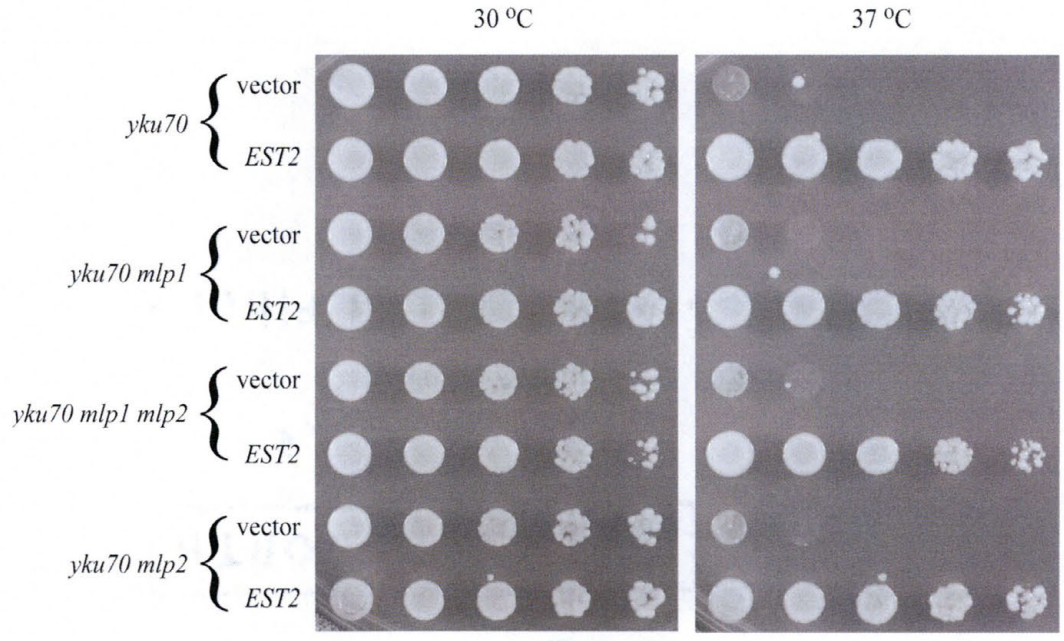


Figure 26. *EST2* overexpression alleviates the temperature sensitivity of *yku70* mutants lacking *MLP1*, *MLP2* or both genes.

In addition to their telomere instability phenotype, *ku* mutants are also defective in one of the two major pathways of DNA double-strand break repair, called nonhomologous end-joining or NHEJ (26). This DNA repair defect causes the mutant cells to have reduced resistance to DNA damaging agents such as bleomycin and to be hypersensitive to expression of the endonuclease *Eco*RI *in vivo* (27, 74).

Recent work has established that overexpression of *TLC1* RNA rescues the *Eco*RI-sensitivity of double-strand break repair mutant *rad50*, *mre11*, and *xrs2* cells (48). However, the possibility that *TLC1* RNA (or Est2 polymerase) overexpression might rescue *Eco*RI-killing of *yku70* or *yku80* mutants has not been tested.

Investigation of *TLC1* and *EST2* rescue in *yku70* cells that are overexpressing *Eco*RI was accomplished by creating *yku70* strains containing *TLC1* and *EST2* plasmids plus another plasmid that had the *GAL1* promoter controlling *Eco*RI expression. In these strains, *TLC1* and *EST2* are producing product constitutively and the *Eco*RI gene can be induced by growing the cells on galactose media. Interestingly, when *yku70* cells containing either pLKL64Y (*TLC1*) or pVL999 (*EST2*) were grown at on galactose and thus overexpressing *Eco*RI, the cells, including wildtype cells, were sensitized to *Eco*RI killing (Figure 27). Thus, high intracellur levels of *TLC1* RNA not only didn't rescue this phenotype of *ku* mutants, it accelerated the killing effect of *Eco*RI. It is not clear why this occurred, but might be due to changes in chromatin structure that make more recognition sites accessible to the nuclease.

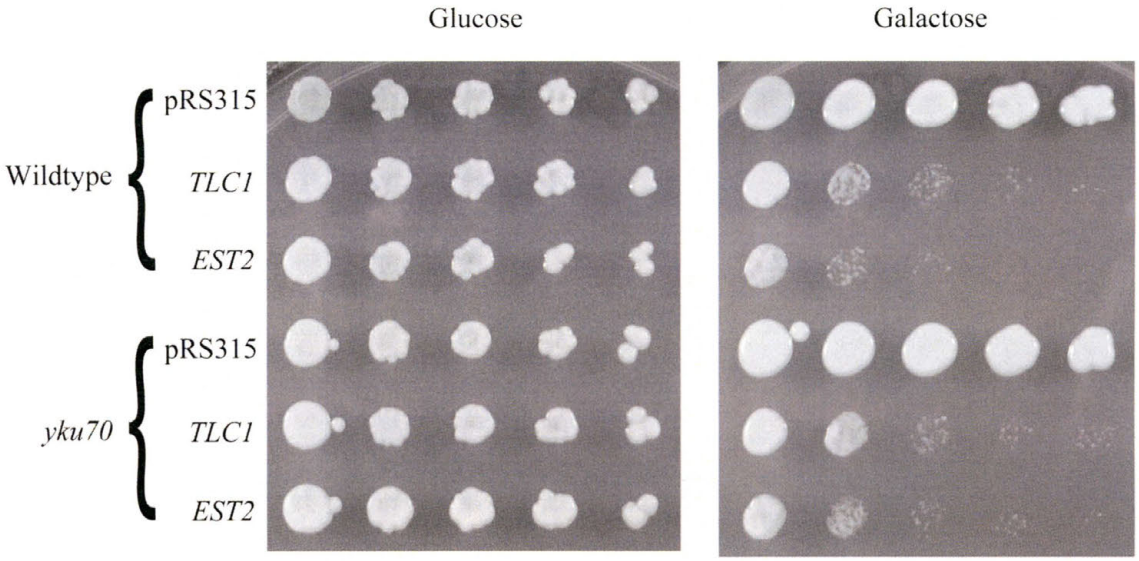


Figure 27. Overexpression of *TLC1* and Est2 sensitizes *yku70* and wildtype cells to *Eco*RI overexpression.

To further investigate the mechanisms by which telomere instability of *ku* cells can be rescued, a cDNA library screen was performed to find novel genes that suppress the temperature sensitivity of *yku70* mutants. The cDNA library is a random mix of plasmids containing approximately every transcribed yeast gene under the control of a *GAL1* promoter. Each plasmid is derived from the yeast cloning vector pRS316 (Figure 28) and therefore renders transformed cells Ura^+.

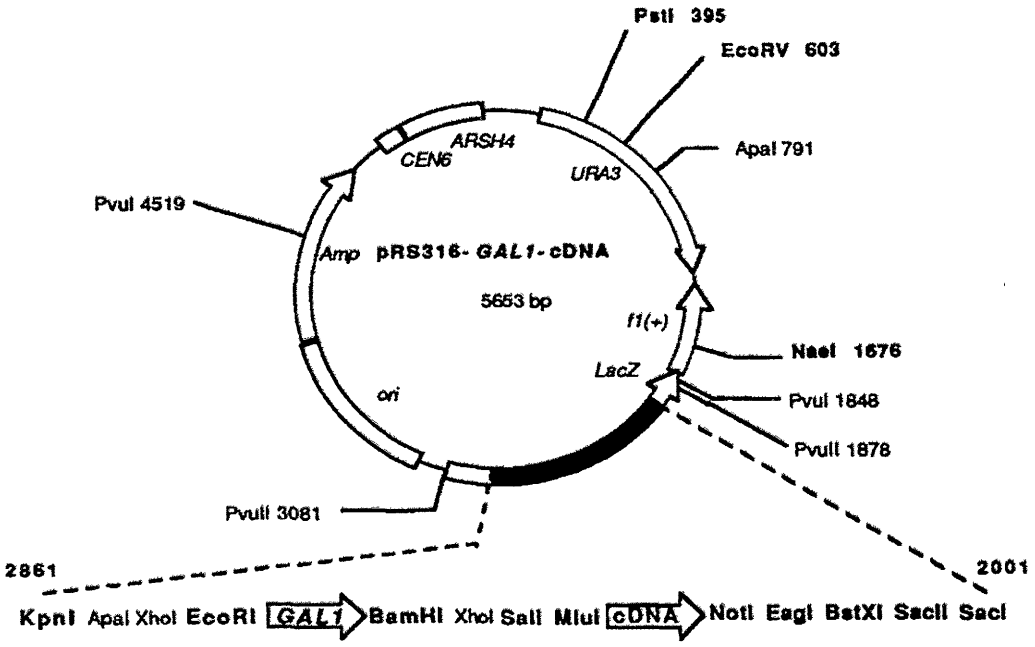


Figure 28. Diagram of the pRS316 cloning vector used to create the pRS316-*GAL1*-cDNA library (75). pRS316 contains *CEN/ARS*, *URA3*, *Amp*^{r}, and a multiple cloning site region which is transcriptionally regulated by a *GAL1* promoter.

The collection of cDNA plasmids was transformed into *yku70* cells. Colonies from the transformation were tested for their ability to grow on glucose minus uracil and galactose minus uracil plates at 37 °C. Of ~20,000 transformants screened, 31 *yku70* temperature sensitivity suppressors were isolated. Each transformant strain was found to grow on galactose plates at 37 °C (conditions where the cDNA is expressed), but not on glucose plates at 37 °C. The plasmids from 25 of the 31 isolates were able to be extracted from the yeast hosts and analyzed on a 0.6% agarose gel (Figure 29). The gel shows plasmids of numerous sizes were observed. The pRS316 vector that the genes were cloned into is the same size in all plasmids. The only difference in sizes would be a consequence of differing gene sizes cloned into the vector.

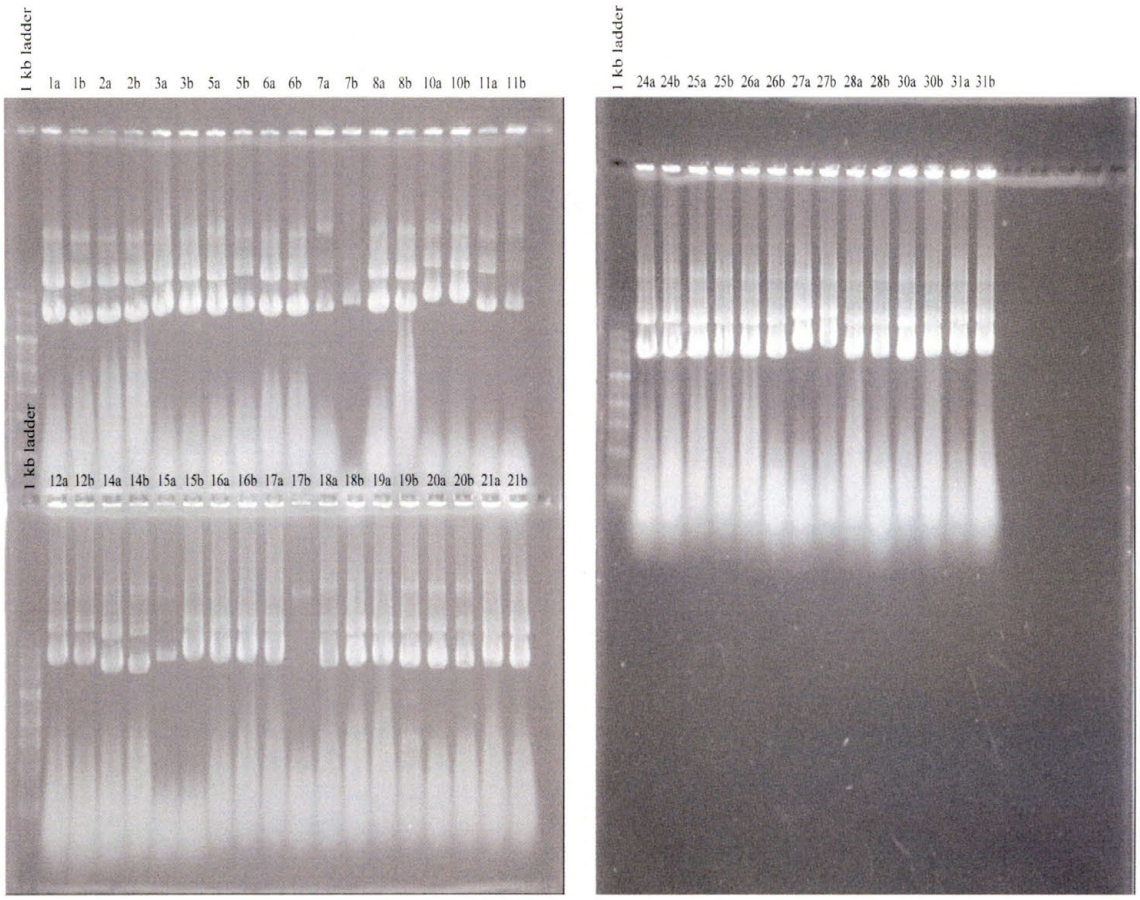
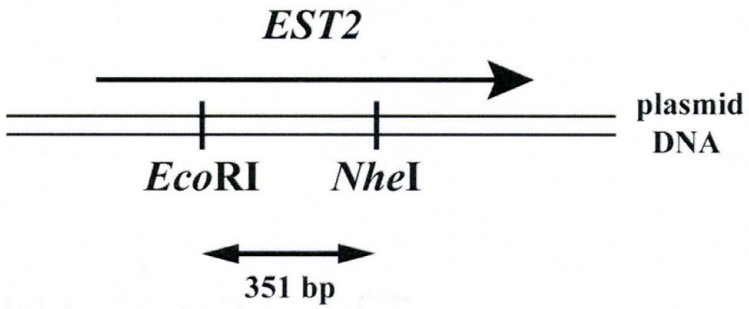


Figure 29. Agarose gel analysis of plasmids that suppress temperature sensitivity of *yku70* cells isolated from the cDNA library screen.

Restriction digestions of the cDNA plasmids were performed to see if the plasmids encoded previously described *yku70* temperature sensitivity suppressing genes (*EST2*, *TLC1*) as well as to test for the presence of *YKU70*. Figure 30A is an example of *EST2* digestion resulting in a 351 bp DNA fragment released after digestion. Positive *TLC1* and *EST2* restriction digest controls included pLKL64Y (*ADH1p*::*TLC1*) and pVL999 (*ADH1p*::*EST2*), respectively. *Afl*II and *Nco*I enzymes were chosen for detection of *TLC1*. Digestion releases a 467 bp fragment if this gene is present on the plasmid. *Eco*RI and *Nhe*I were used for *EST2* gene detection resulting in a 351 bp fragment if this gene is on a plasmid. Figure 30B shows the control *EST1*, *EST2*, and

TLC1 plasmid digestions with appropriate enzymes. *EST1* was reported by Nugent *et al.* (4) to suppress killing of *yku70* cells at 37 °C, though this could not be reproduced in our lab. (Although *EST1* was digested here, it was not screened for in the cDNA library.) The pLKL64Y (*TLC1*), pVL999 (*EST2*), and pVL784 (*EST1*) digests were successful, producing bands of the expected size for each digest. These digests were included as positive controls during the next tests, involving digestion of each of the 25 library plasmids that rescued *yku70* cells.

A



B

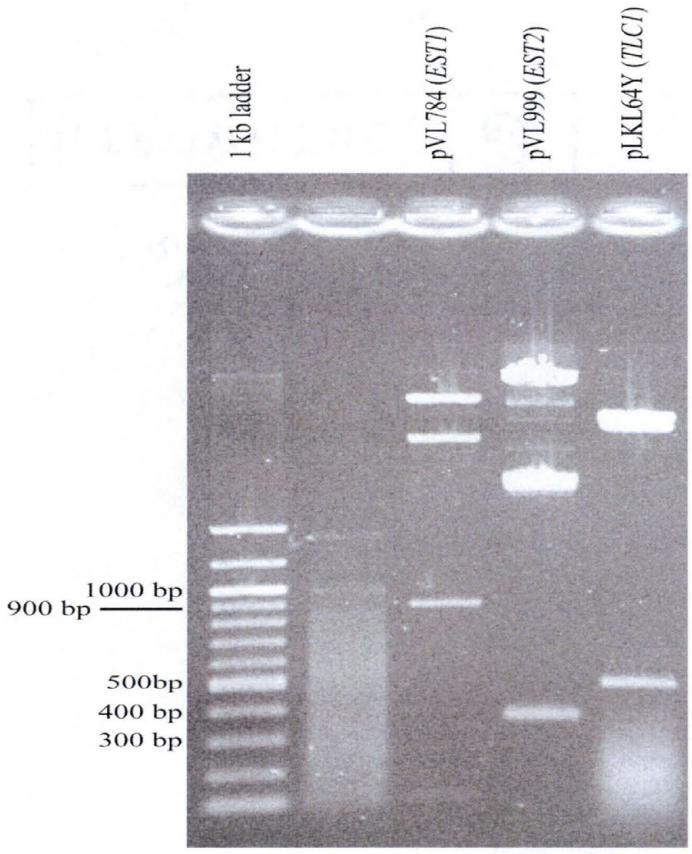


Figure 30. A. Schematic representation of a plasmid encoding *EST2* digested with *Eco*RI and *Nhe*I. B. Control restriction digestions demonstrating the feasibility of screening library plasmids for the presence of *EST1*, *EST2*, and *TLC1*. Restriction fragments released are approximately 901 bp, 351 bp and 467 bp for *EST1*, *EST2*, and *TLC1* respectively.

The cDNA plasmids were first screened to identify any *EST2* genes present in the 25 isolates. The 25 cDNA samples and pVL999 (*EST2*) were digested with *Eco*RI and *Nhe*I and the digestion products were run on a 1.2% agarose gel. Of the 25 samples, no isolate exhibited the same 351 bp digestion product as pVL999, thus suggesting that no *EST2* genes were present in the 25 cDNA isolates (Figure 31). We note that library plasmids containing incomplete cDNAs from the *EST2* gene or rare mutated alleles might not be detected in this assay.

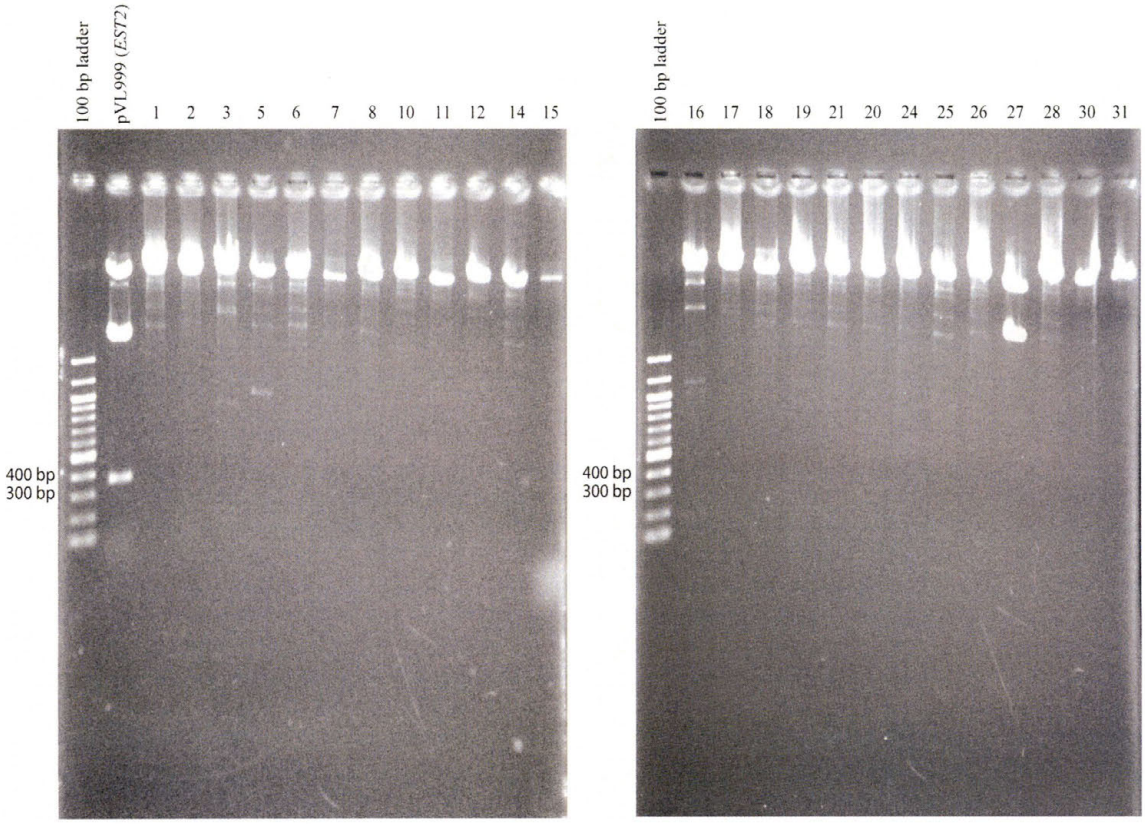


Figure 31. Digestion of cDNA plasmids with *Eco*RI and *Nhe*I to screen for the presence of the *EST2* gene.

To reveal the identity of any *TLC1* genes within the 25 isolates, *Afl*II and *Nco*I were used to digest and release a 467 base pair fragment from *TLC1*. The 25 isolates as well as pLKL64Y (*TLC1*) were digested with *Afl*II and *Nco*I and electrophoresed on a 1.2% gel. Of the 25 isolates, only isolate #16 showed a release of a fragment of the same size as that of the pLKL64Y positive *TLC1* control (Figure 32), thus strongly suggesting that the identity of the gene on plasmid #16 is *TLC1*. None of the other plasmids showed this pattern indicating that these plasmids did not contain the *TLC1* gene (Figure 32).

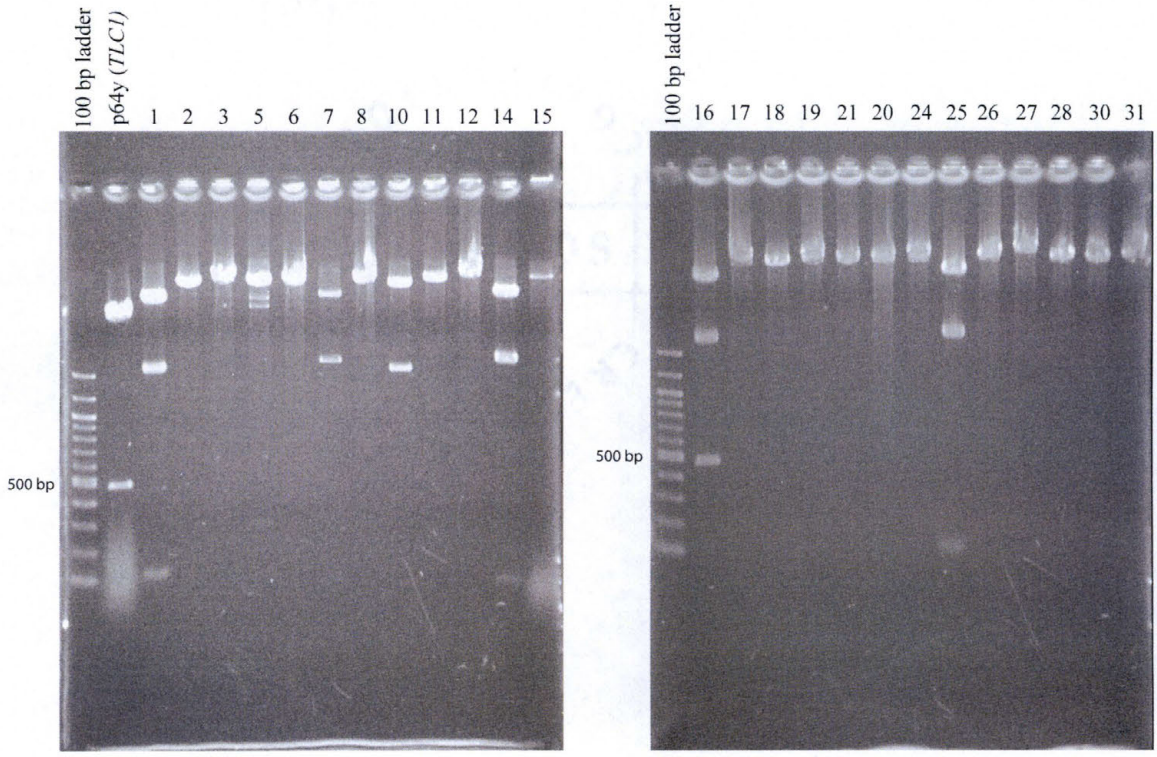


Figure 32. *TLC1* digestion of cDNA plasmids with *Afl*II and *Nco*I digestion.

In addition to *TLC1* and *EST2*, the cDNAs were also screened via restriction digestion for the presence of *YKU70*. Tranforming an expression plasmid containing *YKU70* into a *yku70* mutant would alleviate the temperature sensitivity phenotype in that cell and thus allow the cell to grow at 37 °C. The cDNAs were digested with *Pst*I and

*Xba*I which releases a 530 bp fragment. The results from this digestion indicated that none of the 25 isolates were *YKU70* (data not shown).

Since only one of the cDNA *yku70* temperature sensitivity-suppressing isolates could be identified, the samples were sent to Retrogen Inc. (San Diego, CA) to be sequenced. Sequences obtained for the 25 cDNA isolates were compared to the Sacharomyces Genome Database (SGD) for identification of genes. The sequence comparison showed that one of the 25 isolates was *TLC1* (Figure 33), which was previously determined by restriction digests. According to the SGD, the other 24 genes were identified as genes associated with numerous cellular functions (Figure 33). Many of these genes have no apparent relationship to DNA metabolism. Interestingly, some genes were obtained multiple times. For example, the *HYP2* gene was present in 8 independent isolate, *CPR1* was present in 3 clones, and *URA5* was found twice. Most of the genes have previously been linked to protein synthesis and/or ribosome structure. It is conceivable that overexpression of genes such as *HYP2* and *CPR1*, which are known translation-associated genes, helps to increase the concentration of proteins that are downregulated in heat-shocked yeast cells at high temperatures. Further analysis will determine whether the cDNA isolates only rescue telomere instability of *ku* mutants or favorably alter other cellular metabolism resulting in increasing vitality at high temperatures for all yeast cells. It is worth noting, however, that *REP1*, *YLR003C*, and *URA5* are associated with DNA metabolism and may be most likely to rescue *yku70* cells specifically.

cDNA plasmid	Sequence length (bp)	Gene / ORF ID	Gene size: ATG to STOP (bp)	Function
pKU-S1	998	*ANB1*	473	Translation initiation factor eIF-5A
pKU-S2	996	*HYP2*	473	Translation initiation factor eIF-5A
pKU-S3	976	*URA5*	662	Biosynthesis of pyrimidines
pKU-S5	959	*REP1*	1121	Regulation of plasmid copy number amplification
pKU-S6	996	*HYP2*	473	Translation initiation factor eIF-5A
pKU-S7	1000	*CPR1*	488	Cis-trans isomerization of peptide N-term to proline residues
pKU-S8	1011	*HYP2*	473	Translation initiation factor eIF-5A
pKU-S10	1002	*RTF1*	1676	Subunit of RNA polymerase II-associated Paf1 complex
pKU-S11	1001	*RRP14*	1304	Ribosomal biogenesis and cell polarity
pKU-S12	1002	*UBP6*	1499	Ubiquitin –specific protease
pKU-S14	995	*CPR1*	488	Cis-trans isomerization of peptide N-term to proline
pKU-S15	1001	*UBP6*	1499	Ubiquitin –specific protease
pKU-S16	1013	*TLC1*	1301	RNA subunit of telomerase
pKU-S17	1020	*YLR003C*	875	Uncharacterized protein, may participate in DNA replication
pKU-S18	1004	*BUD21*	644	Component of small ribosomal subunit processosome
pKU-S19	1022	*HYP2*	473	Translation initiation factor eIF-5A
pKU-S20	1007	*HYP2*	473	Translation initiation factor eIF-5A
pKU-S21	993	*HYP2*	473	Translation initiation factor eIF-5A
pKU-S24	990	*RPL9A*	575	Subunit of large (60S) ribosome
pKU-S25	941	*CPR1*	488	Cis-trans isomerization of peptide N-term to proline
pKU-S26	1007	*HYP2*	473	Translation initiation factor eIF-5A
pKU-S27	1012	*NUG1*	1562	GTPase required for 60S subunit export out of nucleus
pKU-S28	1012	*HYP2*	473	Translation initiation factor eIF-5A
pKU-S30	1015	*MPP6*		Nuclear RNA binding protein associated with exosomes
pKU-S31	1004	*URA5*	662	Biosynthesis of pyrimidines

Figure 33. List of genes capable of suppressing the temperature sensitivity of *yku70* mutants at 37 °C. Included in this table are plasmid names, lengths of sequences determined from the 3' end using T7 primer, gene / ORF names, sizes of genes, and a description of each gene's known function.

Summary and Conclusions

The temperature sensitivity of *ku* mutants is caused by telomere instability due primarily to a compromised cap structure leading to increased nuclease degradation of chromosomal DNA ends. In this study, we have investigated the roles of several proteins in maintaining cap structure and affecting rescue of *ku* mutants by telomerase. *yku70* and *yku80* killing at high temperatures was compared to a *yku70 yku80* double mutant in order to assess whether the same temperature sensitive phenotype resulted from deleting either of the subunits of the Ku heterodimer. There was no notable difference between the death of *yku70*, *yku80* or *yku70 yku80* mutants at 37 °C, and all were rescued efficiently by overexpression of *TLC1* RNA or Est2 polymerase.

Exo1 nuclease is primarily responsible for the degradation of 5' telomeric DNA in cells with compromised telomeric caps. This degradation causes *ku* mutants to undergo DNA damage checkpoint response and arrest in G_2 phase of the cell cycle. Previous work has shown that deletion of the *EXO1* gene increases growth of *ku* cells at elevated temperatures (30). In the current study, we observed that overexpression of *EXO1* accelerates the death of *ku* mutants at 37 °C. Overexpression of *TLC1* RNA or Est2 polymerase were shown to rescue the accelerated death of *yku70* mutants, indicating that the mechanism of action of these subunits is to specifically block the degradation of DNA by Exo1.

In addition to Exo1, other nucleases are thought to contribute to the degradation of telomeric DNA. Inactivation of known nuclease genes *RAD1*, *RAD10*, *MMS4*, and *MUS81* had no impact on the death of *ku* mutants at elevated temperatures. Furthermore,

RAD17 and *RAD24*, which encode checkpoint proteins reported to affect telomere stability (33) did not affect killing at 37 °C or suppression by *TLC1* or Est2.

Ku associates with specific subtelomeric and telomeric chromatin proteins. Among these proteins are the Sirs which associate with both subtelomeric and telomeric DNA. The impact of *SIR* genes on the death of *ku* cells and their rescue by *TLC1* RNA was important to test due to the known direct interactions between Ku70 and Sir4. Double mutants of *yku70 sir2*, *yku70 sir3* and *yku70 sir4* grew moderately better compared to *yku70* single mutants. However, *TLC1* RNA could not rescue the death of *yku70* mutants at high temperatures in the absence of Sir2 and Sir4. Also, Est2 polymerase overexpression did not rescue the death of *yku70 sir4* cells at 37 °C either. These results suggest *TLC1* and Est2 function by stabilizing a protein cap structure containing Sir4 and possible Sir2. In the absence of Sir4, this structure cannot form and so telomerase overexpression is ineffective.

In addition to the Sir proteins, the Rif1 and Rif2 proteins are also found with Ku at the ends of chromosomes. *yku70 rif1* mutants showed a wildtype phenotype at 37 °C, though growth was reduced at 39 °C. Interestingly, deletion of either *RIF1* or *RIF2* leads to elongated telomeres, but only removal of *RIF1* was able to rescue the *ku* cells. The *yku70 rif2* strain was also rescued by *TLC1* and Est2 overexpression, thus indicating that Rif2 is not necessary for the mechanism of rescue by *TLC1* or Est2.

Mlp2 protein binds specifically to Yku70 and helps mediate tethering of chromosome ends to the inner site of the nuclear envelope. Increased survival was observed at 37 °C for *yku70 mlp2* and *yku70 mlp1 mlp2* cells and to a lesser degree for *yku70 mlp1* mutants. Ten percent of human cancer cells circumvent telomere instability

by elongating telomeric DNA via homologous recombination. The lack of physical positioning of telomeres to the nuclear periphery may result in higher incidences of recombination between telomeres that would not have access to each other if anchored to the nuclear membrane. Therefore, disrupting telomere tethering by removing Yku70 in addition to Mlp1 or Mlp2 may lead to telomere stability in *ku* mutants by recombination. Additionally, overexpression of *TLC1* RNA and Est2 polymerase rescued all of the *ku mlp* mutants at 37 °C, indicating that their presence is not required for blockage of Exo1 degradation.

The cDNA library screen resulted in isolation of 25 cloned yeast genes that when overexpressed rescue the death of *yku70* cells at 37 °C. Restriction digestion showed that only one plasmid contained *TLC1* and no isolates were revealed to be *EST2* or *YKU70*. The DNA sequences of the 25 genes were sequenced and compared to the Saccharomyces Genome Database of genes. Most of the 25 cloned isolates that suppress the killing of ku mutants at 37 °C have previously been linked to protein synthesis, suggesting that they might increase temperature resistance generally, rather than only *yku70* cells. Other genes such as *REP1*, *YLR003C*, and *URA5* are linked to DNA metabolism and may be more specific.

This study was performed to enhance our knowledge of interactions between Ku, telomeric chromatin, and the telomerase enzyme. Insight into such interactions was achieved by systematically disrupting and overexpressing critical genes. The data obtained has proven critical in unraveling the roles of proteins at the telomeres and is applicable to numerous human diseases.

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