ROLES OF GENES AFFECTING TELOMERE LENGTHS, CHROMATIN REMODELING, AND CELL CYCLE CHECKPOINTS IN MAINTENANCE OF CHROMOSOME STABILITY IN YEAST *YKU70* MUTANTS

by

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A thesis submitted to the Graduate Council of
Texas State University in partial fulfillment
of the requirements for the degree of
Master of Science
with a Major in Biochemistry
August 2016

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ACKNOWLEDGEMENTS

I would like to thank Dr. Lewis for the opportunity to work in his laboratory and his mentorship. Dr. David, and Dr. Du for serving on my committee and guiding me throughout my academic career. The members of the Lewis lab that have been with me throughout this process. The South Texas Bridge to Doctoral Program for supporting me and my research. My friends, family and loved ones who gave me the support and encouragement to complete this degree.

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CHAPTER I

INTRODUCTION

DNA Damage and Repair

The DNA in a cell is constantly under attack by exogenous factors such as ionizing radiation, chemicals, and endogenous factors that occur as a result of normal cellular metabolism (1). These DNA damaging agents can cause lesions such as bases with altered structures and strand breaks (2). Depending on the severity of the damage, transcription and replication machinery may be unable to access the DNA appropriately which may lead to further damage to the cell or lethality. To prevent this, it is necessary for cells to have the ability to repair damage. Cells have specialized pathways for dealing with different kinds of DNA lesions. Major repair pathways in eukaryotes have been characterized including nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) (3, 4). For more lethal damage associated with DNA double-strand breaks (DSBs) two additional pathways, homology directed recombination (HDR) also referred to as homologous recombination (HR) and nonhomologous end-joining (NHEJ) can come into play (5, 6, 7, 8). DSBs can be generated by internal cellular processes such as oxidation, DNA replication forks arresting, nuclease activities, as well as through repair of other DNA lesions and externally by ionizing radiation or clastogenic chemicals. DSBs are the most difficult to repair and can be associated with other lesions as well such as altered or missing bases (4). The major protein complexes involved in the HR and NHEJ pathways are conserved in yeast and vertebrates, including humans (3, 4).

DNA repair in *Saccharomyces cerevisiae*

Yeast DSB repair has been intensely investigated and major members of the HR and NHEJ pathways have been identified and characterized. Though HR and NHEJ pathways are conserved in the yeast *Saccharomyces cerevisiae* and other eukaryotes, HR is the predominant DSB pathway used for repair in yeast, and NHEJ contributes most to the DSB repair in vertebrates. NHEJ is less accurate than HR, but is the simplest mechanism for fixing a DSB because it does not require extensive DSB DNA end processing (5, 9, 10, 11, 12, 13, 14).

In yeast, a key player in the initiation of the NHEJ repair pathway is the Ku complex, a heterodimeric ring comprised of Yku70 and Yku80 that protects damaged and exposed DNA ends from nucleases and recruits other DNA repair proteins (3, 15, 16, 17, 18, 19). In humans the homolog *KU70* is also important for V(D)J recombination in B cells for antibody production and T cells for T cell receptor production. Knocking out *KU70* in this pathway leads to severe combined immunodeficiency (SCID) (17). The Ku complex protects the broken strands from degradation by nucleases and forms a complex that can then recruit DNA ligase IV (encoded by Dnl4/Lig4 in yeast) and accessory proteins Lif1/Xrcc4 and Nej1/Xlf to repair the break (Figure 1) (4, 3, 5, 20, 21, 22, 23).

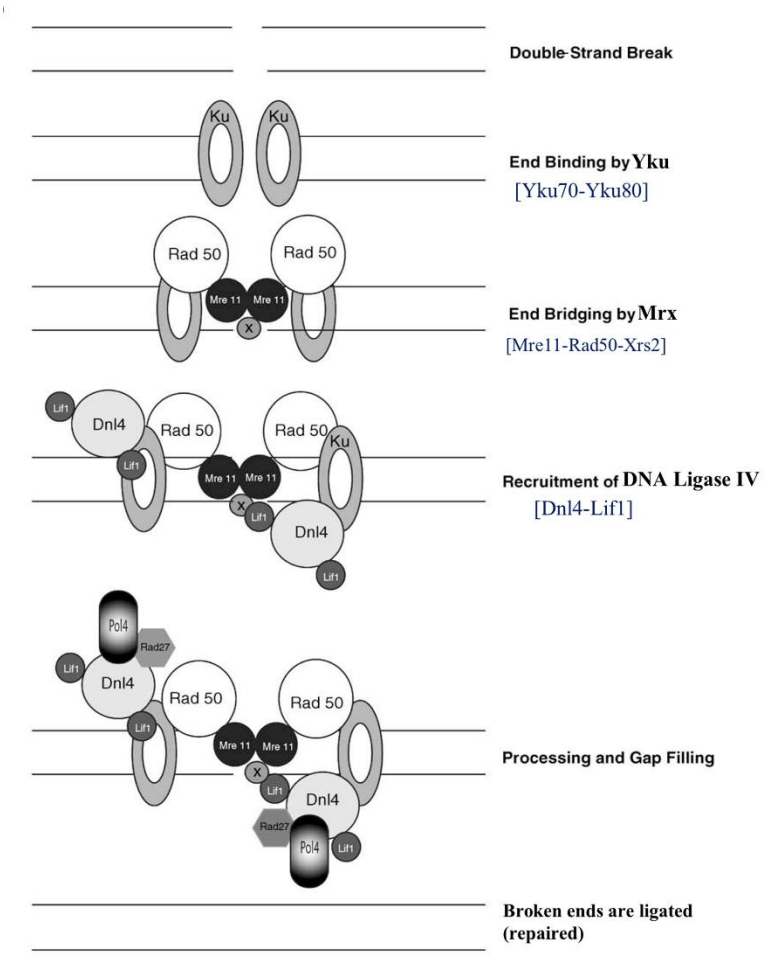

Figure 1. Nonhomologous end-joining in *Saccharomyces cerevisiae.* The Ku, Mrx, and DNA Ligase IV complexes bind to the ends of double-strand breaks for repair (Hefferin and Tomkinson, 2003).

The HR pathway utilizes a sister chromatid or homologous chromosome as a template to repair DSB ends. Several members of the RAD52 group of proteins (Rad51, Rad52, Rad54, Rad55, Rad57, and Rad59) as well as the Mrx complex (Mre11, Xrs2 and Rad50) are required for efficient repair of DSBs and are unique to the HR pathway (8, 24, 25, 26, 27). Strains with mutations in these genes have been shown to be sensitive to ionizing radiation and chemical clastogens.

Several steps must transpire for efficient repair of DSBs by HR. The first step to resect broken DNA ends is initiated by the Mrx complex, which involves the creation of long 3' ended single-stranded tails via the subsequent actions of Exo1, Sae2, Sgs1, Dna2, and possibly other proteins (23). These single-stranded tails are then bound by the ssDNA binding protein complex (Rpa) while the RAD52 group of proteins promote homology search and strand invasion of the homologous unbroken template DNA strand. This exchange of information is followed by resolution of the complex and ultimately leads to repair of the broken strands. Many other cellular processes including DNA damage-induced cell cycle checkpoint responses, chromatin remodeling, sister chromatid cohesion, end processing by nucleases, and chromatin reassembly have been shown to influence the efficiency of the HR pathway (3, 4, 23, 26, 27).

Yku proteins are important for both NHEJ and telomere stability

Telomeres are repetitive sequences at the ends of linear eukaryotic chromosomes that are essential for protecting the genome from being compromised due to degradation by nucleases, end-to-end fusions, or recombination events during each cell division. In *S. cerevisiae*, telomeres of wild type strains are approximately 350-500 bp in length. Ku plays several roles at the telomere regions that are important for maintaining genome stability (Figure 2) (15, 16, 28).

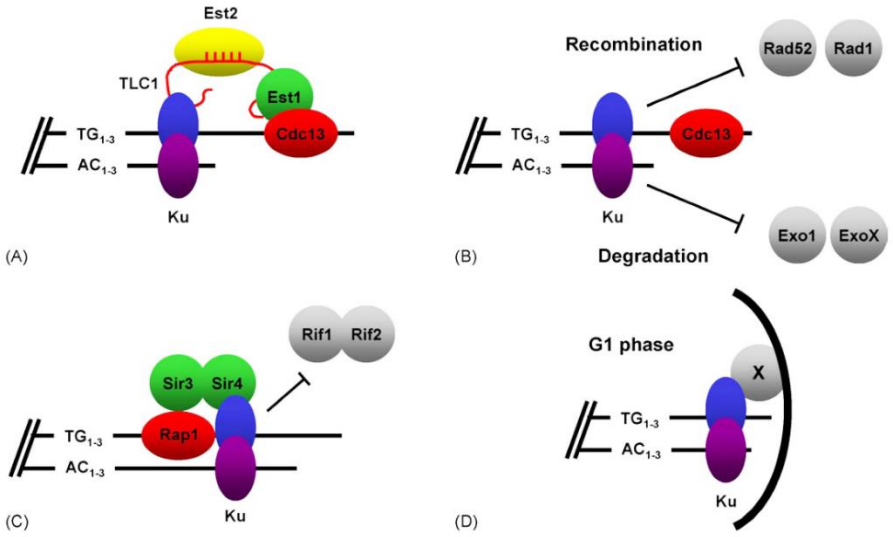

Figure 2. Ku plays several roles in yeast at the telomere region. Ku (A) recruits the telomerase complex and (B and C) protects against degradation and recombination. It is also required for telomere position effects and tethering to the nuclear periphery (D) (16).

yku70 and *yku80* mutants are not only defective in NHEJ, but also have other defects related to the telomere regions. Single-stranded DNA ends are bound by a protein cap to protect chromosomal ends from degradation and end-to-end fusions, as well as to prevent activation of DNA repair or DNA damage checkpoint pathways (16, 17, 29, 30). In *yku70* mutants telomeres have long single-stranded tails at the 3' ends throughout the cell cycle, short but stable telomeres, decreased telomere tethering to the nuclear periphery, and less recruitment of telomerase to DNA ends during S phase when grown at normal temperatures (30^{o}C) (11, 12, 15, 16, 31, 32, 33, 34).

Mutant *yku70* cells have short but stable telomeres that are one third to one half the length seen in wildtype cells at 30°C. However, at 37°C the cells are temperature sensitive. In y*ku70* mutants grown at 37^{o}C the protein cap becomes unstable, the ends become susceptible to degradation by nucleases, and this leads to telomere shortening at a

rate of 10-15 bp per generation (35). At elevated temperatures *yku70* mutants exhibit increased single-stranded DNA at the telomeres and undergo arrest in G_2 phase due to a DNA damage-induced cell cycle checkpoint response and lose viability (17, 36). This response depends on a group of DNA damage checkpoint genes including *RAD9, RAD24, MEC1,* and *CHK1* and is believed to be due to an increase in the amount of single stranded DNA in the telomere region (Figure 3) (19, 36, 37, 38).

Suppression of the temperature sensitive phenotype in *yku70* mutants

Increased accessibility to the DNA ends at 37°C also allows for increased degradation by the 5' to 3' nuclease activity of Exo1 (37, 39). The temperature sensitive phenotype at 37ºC can be strongly reduced by the deletion of *EXO1* (39, 40). Genes that also act to reduce the checkpoint response and temperature sensitivity of *yku70* mutants, at least partially include a negative regulator of DNA synthesis by telomerase called *RIF1,* the spindle checkpoint gene *MAD2,* the DNA checkpoint response gene *CHK1,* and a member of the KEOPS kinase *CGI121* (36, 37, 39). A full list of genes that have been tested for rescue will be discussed later on.

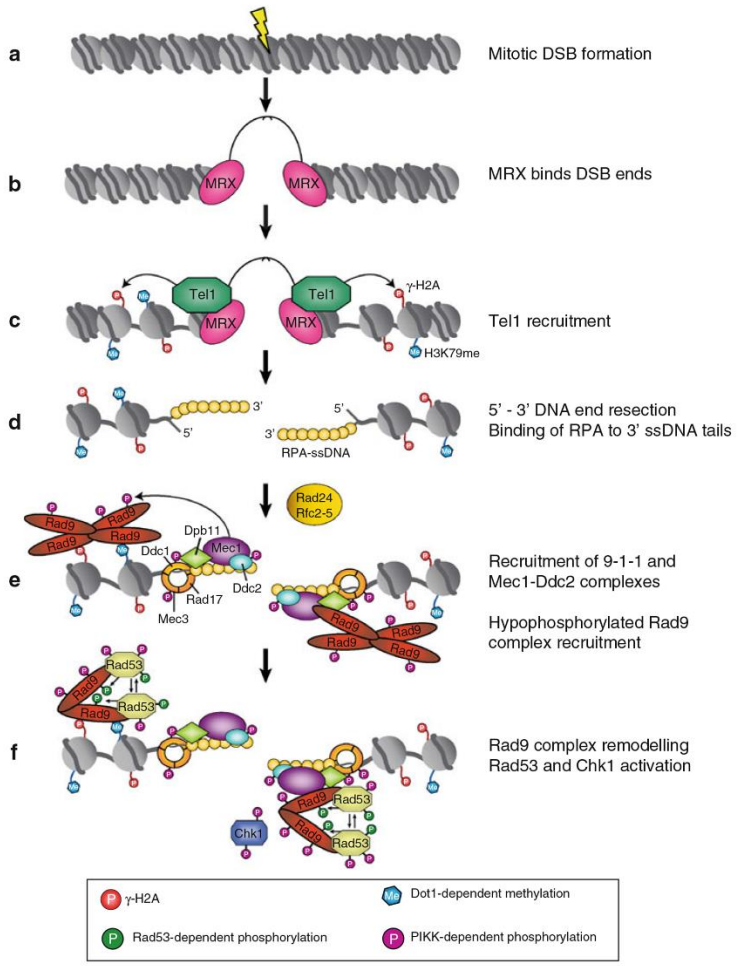

Figure 3. DNA double-strand breaks activate a DNA damage checkpoint response. Checkpoint proteins Rad9, Rad24, Mec1, and Chk1 activate during the double-strand break repair process (19).

Ku has been shown to maintain telomere length by preventing telomere degradation by Exo1 as well as by promoting telomere DNA addition by telomerase. The major mechanism in place to maintain the length of telomere ends is the presence of telomerase. In yeast the telomerase complex consists of three proteins, Est1, Est2, and Est3, as well as an RNA component *TLC1* (Figure 4). Est2 is the catalytic RNA-dependent DNA polymerase (reverse transcriptase) subunit and *TLC1* is the RNA

template that contains the sequence used for extension of the telomeres (25, 30 , 40, 41, 42). In addition to Yku70 and the telomerase complex, several other proteins are required for efficient telomere addition in yeast *in vivo*. This includes the essential single-stranded telomere DNA binding protein Cdc13. Accessory proteins Cdc13, Stn1, and Ten1 form a complex (CST) along with the Ku complex that is important for telomere end protection and telomerase recruitment (44).

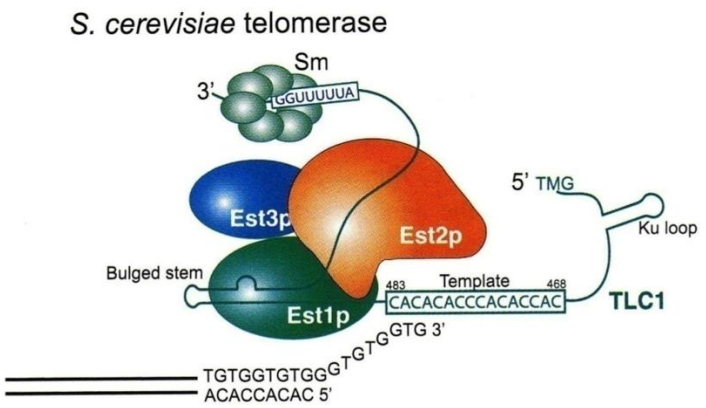

Figure 4. Telomerase complex of *S. cerevisiae.* Telomerase binds to the 3' end of the telomere (43).

Overexpression of some genes whose products are associated with the telomerase complex has been shown to eliminate the temperature sensitivity in *yku70* mutants (16). Overexpression of the telomerase catalytic subunit *EST2* or the RNA template *TLC1* in *yku70* mutants increases stability at the telomeres and eliminates the temperature sensitive phenotype (Figure 5) (12, 25, 38, 45). Our laboratory has previously observed that overexpression of *EST2* and *TLC1* rescues *yku70* mutant cells grown at 37^{o}C, but *EST1* and *EST3* do not (12, 46, 47). These experiments also demonstrated that *CDC13*,

TEN1, and *STN1* overexpression does not rescue the temperature sensitive phenotype. Another gene identified to partially suppress the killing of *yku* mutants at 37ºC is *RTT103*, a gene with roles in DNA repair and transcription termination that are still undefined (48).

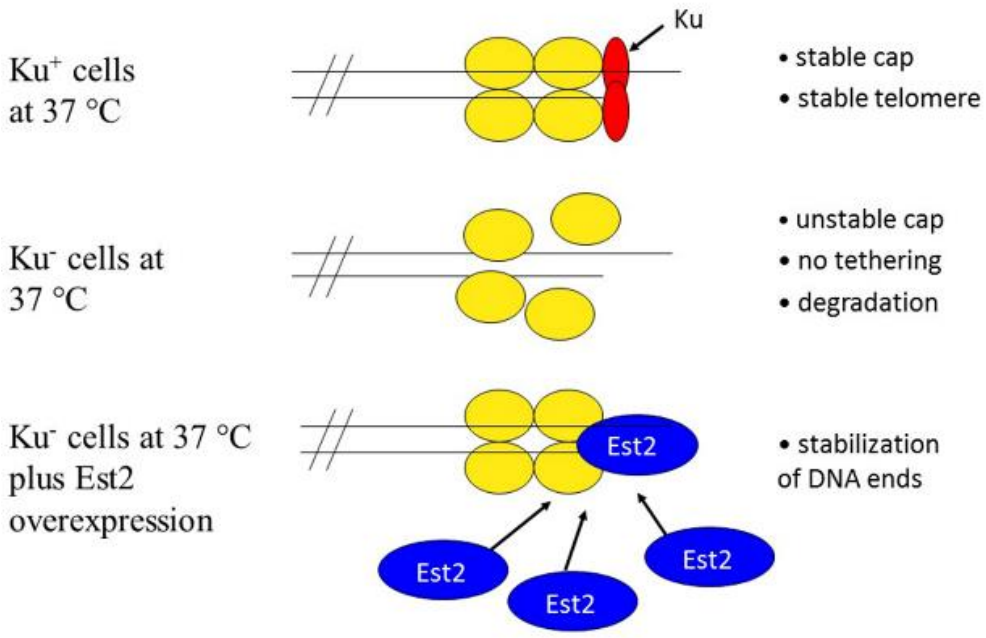

Figure 5. Overexpression of telomerase protein increases stability of DNA ends in Ku-deficient cells. Mutant *yku70* cells grown at 37°C are temperature sensitive with an unstable cap at the telomeres. Overexpression of the telomerase catalytic subunit *EST2* eliminates the temperature sensitive phenotype via increased stability at the telomeres.

Recent work in the laboratory revealed that overexpression of *EST2* rescues *yku70* cells by adding stability to the ends, increasing their resistance to degradation by nucleases, and not through increased reverse transcriptase activity. Overexpression of catalytically inactivated Est2 mutants rescued *yku70* cells at 37°C as efficiently as

overexpression of the wildtype enzyme, supporting the argument that telomerase acts to enhance capping of the telomeres and adds stability and resistance to Exo1 nuclease (36).

The major goal of this project was to improve our understanding of the genes and DNA metabolic pathways required for maintaining telomere stability in *yku70* and *yku80* mutants. Askree *et al*. (2004) and Gatbonton *et al*. (2006) performed genome-wide screens of diploid yeast mutant collections to identify genes affecting telomere lengths (49, 50). These screens identified *ard1, elg1, htl1, pif1, rad27, rif1, rif2, rps17a, rsa1,* and *srb8* mutants as having very long telomeres (VLT phenotype). It is possible that mutants with very long telomeres identified in those studies may provide insight on the role of *YKU70* at the telomeres. Our laboratory has already tested a few genes shown to create very long telomeres when inactivated to see their effect on telomere stability in *yku70* mutants at 37°C. Double knockouts with *yku70* inactivated and these other genes also inactivated were tested (*elg1 yku70*, *htl1 yku70*, *pif1 yku70,* and *rad27 yku70*) but these genes did not strongly affect rescue by *EST2* (57). Three of the VLT mutants (*rps17a*, *rsa1*, and *srb8*) were tested for the first time in the current study. We hypothesize that these mutants and others affecting accessibility of telomerase or nuclease to the DNA in telomere regions may influence the rescue of *yku70* cells at 37°C.

In this project, the survival of *yku70* cells at high temperatures and rescue of the cells by overexpression of telomerase was tested in cells defective in multiple processes. These included (a) VLT mutants, containing very long telomeres, (b) chromatin remodeling mutants, (c) DNA damage checkpoint mutants, (d) a spindle checkpoint mutant, and (e) other mutants known to affect DNA repair and telomere stability. The experiments that were performed involved working with strains from a haploid strain

deletion library as well as strains that were created by having *YKU70* knocked out via homologous recombination. Mutants selected from the strain library included those identified as having very long telomeres by Askree *et al.* and Gatbonton *et al*., genes involved in chromatin remodeling, checkpoint genes, and selected DNA repair genes (49, 50). Next, the effects of inactivation of genes mentioned above on the stability of the telomere cap complex in *yku70* mutant cells were tested via rescue of the temperature sensitivity of *yku70* mutants at 37°C. Double mutants were also transformed with plasmids overexpressing *EST2* and *TLC1* and grown at high temperatures to investigate the genetic requirements for telomerase rescue of *yku70* cells by pronging assays. Southern blots were then done on selected mutants to further understand if changes in telomere lengths in the double mutants could be detected.

CHAPTER II

MATERIALS AND METHODS

Reagents

Ethylenediaminetetraacetic acid (EDTA) and sodium hydroxide (NaOH) were purchased from EMD Chemicals, Inc. (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) and lithium acetate (LiAc) were obtained from Alfa Aesar (Heysham, Lancashire). Sodium dodecyl sulfate (SDS) was obtained from J.T. Baker (Center Valley, PA). Sodium acetate anhydrous, and glacial acetic acid were purchased from Mallinckrodt-Baker, Inc. (Paris, KY). The sonicated salmon sperm carrier DNA was obtained from Agilent Technologies (Santa Clara, CA). Ethidium bromide (EtBr) was obtained from IBI Scientific (Peosta, IA). Sodium chloride, sodium citrate dehydrate, Tris-HCl, and the Qubit dsDNA BR Assay Kit were purchased from ThermoFisher Scientific (Fair Lawn, NJ). Ammonium sulfate, isopropanol, formamide, and Trizma base were purchased from VWR International (West Chester, PA). Boric acid, maleic acid, polyethylene glycol (PEG 4000), Sarkosyl (N-lauryl-sarcosine), and Tween 20 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Agarose LE was purchased from Gold Biotechnology (St. Louis, MO). TAE (50X) buffer was purchased from Omega (Cowpens, SC). Ethanol was obtained from Texas State University (San Marcos, TX). DIG High Prime DNA Labeling and Detection Starter Kit II, PCR DIG Labeling Mix, and DNA Molecular Weight Marker III were purchased from Roche

Applied Science (Penzberg, Germany). RNase A was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Restriction enzymes, dNTPs, $MgCl_2$, Vent polymerase, Taq DNA polymerase, 6X loading dye, and 2 log DNA ladder were purchased from New England Biolabs (Beverly, MA). ExTaq DNA polymerase was obtained from Takara (Madison, WI). All primers (Table 1) were created by Integrated DNA Technologies (IDT) (Coralville, IO). All amino acids, and ampicillin (Amp) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). D-(+)-glucose, soy peptone, and yeast nitrogen base were made by Amresco (Solon, OH). Bacto yeast extract was manufactured by Becton, Dickinson, and Company (Sparks, MD). Agar, molecular biology grade was obtained from Teknova (Hollister, CA). QIAprep Spin Miniprep Kits were purchased from Qiagen (Hilden, Germany).

Equipment

Horizon 11-14 gel rigs were from LabRepCo (Horshan, PA). The Sorvall Lynx 6000 floor centrifuge, Savant DNA 120 SpeedVac Concentrator, Qubit 2.0 Fluorometer, replica-plating tool, and velvet squares were obtained from Thermo Fisher Scientific (Fair Lawn, NJ). The MXX-123 analytical balance was purchased from Denver Instruments (Bohemia, NY). Vortexes and all incubators were purchased from VWR Scientific Products (Radnor, PA). The model 5424 centrifuge was purchased from Eppendorf (Hamburg, Germany). UV Stratalinker 2400 was purchased from Stratagene (La Jolla, CA). The T100 Thermal Cycler, Trans-Blot Turbo Blotting System, and ChemiDoc XRS+ instruments were obtained from Bio-Rad Laboratories (Hercules, CA). The RED imaging system was purchased from Alpha Innotech (San Jose, CA). HB-1000 Hybridizer was purchased from UVP Laboratory Products (Upland, CA). The E-Class

shaker, Orbit Environ Shaker, and Lab Rotator were made by Lab Line Instruments (Tripunithura, India). The orbital shaker and digital dry baths were purchased from BioExpress (Kaysville, UT).

Yeast Strains and Plasmids

Yeast strains YLKL1564, YLKL1565, YLKL1566, YLKL1567, YLKL1568, YLKL1569, YLKL1570, YLKL1572, YLKL1573, YLKL1574, YLKL1575, YLKL1576, and YLKL1577 were constructed by transforming single mutant cells from the single deletion yeast strain library with a DNA fragment made by PCR amplification of the *yku70Δ::HIS3* insertion from *yku70* mutant strain YLKL652. Cells were spread to glucose minus histidine plates for colony growth and then colonies were patched to fresh glucose minus histidine plates for further purification. PCR confirmation using primers Ku70G and 5-HIS-3 was done to verify the *yku70* deletion in all strains. Primers used in this project are listed in Table 1. All single mutants and wild-type strains were available in the strain deletion libraries obtained from Open Biosystems (4). All original library strains and new strains created for this project are listed in Table 2. All plasmids used in these experiments were isolated from *E. coli* cells by alkaline lysis for plasmid DNA (Table 3).

Table 1. List of PCR Primer Sequences. A complete list of primers used.

Primer	**Sequence**
Ku70A	TTGAGATCGGGCGTTCGACTCGCC
Ku70E	CAGTTAATTGACTCTCGGTAGCCAAGTTGGT
Ku70F	GAGTTACAACTATAGATTACTGTCGTGCATA
Ku70G	CATGATATTAGACGGACTCATAATTGAATGGTT
3'HIS3	CGTTCAGAATGACACGTATAGA
5-HIS-3	GGCTTTCTGCTCTGTCATCTTTGCCTTCGTT
M13 forward primer	AGCGCGCAATTAACCCTCACTAAAG
M13 reverse primer	CAGGAAACAGCTATGACC

Table 2. Yeast strain list. A complete list of *S. cerevisiae* strains used for this project.

Strain	**Genotype**	**Source**
BY4741	*MATa his3Δ leu2Δ0 ura3Δ0 met15Δ0*	(51)
BY4742	*MATα his3Δ leu2Δ0 ura3Δ0 lys2Δ0*	(51)
YLKL652	BY4742, *yku70Δ::HIS3*	Laboratory strain
	BY4742, *rsc1Δ::G418^r*	Strain Library (4)
	BY4742, *rsc2Δ::G418^r*	Strain Library (4)
	BY4742, *rps17aΔ::G418^r*	Strain Library (4)
	BY4742, *rsa1Δ::G418^r*	Strain Library (4)
	BY4742, *chk1Δ::G418^r*	Strain Library (4)
	BY4742, *ddc1Δ::G418^r*	Strain Library (4)
	BY4742, *mec3Δ::G418^r*	Strain Library (4)
	BY4741, *srb8Δ::G418^r*	Strain Library (4)
	BY4742, *sgs1Δ::G418^r*	Strain Library (4)
	BY4742, *mad2Δ::G418^r*	Strain Library (4)
	BY4742, *dun1Δ::G418^r*	Strain Library (4)
	BY4742, *cgi121Δ::G418^r*	Strain Library (4)
	BY4742, *rad17Δ::G418^r*	Strain Library (4)
YLKL1564	BY4742, *rsa1Δ::G418^r yku70Δ::HIS3*	This study
YLKL1565	BY4742, *rsc1Δ::G418^r yku70Δ::HIS3*	This study
YLKL1566	BY4742, *rsc2Δ::G418^r yku70Δ::HIS3*	This study
YLKL1567	BY4742, *rps17aΔ::G418^r yku70Δ::HIS3*	This study
YLKL1568	BY4742, *rsa1Δ::G418^r yku70Δ::HIS3*	This study
YLKL1569	BY4742, *chk1Δ::G418^r yku70Δ::HIS3*	This study
YLKL1570	BY4742, *dun1Δ::G418^r yku70Δ::HIS3*	This study
YLKL1572	BY4742, *mad2Δ::G418^r yku70Δ::HIS3*	This study
YLKL1573	BY4742, *srb8Δ::G418^r yku70Δ::HIS3*	This study
YLKL 1574	BY4742, *mec3Δ::G418^r yku70Δ::HIS3*	This study
YLKL1575	BY4742, *ddc1Δ::G418^r yku70Δ::HIS3*	This study
YLKL1576	BY4742, *sgs1Δ::G418^r yku70Δ::HIS3*	This study
YLKL1577	BY4741, *sgs1Δ::G418^r yku70Δ::HIS3*	This study
YLKL1577	BY4742, *rad17Δ::G418^r yku70Δ::HIS3*	This study

Table 3. Plasmid list. A complete list of plasmids used in this study.

Plasmid	**Genotype**	**Source**
pVL799	*ADH1p::TLC1p LEU2 2μ* Amp^r	52
pVL999	*ADH1p::EST2 LEU2 2μ* Amp^r	52
pRS315	*LEU2 CEN/ARS* Amp^r	53
YTCA1	Telomere Repeats Amp^r	54

Cell Culture Solutions and Media

Non-selective YPDA yeast plate growth media contained 1% bacto yeast extract, 2% soy peptone, 2% D-(+)-glucose, 2% agar, and 0.001% adenine. YPDA broth was prepared as YPDA media but without agar. Synthetic dropout media was used for plasmid selection. Dropout mix was composed of 0.17% yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% D-(+)-glucose, 2% agar, and all essential amino acids and bases used for strain selection.

E. coli cells were grown using TB + ampicillin (amp) broth (1.2% bacto tryptone, 2.4% yeast extract, 0.5% glycerol, 10% 0.017 M KH_2PO_4 + 0.072 M K_2HPO_4, 100 µg/mL amp) or for sustained growth on LB + amp plates (1% bacto tryptone, 0.5% yeast extract, 0.5% NaCl, 100 µg/mL amp + 1.5% agar).

Gel Electrophoresis

Gel electrophoresis was performed in Horizon 11-14 gel rigs using 0.7 – 2% agarose gels in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) at ~120 - 150 V or 0.5X TB buffer (45 mM Tris, 45 mM boric acid) at ~250 V. Ethidium bromide (0.5 µg/ml) was used to stain agarose gels and images were captured on the Alpha Innotech Red Imaging system.

Chromosomal DNA Purification

An optimized protocol was designed by the Lewis laboratory as follows. Three mL of yeast cells were pelleted, 300 µL of 6% SET solution (6% SDS, 10 mM EDTA, and 30 mM Tris pH 8.0) was added. This mixture was incubated at 65°C for 15 minutes (7 minutes for Southern blot use) and transferred to a water/ice bath for 5 minutes. 150

µL of cold potassium acetate solution (3.0 M potassium acetate and 2.0 M acetic acid) was added, then inverted for 10 s. The mixture was centrifuged at 21000X g for 10 minutes to pellet protein and cell debris. The supernatant (containing the nucleic acid component) was combined with 500 µL isopropanol and mixed for 30 seconds before being spun at 21, 000X g for 2 minutes. The supernatant was removed and the pellet was washed with 500 µL 70% ethanol, then incubated at room temperature for 1 minutes before the ethanol was removed. The remaining ethanol was removed through the use of a speedvac for 10 – 15 minutes until dry. The pellet was suspended in 50 µL TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA), and 1 µL of 2 mg/mL RNase A was added to degrade RNA contaminants. This mixture was incubated at room temperature for 10 minutes. The pellet was allowed to dissolve at 4°C then stored at -20°C.

Plasmid DNA Isolation

An improved alkaline lysis protocol for *E. coli* plasmid DNA minipreps method from the Lewis laboratory was used that is very similar to the alkaline lysis method described by Green and Sambrook (55). Bacterial colonies were grown overnight in 2-5 mL of TB broth + Amp in a tall tube overnight in a 37°C shaker. 1.5 mL of the culture was added to a microfuge tube and quick spun at 21, 000X g for 15-20 seconds. The sample was briefly spun again and the supernatant was removed. The bacterial pellet was resuspended in 100 µL of Solution I (50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0) by scraping the bottom of each tube against a wire test tube rack until the pellet was thoroughly mixed. The mixture was kept on ice to reduce the amount of nicked open circular plasmid DNA (Carbone et al). Two hundred µL of freshly prepared Solution II (0.2 M NaOH + 1% SDS) kept at room temperature was added. Tubes were

closed and inverted rapidly five times to mix, and stored on ice for 1 minute. One hundred and fifty µL of ice-cold Solution III (3 M KOAc + 5 M glacial acetic acid + H_2O) were added and the tubes were inverted rapidly several times to mix. The sample was stored on ice for 3 minutes before centrifugation at 21, 000X g for 10 minutes. The supernatant was transferred to a new tube except for the last 4-5 mm above the cell debris pellet. The DNA was precipitated by adding 400 µL of isopropanol, inverting several times to mix, and then spinning for 5 minutes at 21, 000X g. The supernatant was removed using a 1000 µL pipettor, and the pellet was washed with 500 µL of 70% EtOH without disturbing the pellet. The sample was then centrifuged for 2 minutes at 21, 000X g with pellets facing the same direction as in the first centrifugation so they were not dislodged. The supernatant was removed with a 1000 µL pipettor and put in the Speed-vac for ~10 minutes or until the pellet was dry. The pellet was resuspended in 50 µL of TE plus 2 µL of 2 mg/mL RNase A and vortexed. The pellet was incubated at room temperature for 10 minutes and then stored at -20°C.

PCR Amplification of *yku70Δ::HIS3* Insert for Gene Disruption

Reactions were done in 0.2 mL PCR tubes with 10 µM Ku70E and 10 µM Ku70F primers, 1X Taq Polymerase buffer, 0.25 mM dNTPs, Taq Polymerase (5 units), ddH_2O and chromosomal DNA isolated from YLKL652 used as a template in a final volume of 50 µL. Five µL (10 µL for weak primer sets) of each sample were combined with 5 µL of ddH_2O and 2 µL 6X loading dye, mixed, and loaded onto a 0.8% agarose gel. Gels were stained with ethidium bromide for 15 minutes and imaged. Samples that produced strong bands were later mixed together and concentrated.

PCR cycles were performed using 94°C for 30 seconds, annealing temperatures from 40°C – 53°C for 40 seconds, and an extension temperature of 72°C for 120-180 seconds (1 minute for the first kb with an additional 30 seconds added for each kb after). PCR was conducted in an Applied Biosystems 2720 Thermal Cycler or a Bio-Rad T100 for 34 cycles.

Concentrating PCR Samples

PCR reactions were combined into one tube. The approximate volume was calculated and 1/10 of the total volume of 3 M NaOAc was added and the solution was vortexed. Then 2.5 volumes of cold (-20°C) 100% EtOH was added and the tube was vortexed again. The sample was put at -20°C for ≥15 minutes, spun at 21, 000X g for 15 minutes and then the supernatant was removed. The remaining pellet was rinsed with 500 µL of 70% EtOH and centrifuged at 21, 000X g for 3 minutes. The supernatant was removed and the tube was placed in the Speedvac for 10 minutes. Once dry, the pellet was resuspended in 1/5 of the original volume in H_2O.

Qiagen Cleaning Up Chromosomal DNA and PCR Fragments

Five volumes of Buffer PB was added to 1 volume of DNA solution and mixed. The sample was added to a spincolumn and centrifuged for 30 seconds at 17, 000X g. The flowthrough was discarded and a wash of 750 µL Buffer PE was added to the column. The sample was centrifuged for 30 seconds at 17, 000X g and the flowthrough was discarded. The sample was centrifuged an additional 1 minute at 17, 000X g to remove residual wash buffer. The column was placed into a clean 1.5 mL tube and 40 µL

of water was added to the center of the column and left to stand for 3 minutes before centrifuging for 1 minute at 17, 000X g.

DNA Transformations

Yeast strains were transformed using a modified rapid lithium acetate/DMSO transformation method described by Tripp *et al.* (56). Cells were grown overnight in 4 mL YPDA broth. One-and-a-half mL of the liquid culture was spun down and the growth media was removed. Cells were incubated in 0.2 M DTT at 42°C for 20 minutes. Cells were spun down and the supernatant was removed. Carrier DNA (50 µg) and transforming DNA (50-300 ng) were added to the pellet and mixed. A master mix composed of 40% PEG 4000, 0.1 M LiAc, 1.0 mM EDTA, 0.1 M Tris (pH 7.5) was added to the pellet and DNA. A volume of 1/10th of DMSO was then added to the mixture. This mixture was shaken for 15-30 minutes at 30°C, then at 42°C for 15 minutes. The mixture was spun down, the supernatant discarded, and 1 mL of YPDA broth was added. The samples then incubated in a shaker for 30 minutes at 30°C. Cells were spun down and the broth removed. The pellet was resuspended in 200 µL ddH_2O and aliquots of the cells were plated to selective media at 30°C for colony formation.

Double Imprint Replica Plating

At least 2 independent PCR confirmed isolates from each *yku70* double mutant were patched onto fresh synthetic dropout media plates and grown for 2 days at 30°C. The first plate with patches of cells grown on it would then be used as a master that was used to transfer the same pattern of cells onto to a sterile velvet. A second plate was placed on the velvet and cells would be transferred. A new velvet was obtained and the

second plate was used as a master plate for cells to transfer to the velvet. A third and fourth plate were placed on the new velvet and cells were transferred. The second plate was put into an incubator at 30°C and the third and fourth plates were put into an incubator at 37°C. Cells were grown for 2 days and temperature sensitivities at 37°C were compared and considered valid when at least 2 independent isolates showed the same phenotype.

Dilution Pronging Survival Assay

Cells were harvested into sterile ddH_2O, diluted 1/40, then sonicated for 8 seconds at 2-3 watts or 20% amplitude using a Sonics Vibracell VCX130 sonicator (Newton, CT). After sonication, cells were quantitated on a Reichert (Buffalo, NY) hemocytometer using a Comcon (Russia) LOMO phase contrast microscope. Yeast cells were added to a microtiter plate at a concentration of 0.5X 10^7 – 1.7X 10^7 cells per 220 µL. These cells were serially diluted 5-fold, 5 times across the columns of the plate. The cells were then pronged onto either synthetic glucose complete, glucose minus histidine, or glucose minus leucine plates depending on the requirements of the assay. Pronged cells were incubated at 30°C as a permissive control temperature and also incubated at 37°C or 40°C based on the requirements of the assay. Cells were allowed to grow for 2-5 days and images were taken of the plates using a Canon Powershot G3 digital camera and saved as JPEG files.

Qubit Fluorometry for Quantification of DNA

Two assay tubes for the standards and one tube per sample that was tested were labeled. The Qubit working solution was prepared by diluting Qubit BR dsDNA reagent

1:200 in Qubit BR dsDNA buffer and vortexing. Two hundred µL of Working Solution was made for each standard and sample, plus an additional 200 µL. The standards were prepared by adding 190 µL of Working Solution to 10 µL of Standard #1 (0 ng/µL dsDNA) and 10 µL of Standard #2 (100 ng/µL dsDNA). Samples were prepared by adding 2 µL of DNA to 198 µL of Working Solution. One sample with a previously measured concentration was always included as well. Once standards and samples were prepared all tubes were vortexed for 2-3 seconds and incubated at room temperature for 2 minutes. Tubes were inserted into the Qubit 2.0 Fluorometer and readings were taken.

XhoI digestion

Chromosomal DNA was purified, ethanol precipitated, and quantified. Approximately 5 µg of DNA was added to 1X CutSmart buffer, 1.2 units of XhoI, and ddH_2O for a final volume of 600 µL and digested overnight at 37°C. The XhoI-digested DNA was concentrated by ethanol precipitation and quantified by fluorometry. One µg of each sample was loaded onto a 1.2% agarose gel and visualized using ethidium bromide.

Southern Blots: PCR Amplification of nonradioactive DIG probe.

Probes for Southern analysis were synthesized by PCR with Taq polymerase using 1 µM M13 forward primer, 1 µM M13 reverse primer, 1X PCR DIG labeling mix (0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.13 mM dTTP, 0.07 mM digoxigenin-11-dUTP), and 2 µL 1:30 diluted YTCA1 plasmid DNA per 50 µL reaction.

PCR cycles for DIG primers were performed using an initial denaturation temperature of 94°C for 2 minutes followed by a repetition of 94°C for 30 seconds, annealing temperatures from 51°C for 40 seconds, and an extension temperature of 72°C

for 30 seconds. PCR was conducted in an Applied Biosystems 2720 Thermal Cycler for 34 cycles.

DNA Probe

The probe solution was made in a 1.5 mL tube with 20 µL of PCR probe DNA, 50 µL of formamide, and 30 µL of ddH_2O. Once reagents were combined the solution was vortexed and placed in a 100°C heating block for 5 minutes and then transferred to wet ice for 2 minutes.

Southern Blot Solutions

Prehybridization/Hybridization solution, 0.5X SSC + 0.1% SDS solution, and 2X SCC + 0.1% SDS solution were prepared on the day of the experiment.

Southern Blots

Gels were made using 55-60 mL of 1.2% agarose gel solution in 0.5X TB buffer and a 10 well comb. XhoI digested DNA was heated at 65°C for 5 minutes and put in wet ice for 5 minutes before loading. The first lane was loaded with 2-Log ladder, the second lane was loaded with Digoxigenin-labeled DNA Molecular Weight Marker III, and each lane after was loaded with the same amount of each DNA sample (400-1000 ng) per lane. After loading, the gel was run at ~250 V until the dye was ~2/3-3/4 of the way down the gel. The gel was stained in ethidium bromide for 10 minutes and then destained in dH_2O for 10 minutes. The gel was imaged, with minimal exposure time to UV light to confirm that the digest was complete and the amount of DNA in each smear looked ~equal. The gel was then transferred into a dish with enough denaturation buffer (8.7% sodium chloride, 2% sodium hydroxide) to cover it and put on a shaker for 5 minutes at room

temperature. The liquid was then poured off and replaced with fresh denaturation buffer and shaken gently for 30 minutes. The denaturation buffer was poured off and enough neutralization buffer (8.7% sodium chloride, 6.1% Tris base) to cover the gel was added. The gel shook in the buffer for 5 minutes at room temperature before the buffer was removed and replaced with fresh neutralization buffer and left to shake for 30 minutes. A piece of Hybond+ nitrocellulose membrane and 6 pieces of blotting sheets were cut to the size of the gel and submerged in a dish with 0.5X TB buffer. The gel was added to a separate dish with 0.5X TB buffer to equilibrate for 10 minutes. Both the gel and the membrane had the bottom left corner cut. The transfer of DNA to the membrane occurred via the BioRad Transblotter. Three of the blotting sheets were placed down at the bottom of the cassette, followed by the gel, membrane, and then the three remaining blotting sheets. Air bubbles were removed from the set up with a roller and the cassette was closed and locked before being put into the Transblotter. The Transblotter was set for 25 minutes with a 25 V limit and constant 1.3 mA.

After the transfer the gel was restained in ethidium bromide and imaged to determine how much DNA had stayed in the gel. The membrane was placed on 2 pre-wetted blotting sheets and put into the Stratalinker with energy set to 1200 (120, 000) and auto cross selected for up to 50 seconds to covalently crosslink the DNA to the membrane. After crosslinking, the membrane was soaked in 10X SCC prepared from 20X SSC stock solution (35.1% NaCl and 17.6% sodium citrate) with the DNA side up while shaking for 5 minutes. The solution was removed and fresh 10X SCC was added for an additional 5 minutes. The 10X SCC solution was removed and the Prehybridization/Hybridization solution (50% v/v formamide, 5X SSC, 0.1% sarkosyl,

0.02% SDS, 1X blocking agent) was added to the membrane and shook for 5 minutes. The membrane was rolled and then transferred to a glass tube and 15 mL of Prehybridization/Hybridization solution was added. The tube was placed in the UVP HB-1000 Hybridizer for 1 hour at 40°C. The solution was poured off and 15 mL of fresh Prehybridization/Hybridization solution with DIG-dUTP-labeled DNA probe (2 µL probe/mL hybridization buffer) was added and rolled at 40°C overnight.

The next day the Prehybridization/Hybridization solution with probe was poured into a 50 mL screw cap tube and placed in 4°C. The membrane was put into a small dish and covered with 2X SSC/ 0.1% SDS to shake for 5 minutes at room temperature. The liquid was removed and replaced with fresh 2X SSC/ 0.1% SDS to shake for 15 minutes at 55°C. This step was repeated once more. The liquid was removed and the membrane was covered in Wash buffer for 1 minute at room temperature. The Washing buffer was removed and ~100 µL of Blocking Solution (diluted 1:10 in Washing Buffer) was added to the membrane and it was left to shake for 30 minutes. The blocking solution was poured off and 20 mL of antibody-enzyme conjugate solution (4 µL antibody-enzyme solution in 20 mL 1X blocking solution) was added to the membrane and it was incubated for 30 minutes on a shaker at room temperature. The antibody solution was poured off and ~100 mL of washing buffer was added and it was shaken for 15 minutes. The washing buffer was removed and replaced with 15 mL of fresh washing buffer for an additional 15 minutes. Then the Washing buffer was removed and 20 mL of Detection buffer (0.1 M Tris-HCl, 0.1 M NaCl; pH 9.5) was added for 5 minutes. The membrane was placed on a piece of plastic wrap and 1 mL of CSPD-Ready to Use was added. The plastic wrap was folded over the membrane and the substrate solution was spread evenly

over the membrane and allowed to incubate at room temperature for 5 minutes. Excess liquid was pushed off of the membrane before placing it into a 37°C incubator for 10 minutes.

The membrane was then inserted into the ChemiDoc. To capture the image a new protocol was selected within the ChemiDoc software and under type of blot, chemiluminescent was selected. Next, the filter was manually adjusted to the off position. Then the camera was adjusted to fit the area of the membrane. Image capture times were adjusted to 10 minutes with images taken every 30 seconds. Once the blot had been imaged for 10-30 minutes all files were saved, and selected images were exported for publication quality use.

CHAPTER III

RESULTS AND DISCUSSION

This project expands upon previous work in the Lewis lab regarding *yku70* mutants completed by Brian Wasko, Cory Holland, and James Titus to better understand the loss of viability of *yku70* mutants at 37°C and their rescue by telomerase (47, 36, 57, 58). Wasko and Holland investigated overexpression of Est2, the polymerase component of telomerase, as well as *TLC1* the RNA component of telomerase. Both telomerase components were shown to alleviate temperature sensitivity and death at 37°C when overexpressed in *yku70* mutants (25, 38, 47, 58). This increased stability of the telomeres at elevated temperatures is likely to be due to telomerase physically blocking exonuclease activity by the 5'-3' exonuclease Exo1 (36).

To further understand this phenomenon, the roles of genes in DNA metabolic pathways affecting telomere maintenance and stability in *yku70* double mutants were tested at elevated temperatures. Some of the genes tested in the current project have already been shown to influence survival of *yku70* cells at 37°C and this new work has helped to validate previous findings in the literature (57). Additionally, these experiments have gone beyond what has been tested in the literature by investigating the influence of Est2 and *TLC1* RNA overexpression on survival of *yku70* double mutants.

The genes that have been tested for this project are divided into 6 groups. These groups include genes associated with (a) the very long telomere (VLT) phenotype (*RSA1*, *RPS17a*, and *SRB8*), (b) chromatin remodeling (*RSC1* and *RSC2*), (c) DNA damage checkpoint response (*DUN1*, *DDC1*, *MEC3*, and *CHK1*), (d) mitotic spindle checkpoint (*MAD2*), (e) a protein kinase believed to affect telomere cap structure (*CGI121*), and (f) a DNA repair-associated helicase enzyme known to affect telomeres (*SGS1*).

Double mutants were created using haploid strains from a yeast deletion library by transforming single mutants with a PCR-amplified *yku70Δ::HIS3* DNA fragment to knock out *YKU70*. After entering the cells, the *yku70Δ::HIS3* gene deletion fragment undergoes genetic exchange with the *YKU70* gene while inserting a functional *HIS3* gene into it (Figure 6).

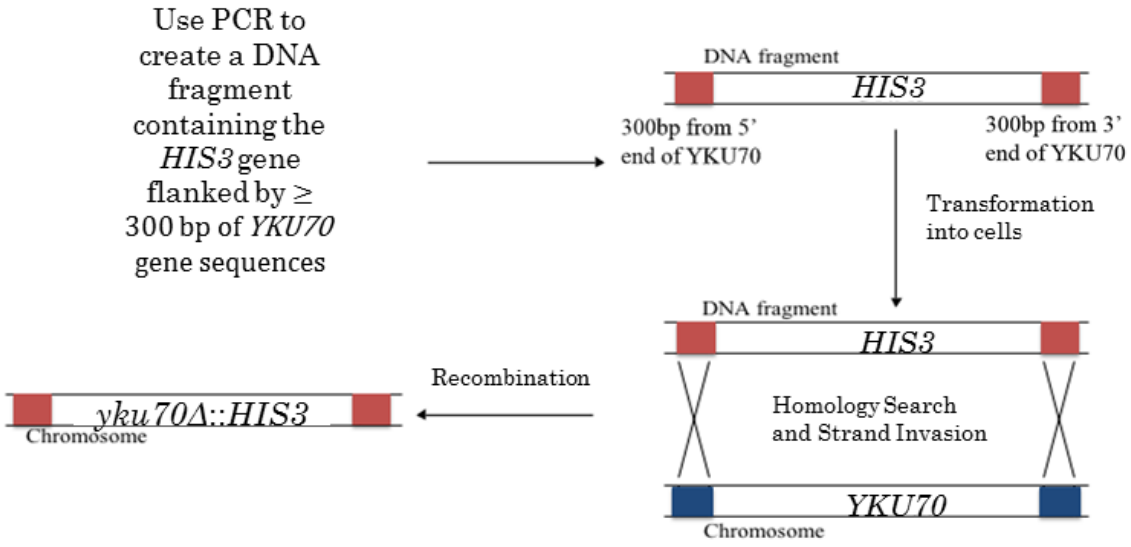

Figure 6. A DNA fragment containing the *HIS3* gene and flanked with 300 bp of the *YKU70* gene was created by PCR and transformed into cells. *HIS3* replaced the *YKU70* gene via homologous recombination.

YKU70 inactivation was accomplished by flanking the *HIS3* gene with ~300 bp corresponding to the 5' and 3'ends of the *YKU70* gene (Figure 7). The DNA fragment was created by PCR amplification of chromosomal DNA purified from yeast strain YLKL652. This strain already has *HIS3* inserted into the *YKU70* gene. The PCR reaction involved amplification of this region of chromosome XIII.

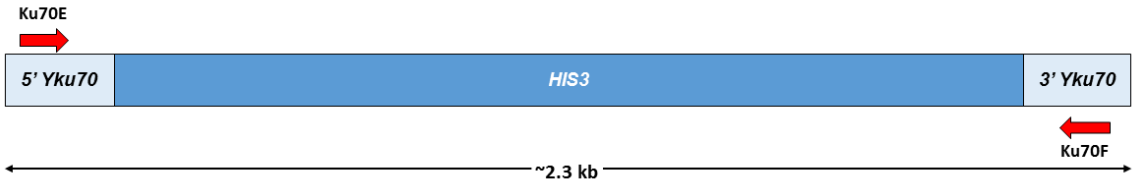

Figure 7. PCR primer binding sites on yeast chromosome XIII. Ku70E and Ku70F amplified a 2.3 kb product containing the full *yku70Δ::HIS3* sequence from chromosomal DNA extracted from yeast strain YLKL652.

The fully amplified insertion with the *HIS3* gene and *yku70* flanking regions is approximately 2.3 kb in size. This product was confirmed by gel electrophoresis in (Figure 8).

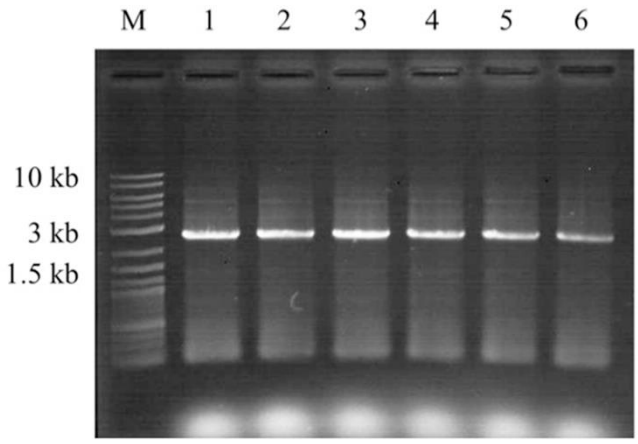

Figure 8. Example of confirmation of PCR products by gel electrophoresis. PCR products from 6 separate reactions are shown in lanes 1-6. Each product showed the expected size of *yku70Δ::HIS3* fragment at 2.3 kb.

The flanking regions possessed a high amount of homology to the *YKU70* gene allowing the amplified DNA to insert into the genome via homologous recombination (Figure 6). This recombination event disrupts the functioning *YKU70* gene, and allows for selection by growth on plates deficient in histidine: only cells that have incorporated the fragment into the chromosome gain the His^+ genotype. To ensure selection of colonies with the *yku70Δ::HIS3* insertion, PCR confirmation was required because in some transformants *HIS3* may have integrated into the wrong locus. Two primer sets were used to screen for isolates and confirm their genotype. Figure 9A shows the approximate locations of the primer sets and their corresponding PCR products run on a gel. In Figure 9B primers Ku70A and 3'HIS3 were used to generate a 1.3 kb product if *HIS3* was properly inserted into *YKU70*. In part C, primer Ku70G and 5-HIS-3 were used to create a 0.9 kb product if *HIS3* was integrated correctly.

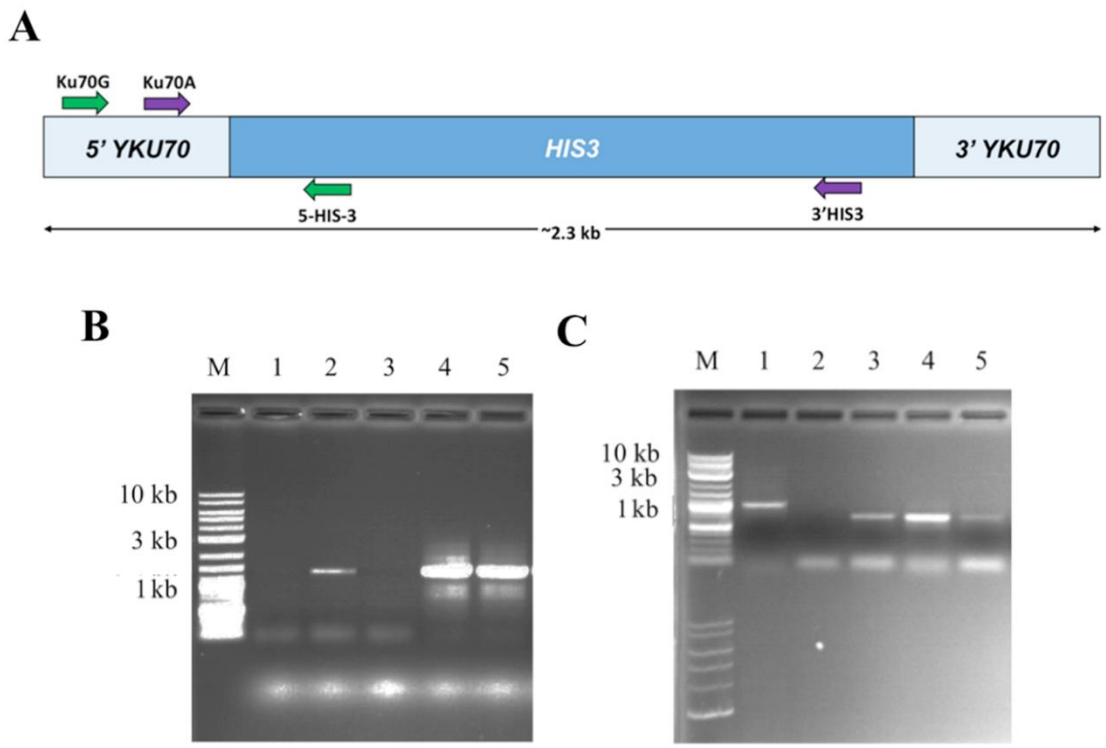

Figure 9. Example of confirmation of *yku70Δ::HIS3* insertion by PCR and gel electrophoresis. (A) Locations of primers used for PCR tests. (B) Ku70A and 3'HIS3 (purple) primers were used for initial screens of strains containing *yku70Δ::HIS3*. Lanes 1-5 were PCR products of chromosomal DNA tested for the *yku70Δ::HIS3* insertion. Lanes 2, 4, and 5, show isolates that did have the correct *yku70Δ::HIS3* insertion. (C) Ku70G and 5-HIS-3 (green) were used to confirm the strains were true *yku70* mutants. Lane 1 is a PCR reaction control. Lanes 2-5 were PCR products of chromosomal DNA tested for the *yku70Δ::HIS3* insertion. Bands indicating insertion in B are 1.3 kb and bands confirmed in C are 0.9 kb bands. Each gel has a 2-Log DNA Ladder as a marker indicated as M in the first lane.

Once a minimum of three independent colonies were confirmed by PCR, cells were patched onto plates for double imprint replica plating tests to assess their growth at 37°C. After the double imprint replica plates were grown for 2 days, isolates from each strain were compared to ensure similar growth patterns and/or sensitivity to high temperatures. Independently created knockout strains for each gene usually gave the same phenotype, i.e, they all grew at 37°C or they all did not grow at 37°C. However, if

an isolate did not have a similar growth pattern to 2 or more other isolates, it was presumed to be due to a random mutation and not used in any further experiments. Figure 10 shows an example of double imprint replica plating of 2 independent isolates of 4 new double mutants as well as wild type and *yku70* cells. The results in the bottom panel show that only wildtype BY4742 and *rsc1 yku70* double mutant cells were able to grow strongly at 37°C.

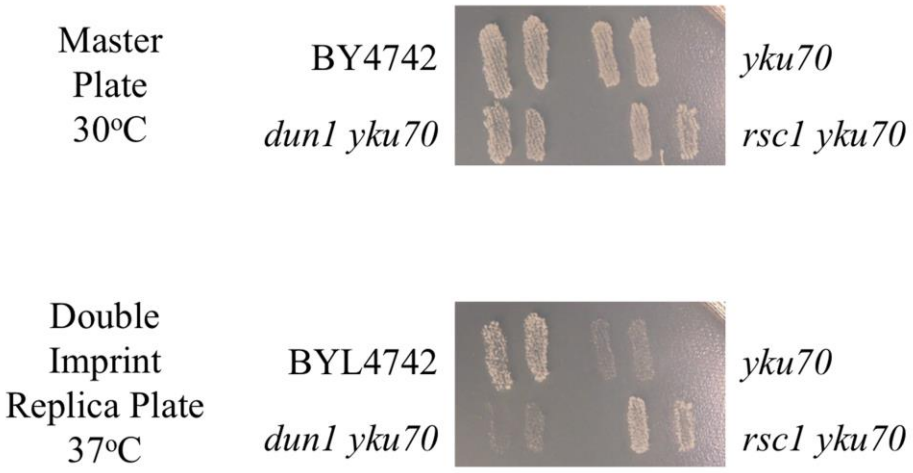

Figure 10. Examples of double imprint replica plating. Two independent isolates of each strain grown side-by-side as single streaks of cells on glucose complete plates exhibited similar phenotypes for growth at 37°C.

Double imprint replica plating is a qualitative method for assessing cell survival. To assess survival more quantitatively, dilution pronging survival assays were performed. After adding cells to water and sonicating to separate the cells, a hemocytometer was used to count cell titers, which were then used to make dilutions of known concentrations. Aliquots were added to a 96-well microtiter plate, where they were diluted 5-fold, 5 times, and then pronged to selective media plates (Figure 11).

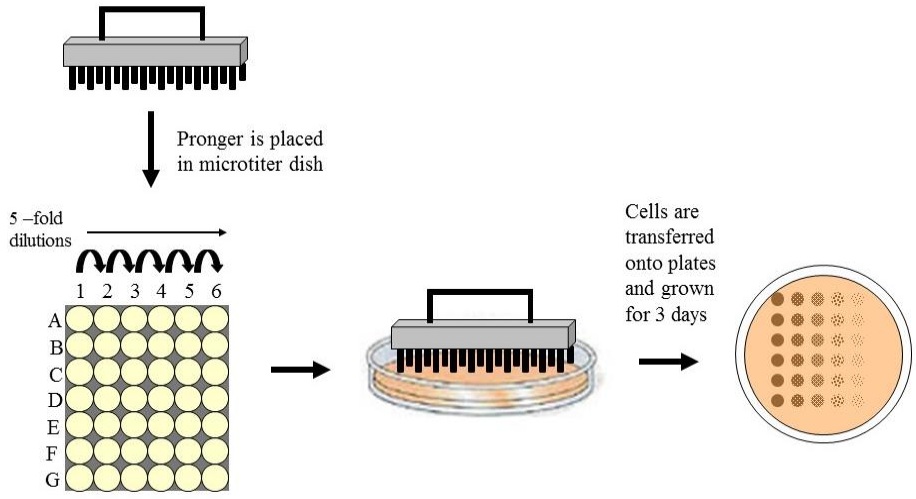

Figure 11. Dilution pronging assays for quantitation of cell survival. Dilutions of cells were made across the columns. The pronging instrument was placed into the microtiter plate and then cells were transferred onto a synthetic media plate and grown for 3-5 days to form colonies.

Plates were incubated at 30°C or 37°C for 3-5 days to ensure complete growth. A beaker of water containing a thermometer and a cover to reduce evaporation was kept in the 37°C incubator on the same shelf as the plates to ensure that the internal temperature was constantly maintained at 37°C. Pronging of multiple PCR-confirmed isolates of a double mutant strain is shown as an example in Figure 12. In this particular test 4 independent isolates created by knocking out *YKU70* in an *rsa1* strain were pronged to glucose plates lacking histidine and grown at 30°C or 37°C. Three of the isolates grew strongly at 37°C and one did not (*rsa1 yku70-9*). The isolate that did not grow likely had a spontaneous secondary mutation in an unknown gene that caused it to not grow at 37°C and it was discarded. For most of the double mutants created in this study, all independent isolates that were tested displayed the same phenotype at 37°C.

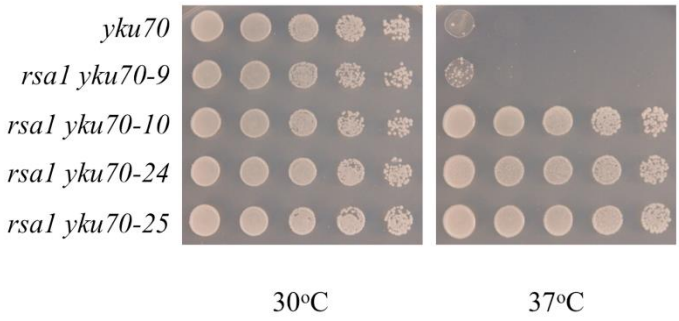

Figure 12. Pronging confirmation of double mutant phenotypes. Strains with PCR-confirmed isolates that displayed variance in ts phenotype could be distinguished quantitatively by pronging. Multiple isolates of *rsa1 yku70* strains were pronged to confirm the rescue of the *yku* ts phenotype.

Very Long Telomere (VLT) Mutants

The first group of genes to be tested for their effect on survival of *yku70* mutants at 37°C were originally characterized by Askree *et al*. and Gatbonton *et al*. as having very long telomeres (VLT phenotype) (49, 50). They identified 10 genes that caused this phenotype when inactivated. These genes included *ARD1, ELG1, HTL1, PIF1, RAD27, RIF1, RIF2, RPS17A, RSA1,* and *SRB8.*

Telomere shortening and degradation is the major cause of death of *yku70* mutants at 37°C. It is possible that mutants with unusually long telomeres may counteract or suppress the death of *yku70* cells at high temperature. Previous experiments in this laboratory and elsewhere tested some of the mutants having extremely long telomeres according to the Askree *et al.* and Gatbonton *et al.* lists in combination with a *yku70* mutation to determine rescue of the temperature sensitive phenotype at 37°C (36, 46, 47, 57, 58, 59). The remaining previously untested mutants were *RSA1* (involved in ribosomal assembly), *RPS17a* (ribosomal protein S17A), and *SRB8* (subunit of RNA Polymerase II mediator complex). Single *rsa1*, *rps17a,* and *srb8* mutants, as well as

double mutants that also had the *yku70Δ::HIS3* insertion were pronged to glucose complete plates at both 30°C and 37°C and allowed to grow for 3 days. At 30°C the wild-type BY4742 cells and single mutants (*yku70* and *rsa1*), and the double mutant *rsa1 yku70* strains grew normally (Figure 13, left side). At 37°C *yku70* cells grew poorly as expected. *rsa1 yku70* cells were able to grow as well as wildtype cells, indicating that inactivation of *rsa1* suppresses the ts^- phenotype.

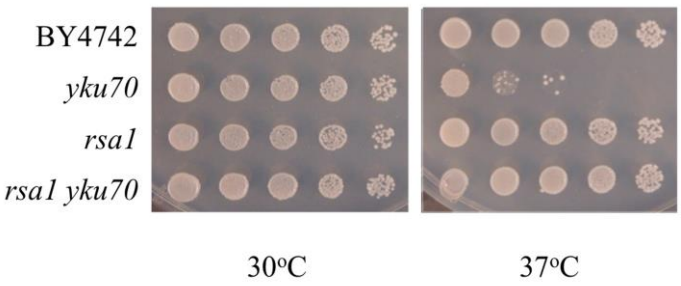

Figure 13. Pronging survival assay of *yku70* cells, an *rsa1* mutant identified as having very long telomeres, and a *yku70 rsa1* double mutant. The double mutant exhibits survival at 37°C.

To test the other two genes, cells were pronged to glucose complete plates at both 30°C and 37°C and allowed to grow for 3 days. At 30°C the *yku70*, *rps17a,* and *srb8* single mutants, and the *rps17a yku70* and *srb8 yku70* strains grew normally (Figure 14).

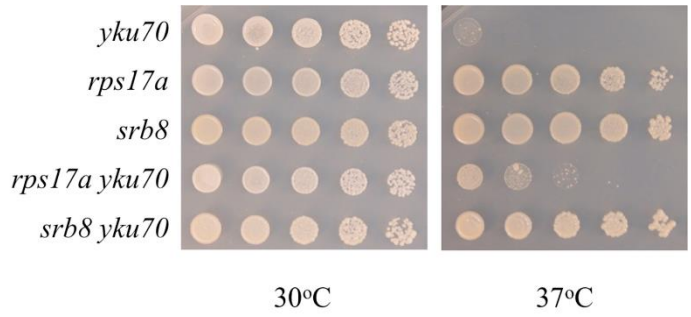

Figure 14. Pronging survival assay of *yku70* double mutants with VLT phenotype. *rps17a yku70* double mutants did not exhibit survival at 37°C. However, *srb8 yku70* double mutants were viable.

srb8 yku70 double mutants grew as well as *rps17a* and *srb8* single mutant cells. In contrast, *rps17a yku70* double mutants showed strong temperature sensitivity. These data indicate that 3 of the 10 mutants with very long telomeres are able to add stability to *yku70* mutants that normally have short and unstable telomeres at elevated temperatures (Table 4). These suppressors include two genes tested in the current project (*RSA1* and *SRB8*) plus one gene characterized in past work (*RIF1*).

Table 4. List of VLT and *yku70* double mutants. Effect of co-inactivation of genes affecting telomere length on survival of *yku70* cells at 37°C.

Double mutant	**Suppression of *yku* 37°C ts phenotype**	**Reference**
rif1 yku70	**Yes**	36, 46, 58
rsa1 yku70	**Yes**	This work
srb8 yku70	**Yes**	This work
ard1 yku70	No	57
elg1 yku70	No	57
htl1 yku70	No	57
pif1 yku70	No	46, 57
rad27 yku70	No	36, 46, 57, 58
rif2 yku70	No	36, 46, 58
rps17a yku70	No	This work

Chromatin remodeling mutants

The RSC complex consists of Rsc1, Rsc2, and several other Rsc proteins that are involved in chromatin remodeling. These two subunits have been associated with DSB repair and physically interact with Yku80 (60). Since Yku70 and Yku80 have roles in creation of the telomere cap structure, and may physically associate with several histone proteins, it is possible that members of the RSC complex may have a role in telomere maintenance as well (60). Furthermore, changes in the chromatin structure of the telomeres may also influence recruitment of telomerase. Changing the ability to remodel the structure and the affinity of nucleosomes may have an impact on exonuclease accessibility and overall survival of *yku70* mutants at 37°C.

Cells were pronged to glucose complete plates at both 30°C and 37°C and allowed to grow for 3 days as before. At 30°C all strains grew normally (Figure 15). At 37°C *yku70* cells grew poorly as expected. Both *rsc1 yku70* and *rsc2 yku70* cells were able to rescue the temperature sensitive phenotype and grew as well as *rsc1* and *rsc2* mutants. A major determinant of survival of *yku70* cells at 37°C is whether the telomeres are degraded by Exo1 nuclease or not. This result implies that the structure of the chromatin in the telomere region may contribute to the accessibility by Exo1. It may also affect its ability to recruit telomerase to the telomeres during S phase.

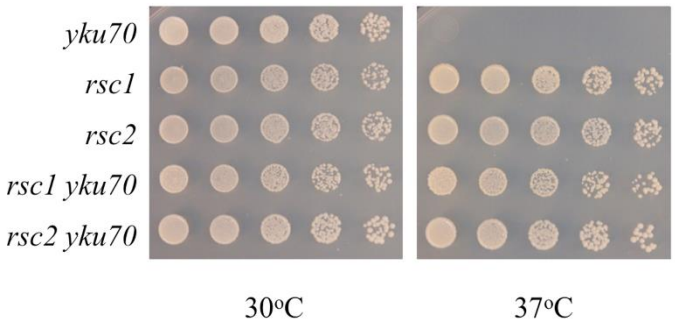

Figure 15. Mutations in genes affecting chromatin remodeling rescue survival of *yku70* cells at 37°C.

DNA damage checkpoint response mutants

yku70 mutants undergo arrest in G_2 phase due to a DNA damage-induced cell cycle checkpoint response and lose viability at 37°C (17, 36). This response is caused primarily by Exo1 degradation and depends on a group of DNA damage checkpoint genes. It occurs due to an increase in the amount of ssDNA in the telomere regions. Deficiencies in the checkpoint response allow for continuous cell division, without pausing in G_2 phase, despite the accumulation of ssDNA (36).

yku70 cells that were also deficient in DNA damage checkpoint response genes *DUN1*, *DDC1*, *MEC3*, or *CHK1* were pronged to glucose complete plates at both 30°C and 37°C and allowed to grow for 3 days. At 30°C all strains grew across each column (Figure 16). Both *mec3 yku70* and *chk1 yku70* cells were able to rescue the temperature sensitive phenotype and grow as well as the single mutants (right panel, bottom 2 rows). However, *dun1 yku70* and *ddc1 yku70* cells retained strong temperature sensitivity at 37°C. One interesting observation is that *ddc1 yku70* and *mec3 yku70* cells gave different

results, even though both Ddc1 and Mec3 have a role in the same complex along with Rad17 (19).

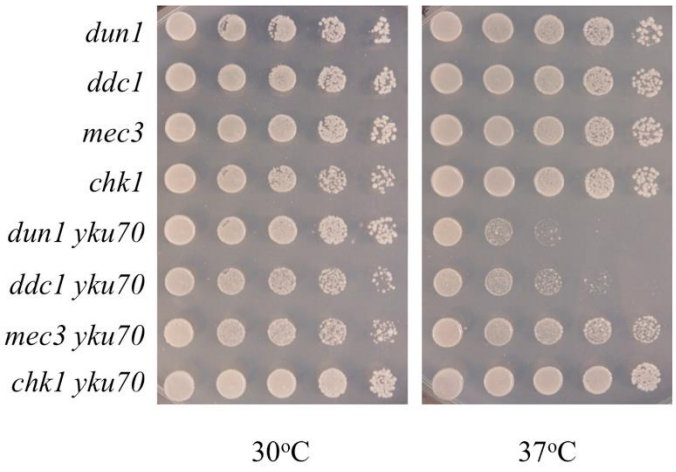

Figure 16. *dun1 yku70* and *ddc1 yku70* double mutants did not exhibit survival at 37°C. However, *mec3 yku70* and *chk1 yku70* double mutants remained viable.

Mitotic spindle checkpoint mutants

Sister chromatid separation occurs as metaphase progresses to anaphase during M phase of the cell cycle. Cells have a spindle checkpoint that serves as a mechanism to prevent chromosome segregation if the spindle apparatus and the cell are not ready, which may lead to errors. Inactivation of the spindle checkpoint gene *MAD2* in *yku70* mutants has been shown to partially rescue temperature sensitivity at 37°C (36). Testing in our laboratory was done to validate this finding and then later to determine if the spindle checkpoint also affects rescue of *yku70* cells by overexpression of telomerase. Cells were pronged to glucose complete plates at both 30°C and 37°C and allowed to grow for 3 days. At 30°C all cells grew across each column (Figure 17). *mad2 yku70* cells showed moderately better growth at 37°C than *yku70* single mutants, confirming the partial suppression seen in the previous study (rows 3 and 6) (36).

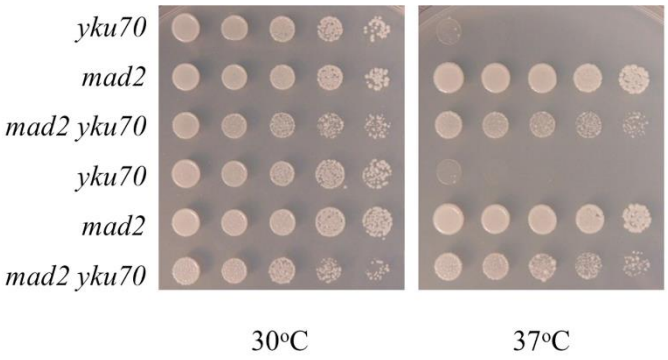

Figure 17. Pronging survival assay of mutants involved in spindle checkpoints. Inactivation of *MAD2* partially rescues survival at 37°C.

KEOPS kinase and telomere cap stability mutants

CGI121 is an evolutionarily conserved gene in yeast and humans whose protein product was identified as having a role in the KEOPS kinase complex. This complex was identified as a telomere regulator and consists of five subunits (Cgi121, Bud32, Kae1, Gon7, and Pcc1). In yeast, Cgi121 protein was shown to promote telomere uncapping and ssDNA accumulation triggered by *cdc13-1* mutations (36, 59). *CGI121* mutations have also been shown to suppress temperature-sensitive defects associated with loss of telomere capping in *yku70* mutants, as well as a defect in telomeric recombination, but its precise role in maintenance of telomere stability is not known (Downey et al 2006; Peng et al 2015). Cells were pronged to glucose complete plates at both 30°C and 37°C and allowed to grow for 3 days. At 30°C the *yku70* and *cgi121* single mutant strains grew across each column but the *cgi121 yku70* double mutants grew poorly (Figure 18, left panel, rows 3 and 6). Two independent isolates of the *cgi121 yku70* double mutant strains, surprisingly, grew consistently better at 37°C than at 30°C. Thus, *cgi121* mutations were confirmed to suppress the ts^- phenotype of *yku70* strains.

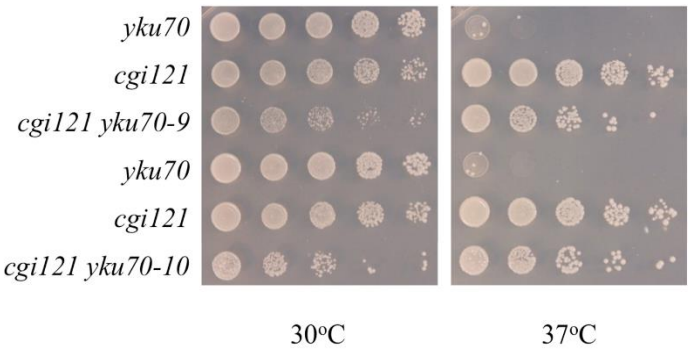

Figure 18. Inactivation of the *CGI121* KEOPS kinase gene partially rescues survival of *yku70* at 37°C.

DNA repair and telomere structure

Sgs1 is a member of the RecQ helicase family and deficiency is associated with Werner syndrome, Rothmund-Thomson syndrome, and Bloom's syndrome in humans (61, 62, 63). Similar to Yku70, previous studies have shown that Sgs1 has a role in DNA repair as well as telomere maintenance. Sgs1 affects telomere structure in the absence of telomerase components Est1, Est2, Est3, or TLC1 RNA (64, 65). Additionally, *sgs1* mutants display elevated levels of recombination, especially in the subtelomeric Y' element regions near the ends of chromosomes (66, 67).

sgs1 yku70 prongings showed moderate growth at 37°C with many small colonies and consistently, several "paps" among the colonies (Figure 19). Paps are large colonies that can be seen growing in a field of slower growing cells. Their frequency was very high, higher than mutation rates typically seen in yeast cells and therefore may involve epigenetic effects. The results of all of the double mutant prongings are tabulated in Table 5 and summarized in the Summary and Conclusions section at the end of this chapter.

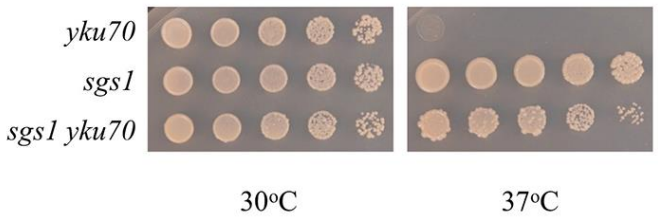

Figure 19. Pronging survival assay of *yku70 sgs1* mutants. The *sgs1 yku70* double mutants exhibit increased survival at 37°C.

Rescue of *yku70* single and double mutants by overexpression of telomerase subunits

Production of supraphysiological levels of Est2 protein or *TLC1* RNA in *yku70* or *yku80* mutants rescues growth of the cells at 37°C, but the exact mechanism involved has not been determined.

None of the double mutants created for this project had been previously tested for rescue at 37°C by overexpression of the telomerase subunits *EST2* or *TLC1*. Double mutants with the *yku70Δ::HIS3* insertion and control *yku70* single mutants were transformed with a vector or a plasmid that overexpressed *EST2* and pronged to glucose minus leucine plates at both 30°C and 37°C and allowed to grow for 3 days. At 30°C vector and *EST2* transformed *yku70* cells and the *rps17a yku70*, *srb8 yku70*, and *rsa1 yku70* double mutants grew across all columns (Figure 20). At 37°C *yku70* and *rps17a yku70* cells with the vector did not grow (rows 1 and 3, right panel). When *EST2* was overexpressed, the growth of *rps17a yku70* mutants was rescued (row 4). *srb8 yku70* and *rsa1 yku70* mutants with *EST2* overexpressed grew similar to the vector controls and rescue by overexpression of *EST2* could not be determined by this assay (see the last 4 rows in Figure 20). These results indicate that *srb8* and *rsa1* mutations suppress the ts$^-$

phenotype of *yku70* cells strongly. The cells grew so well that it was not possible to detect improvements in growth rates or survival caused by *EST2* overexpression at 37°C.

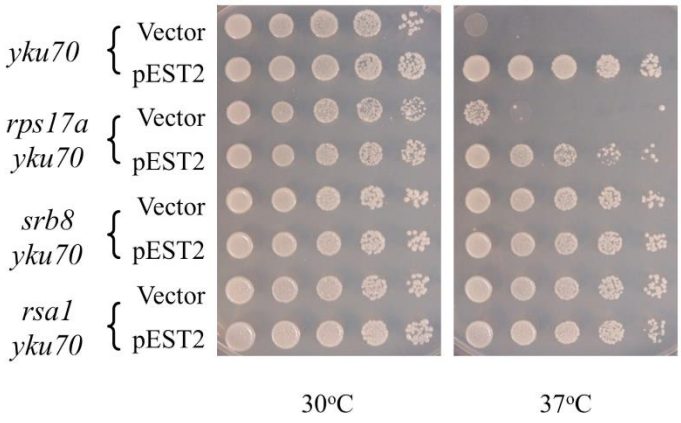

Figure 20. Pronging survival assay of double mutants with inactivated *yku70* and other genes identified as having very long telomeres with and without *EST2* overexpressed. Overexpression of *EST2* in *rps17a yku70* mutants rescues survival at 37°C. Differences in survival with overexpression of *EST2* in *srb8 yku70* and *rsa1 yku70* cells could not be detected in this assay.

Double mutants with the chromatin assembly genes *RSC1* or *RSC2* inactivated were transformed with a vector or a plasmid that overexpressed *EST2* and pronged to glucose minus leucine plates at both 30°C and 37°C and allowed to grow for 3 days. At 37°C, *yku70* cells with the vector grew poorly, but *EST2* rescued the temperature sensitive phenotype (Figure 21, top 2 rows). When *EST2* was overexpressed *rsc1 yku70* cells grew more quickly, leading to larger colony sizes compared to the vector control, revealing that growth rates were enhanced by *EST2* overexpression (rows 3 and 4). In contrast to the *rsc1 yku70* cells, *rsc2 yku70* mutants with *EST2* overexpressed exhibited poor growth at both 30°C and 37°C, indicating that excess Est2 protein was deleterious in these mutants (Figure 21, bottom 2 rows). *rsc2 yku70* cells are the first mutants found to

display inhibited growth due to overexpression of a telomerase subunit. The results suggest that, through an unknown mechanism, inactivation of *RSC2* changes telomeres so that they are resistant to degradation by Exo1 nuclease, but overexpression of telomerase may destabilize them again.

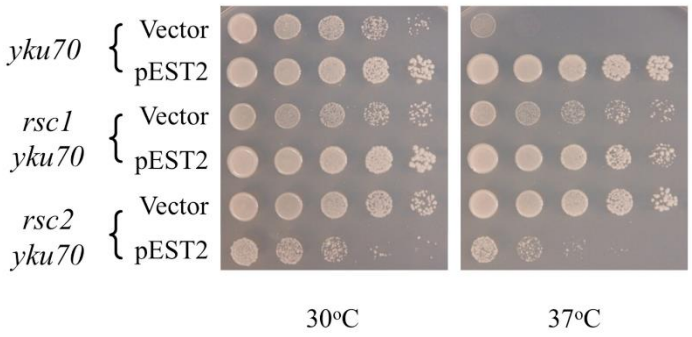

Figure 21. Pronging survival assay of double mutants with mutations in genes involved in chromatin remodeling with and without *EST2* overexpressed. Overexpression of *EST2* in *rsc1 yku70* mutants rescues survival at 37°C, but growth of *rsc2 yku70* cells was strongly inhibited.

Double mutants with DNA damage checkpoint genes inactivated were transformed with a vector or a plasmid that overexpressed *EST2* and pronged to glucose minus leucine plates at both 30°C and 37°C and allowed to grow for 3 days. At 37°C, *dun1 yku70* and *ddc1 yku70* cells were rescued strongly when *EST2* was overexpressed (Figure 22, top 4 rows). *mec3 yku70* and *chk1 yku70* cells with *EST2* overexpressed grew strongly, similar to the vector controls, and therefore effects caused by overexpression of *EST2* could not be determined by this assay (last 4 rows of Figure 22).

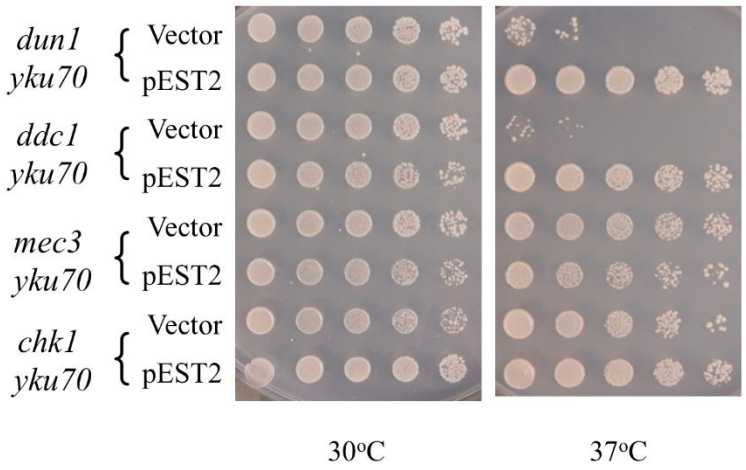

Figure 22. Pronging survival assay of double mutants with inactivated DNA damage checkpoint response genes with *EST2* overexpressed. Overexpression of *EST2* in *ddc1 yku70* and *dun1 yku70* mutants rescues survival at 37°C. Differences in survival with overexpression of *EST2* in *mec3 yku70* and *chk1 yku70* were indistinguishable in this assay.

Double mutants with the spindle checkpoint gene *MAD2* or the KEOPS kinase gene *CGI121* inactivated were transformed with a vector or a plasmid that overexpressed *EST2* and pronged to glucose minus leucine plates at both 30°C and 37°C and allowed to grow for 3 days. The vector and *EST2* transformed *mad2 yku70* and *cgi121 yku70* mutants grew across all columns at both 30°C and 37°C (Figure 23). Thus, rescue by overexpression of *EST2* could not be determined by this assay at 37°C.

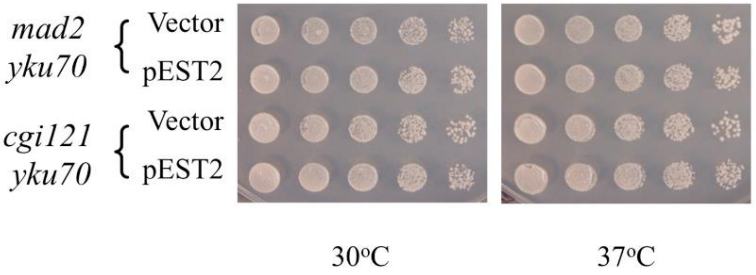

Figure 23. Pronging survival assay of *yku70* double mutants with spindle checkpoints and the KEOPS kinase genes inactivated. Differences in survival with overexpression of *EST2* in *mad2 yku70* and *cgi121 yku70* were indistinguishable in this assay.

Double mutants with the *SGS1* helicase gene inactivated were transformed with a vector or a plasmid that overexpressed *EST2* and pronged to glucose minus leucine plates at both 30°C and 37°C. The vector and *EST2*-transformed double mutant showed modest growth at 37°C (Figure 24A, rows 3 and 4) that was not as strong as seen in *yku70* cells when *EST2* was overexpressed (Figure 24A, row 2). *sgs1 yku70* cells are the first double mutants identified that clearly do not have their temperature sensitive phenotype rescued with telomerase overexpression. To add confidence to the results seen in *sgs1 yku70* cells with *EST2*, another independent PCR-confirmed isolate of *sgs1 yku70* cells was tested and it produced similar results (Figure 24B).

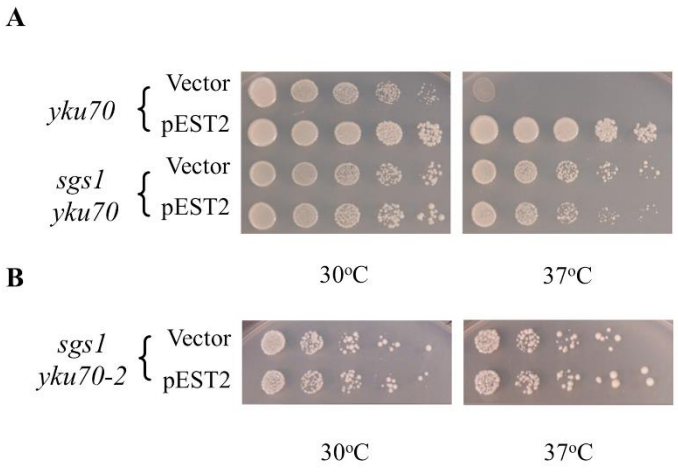

Figure 24. Pronging survival assay of *yku70* double mutants involved in DNA damage repair with *EST2* overexpressed. *sgs1 yku70* mutant survival was not influenced by overexpression of *EST2*.

In the next set of experiments, *TLC1*, the RNA subunit of telomerase, was overexpressed. At 37°C, *yku70 rps17a* cells were rescued but no effects of *TLC1* on

yku70 srb8 and *yku70 rsa1* mutants could be detected (Figure 25). This result is similar to what was seen when *EST2* was overexpressed.

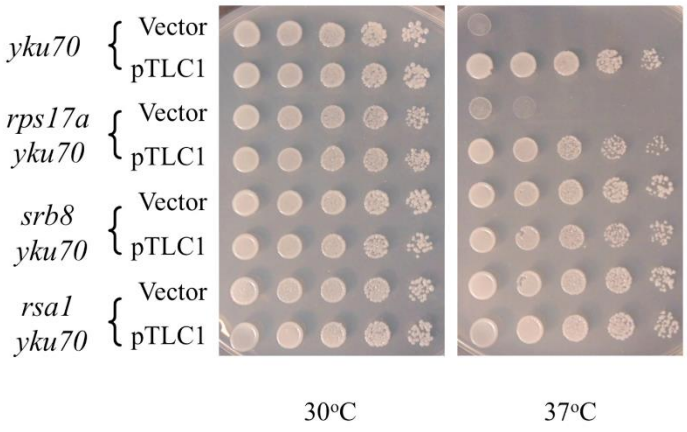

Figure 25. Overexpression of *TLC1* RNA in *rps17a yku70* mutants rescues survival at 37°C. Differences in survival with overexpression of *TLC1* in *srb8 yku70* and *rsa1 yku70* cells were indistinguishable in this assay.

Double mutants with the *yku70Δ::HIS3* insertion in *rsc1* and *rsc2* chromatin assembly mutants were tested next. Results with *TLC1* overexpression were similar to those seen with *EST2* overexpression (Figure 26). *TLC1* rescued the temperature sensitive phenotype of *rsc1 yku70* cells, but not *rsc2 yku70* cells. When *TLC1* RNA was overexpressed *rsc1 yku70* cells grew more quickly, leading to larger colony sizes compared to the vector control, but differences in rescue could not be determined by this assay.

rsc2 yku70 cells with *TLC1* overexpressed showed increased sensitivity to 37°C compared to the vector controls. *rsc2 yku70* cells increased sensitivity due to overexpression of a telomerase subunit in this assay supports what was seen with *EST2*.

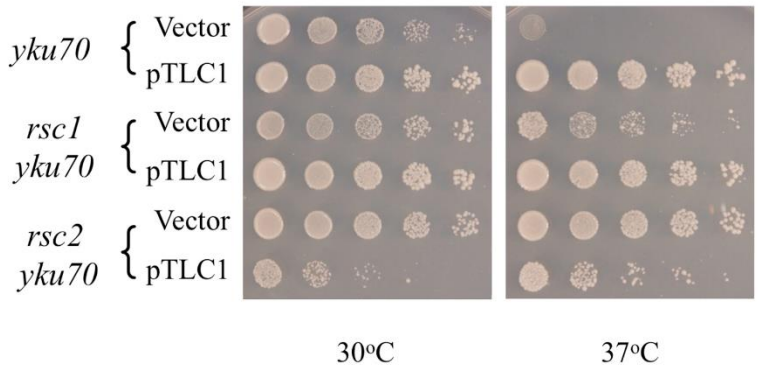

Figure 26. Pronging survival assay of *yku70* double mutants with chromatin remodeling inactivated and *TLC1* overexpressed. Overexpression of *TLC1* in *rsc1 yku70* double mutants increased growth rates at 37°C, while *rsc2 yku70* cell growth was inhibited.

Double mutants with the *yku70Δ::HIS3* insertion and DNA damage checkpoint genes inactivated were analyzed next. As shown in Figure 27 results were similar to those seen with *EST2* overexpression. *dun1 yku70* and *ddc1 yku70* cells were rescued by *TLC1*, but rescue of *mec3 yku70* and *chk1 yku70* mutants could not be detected under these conditions.

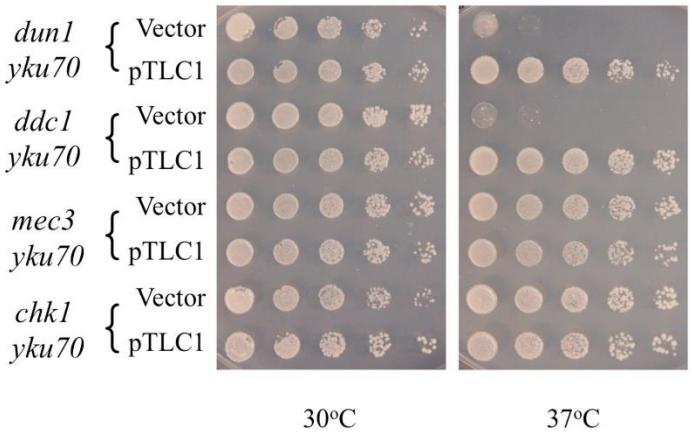

Figure 27. Overexpression of *TLC1* RNA in *ddc1 yku70* and *dun1 yku70* mutants rescues survival at 37°C. Differences in survival with overexpression of *TLC1* in *mec3 yku70* and *chk1 yku70* cells were indistinguishable in this assay.

The effects of *TLC1* overexpression in double mutants with the *yku70Δ::HIS3* insertion combined with knockouts of *MAD2* and *CGI121* were tested. *mad2 yku70* and *cgi121 yku70* cells with *TLC1* overexpressed grew similar to the vector controls and rescue by overexpression of *TLC1* could not be determined (Figure 28).

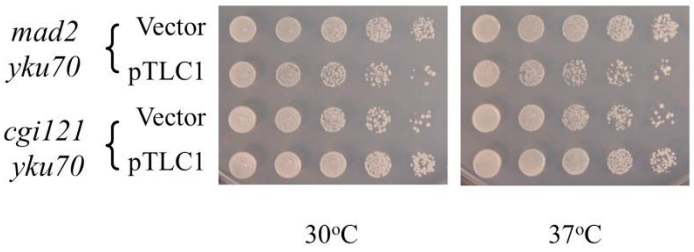

Figure 28. Pronging survival assay of *yku70* double mutants involved with spindle checkpoints and the KEOPS kinase with *TLC1* overexpressed. Differences in survival with overexpression of *TLC1* in *mad2 yku70* and *cgi121 yku70* were indistinguishable in this assay.

The effects of overexpression of *TLC1* RNA in *yku70 sgs1* mutants are depicted in Figure 30. As previously observed with *EST2* overexpression, high intracellular levels of *TLC1* RNA did not strongly affect growth rates or survival at 37°C in two independent isolates of the *yku70 sgs1* mutants (Figure 29A and B).

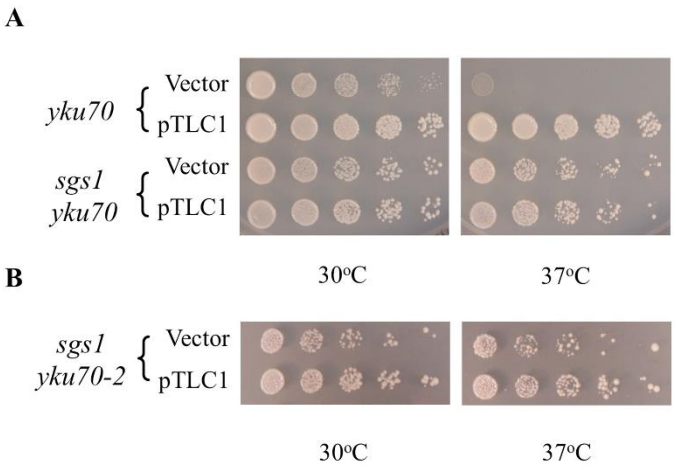

Figure 29. Overexpression of *TLC1* RNA did not strongly affect growth at 37°C in *yku70 sgs1* double mutants.

Some *yku70* double mutants could not be tested for rescue by *EST2* or *TLC1* overexpression at 37°C because the double mutant grew too robustly at 37°C, even without overexpression of telomerase. Tests were performed to determine if the growth of the vector controls could be inhibited more strongly at the higher temperature of 40°C. This temperature was the highest that could be tested before growth of all strains, including wildtype BY4742 cells, became strongly inhibited. Additionally, the number of cells pronged to each plate was reduced to make differentiation between survival of different strains more obvious. Double mutants were pronged to glucose complete plates at 30°C, 37°C, and 40°C and allowed to grow for 3-5 days. At 30°C wild-type BY4742 and all mutant strains grew normally with the exception of *cgi121 yku70* cells (Figure 30). At 37°C, *yku70* cells grew poorly and all the double mutants exhibited strong suppression of the ts^- phenotype seen in *yku70* cells. Only one double mutant (*rsc2 yku70*) showed reduced growth at 40°C relative to that seen at 37°C (compare far right panels to

middle panels in Figure 30). Future studies of *EST2* and *TLC1* expression effects can be performed at the higher temperature of 40°C in this mutant.

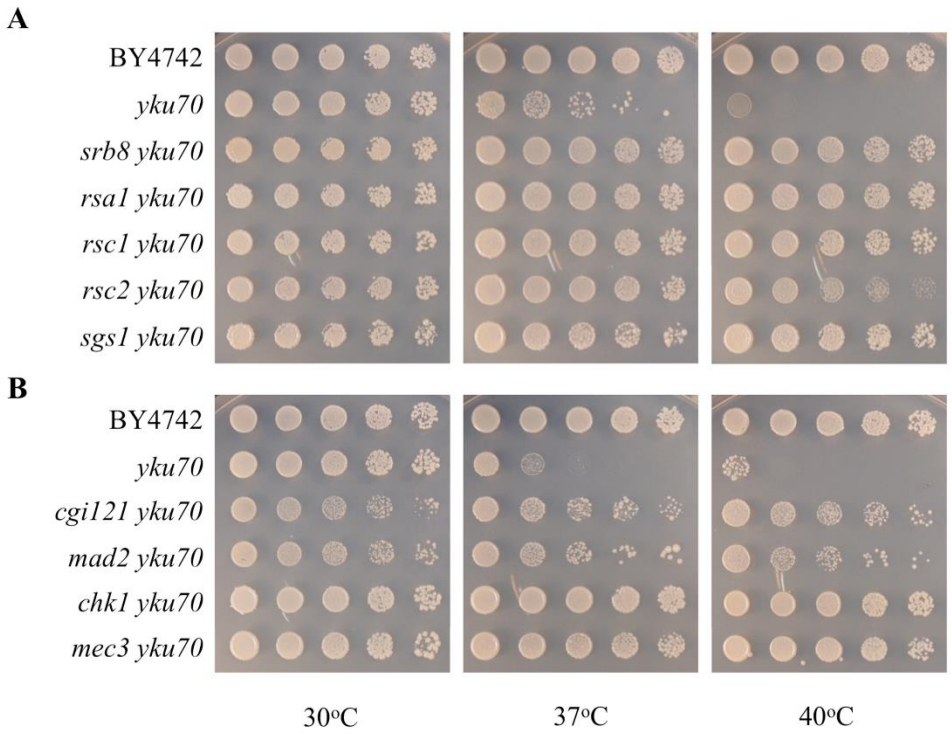

Figure 30. Pronging survival assays of *yku70* double mutants that showed strong growth at 37°C in the previous assays. *cgi121 yku70*, *mad2 yku70*, and *rsc2 yku70* cells showed moderate temperature sensitivity at 40°C than at 37°C.

Southern blot analysis of telomere lengths in *sgs1* mutants

The telomeres of *yku70* mutants are short but stable at 30°C, and undergo rapid shortening at 37°C. Overexpression of telomerase adds stability at the telomeres and eliminates the temperature sensitivity at 37°C, though the exact mechanism remains unknown. Knocking out *SGS1* moderately rescued the temperature sensitive phenotype of *yku70* cells. Surprisingly, in *sgs1 yku70* mutants overexpression of telomerase components *EST2* and *TLC1* did not rescue the temperature sensitive phenotype,

implying that it is required for stabilization of telomeres by telomerase. It was important to determine if inactivation of *SGS1* in *yku70* cells rescue may affect telomere length. This was done by Southern blot analysis. Chromosomal DNA was isolated from wildtype, *yku70*, *sgs1*, and *sgs1 yku70* strains. The chromosomal DNA concentration was measured and 5 µg of each sample was digested with XhoI. There are XhoI cut sites near the ends of each of the 16 chromosomes in haploid yeast cells. Cutting the DNA at these sites allows for separation of the 32 telomere ends from whole chromosomal DNA as well as the ability to distinguish between telomere lengths within different yeast strains. Purified, uncut chromosomal DNA shows a heavy band above 10 kb (Figure 31A), while XhoI-cut DNA has a heavy band above 10 kb accompanied by a smear of DNA fragments to approximately the 5 kb band (Figure 31B).

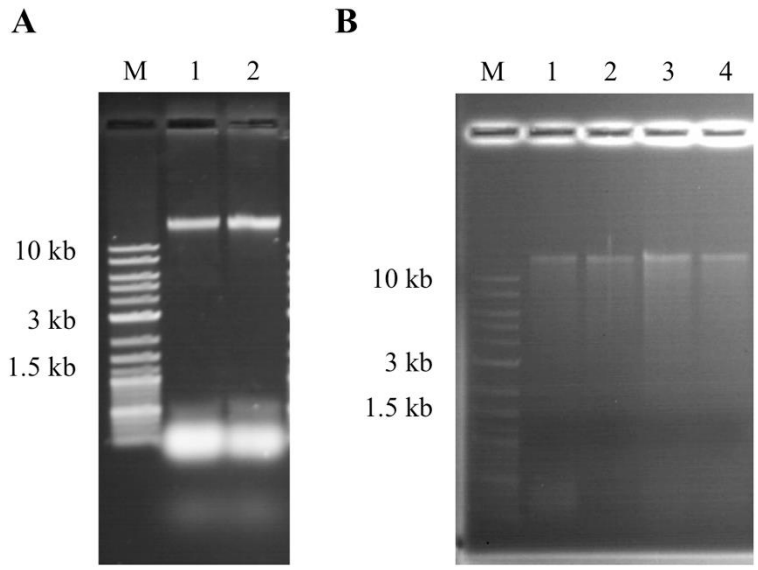

Figure 31. Confirmation of XhoI DNA digestion products by gel electrophoresis. Uncut DNA appears as a strong band above 10 kb (A) while XhoI digested DNA appears as a strong band above 10 kb and produces a smear down to approximately 5 kb (B).

XhoI-digested DNA was quantified so that approximately the same amount could be run on a 1.2% agarose gel using 0.5X TB as buffer. The Southerns were done using two different protocols that differed by experiment time and method of transfer of DNA to a membrane. One method has been used by previous lab members and takes 3 days to complete and transfer of DNA from the gel to a membrane occurs via capillary method overnight. The second method that was developed takes 2 days to complete, and utilizes a Trans-Blot Turbo blotting apparatus (BioRad). Two separate agarose gels (S1 and S2) were run with a 2-Log DNA ladder, DIG-labelled ladder and XhoI-digested DNAs, then stained in ethidium bromide and imaged (Figure 32).

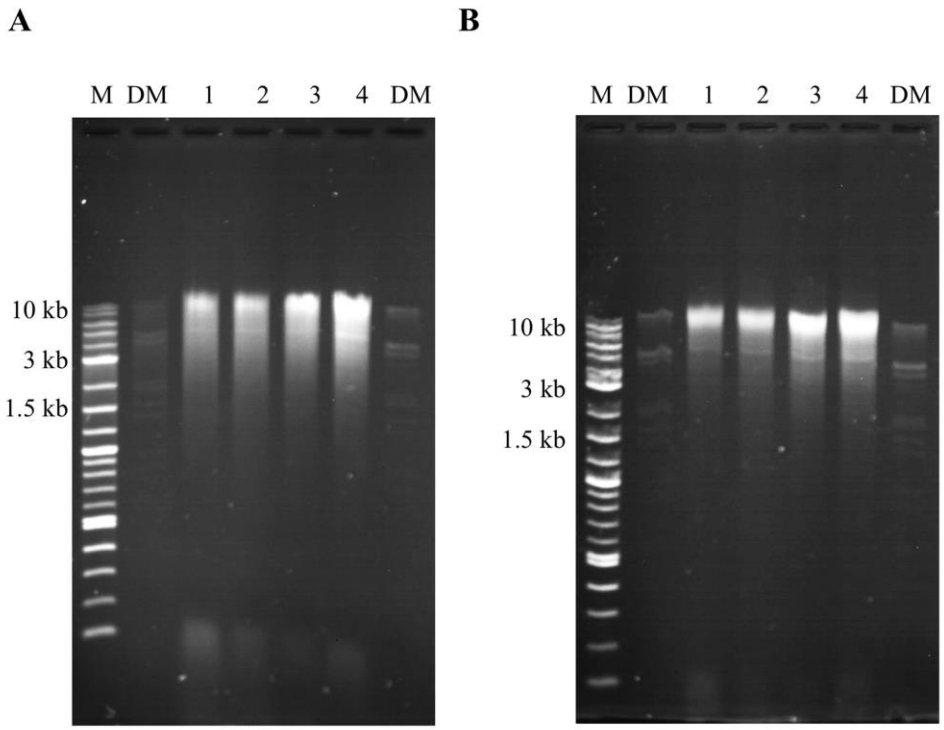

Figure 32. Gel electrophoresis for Southern blots. Agarose gels (A, B) showing the same loading order and similar sample amounts loaded. The lanes labeled M have the 2-log DNA ladder as a marker, and lanes with DM have the Digoxigenin-labeled DNA Molecular Weight Marker III.

Ethidium bromide staining alone cannot distinguish telomere lengths. Once the gel was run, the DNA from S1 was transferred overnight to a Hybond+ membrane by capillary transfer, where the DNA was moved onto the membrane as the SSC buffer was drawn up through the gel as shown in Figure 33A. The DNA from S2 was transferred to the membrane by electric current in 25 minutes using the Trans-Blot Turbo blotting apparatus as diagrammed in Figure 33B.

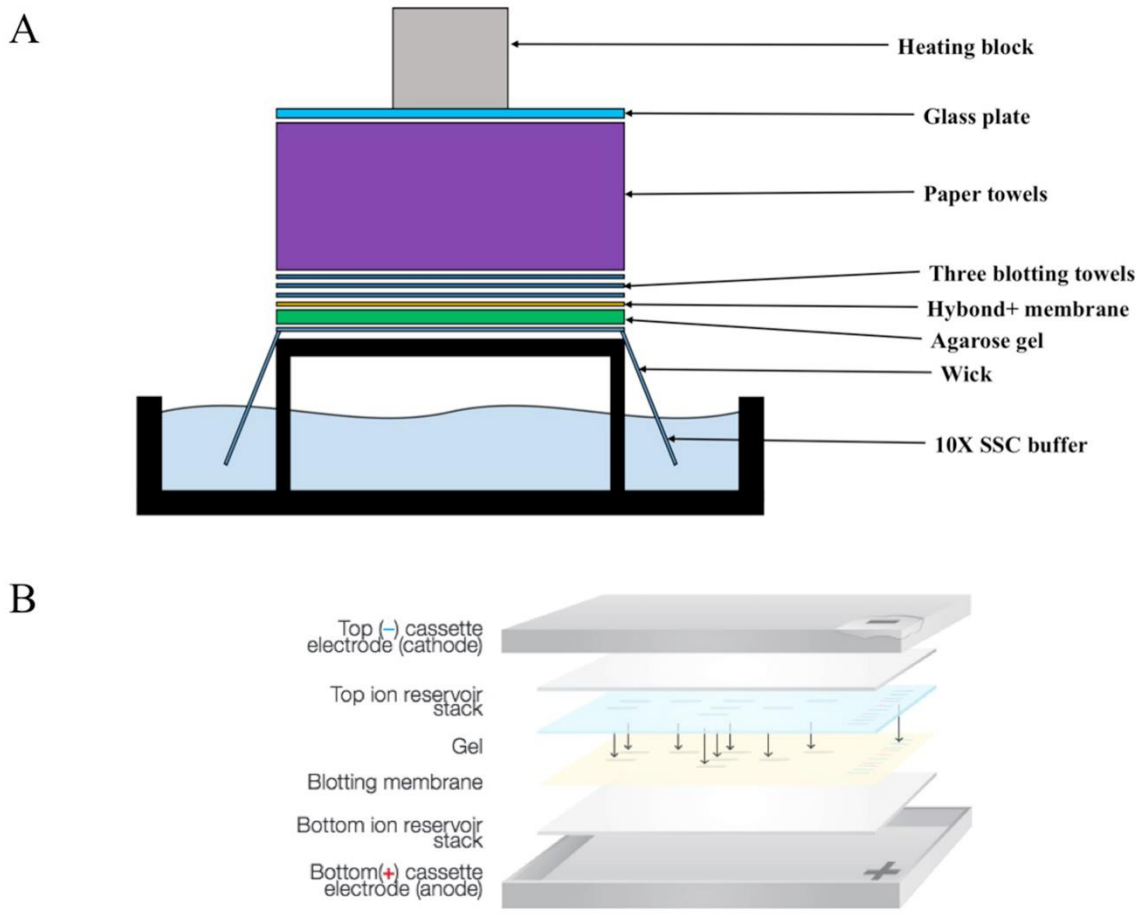

Figure 33. Transfer of DNA from an agarose gel to a Hybond+ membrane. Setup of capillary apparatus (A) and Trans-Blot Turbo blotting apparatus (B) (Bio-Rad).

The dehydrated gels were stained in ethidium bromide and imaged after the transfer to see how much DNA was left in the gels to compare the efficiency of transfer methods. The capillary transfer method showed some remaining DNA above 5 kb in size. However, most telomere fragments are smaller than this and within the region that transferred (Figure 34A). The Trans-Blot Turbo transfer method showed poor transfer efficiency with lots of remaining DNA, and very little difference compared to the gel image taken prior to the transfer (Figure 34B).

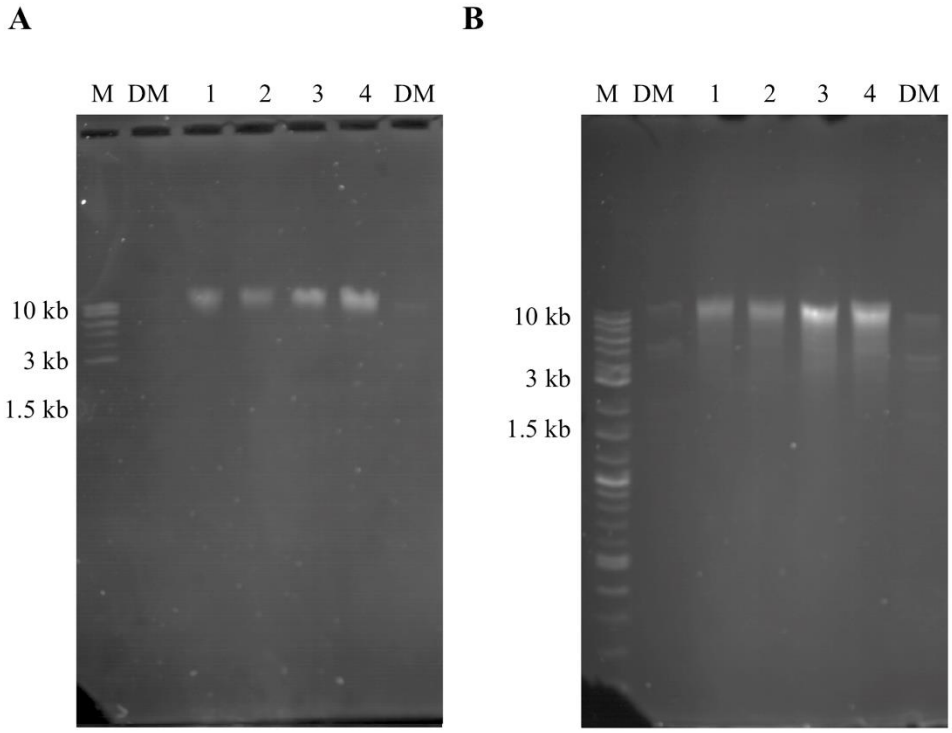

Figure 34. A side-by-side comparison of two agarose gels re-stained after transfer. Both gels started with the same loading order and similar sample amounts loaded after use of capillary transfer (A) and the Trans-Blot Turbo transfer method (B).

Once the DNA was transferred to each membrane, both experiments continued in the same way. To specifically identify the telomere repeat fragments within the total chromosomal DNA, complementary telomere probes were created by PCR. To enable the

probed DNA to be selected for, the probes were designed to be able to be detected by an antibody. By using a dNTP mixture that included a digoxigenin (DIG) labeled dUTP, the PCR product contained a molecule that is not found in yeast DNA and can be targeted with anti-DIG antibodies (Figure 35).

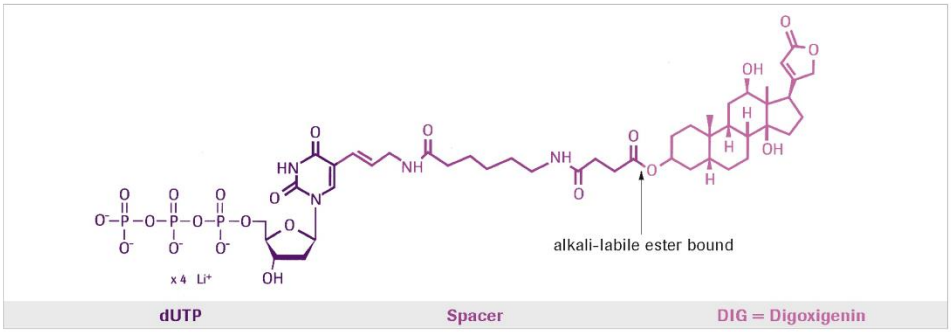

Figure 35. Structure of DIG labeled dUTP. A digoxigenin molecule is attached to a dUTP with a spacer in between (Roche Diagnostics).

The telomere repeat sequence used for the probe was amplified from a telomere repeat containing plasmid. The reaction was done using either dNTPs or DIG DNA Labeling Mix as the substrate in PCR. The approximate size of the amplified product with dNTPs was 0.2 kb and the DIG labeled product was very close to the 0.2 kb mark, though it did not move as far down the gel due to the added weight of the DIG molecules (Figure 36).

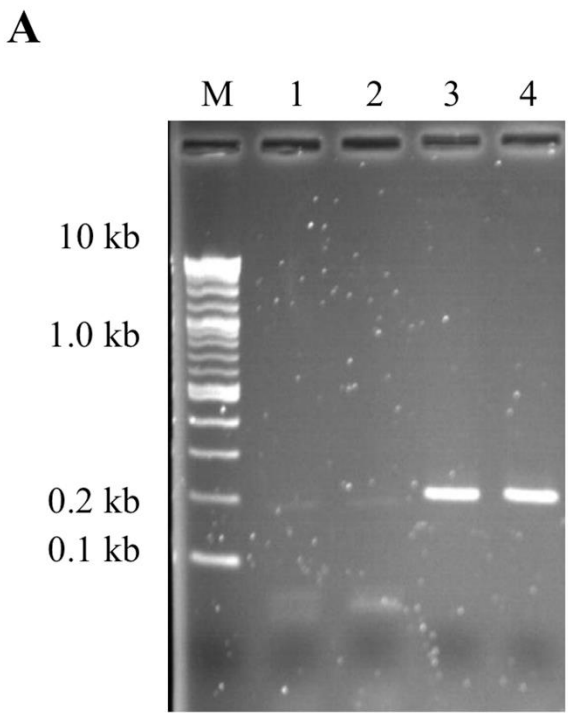

Figure 36. Confirmation of DIG-labeled PCR products by gel electrophoresis. PCR products with telomere repeat sequences show at the expected size of 0.2 kb, while DIG labeled products show a slightly higher band. Lanes 1 and 2, PCR with standard dNTPs. Lanes 3 and 4, PCR with DIG labelled dUTP.

The DIG labeled probe that hybridized to the telomere repeat regions could be detected using an anti-DIG antibody that had an enzyme (alkaline phosphatase) attached to it (Figure 37). As an alternative to using radioactive labeling, the enzyme-linked antibody reacted with a substrate (CSPD) to produce a chemiluminescent signal. This chemiluminescence was then detected using a ChemiDoc XRS+ instrument.

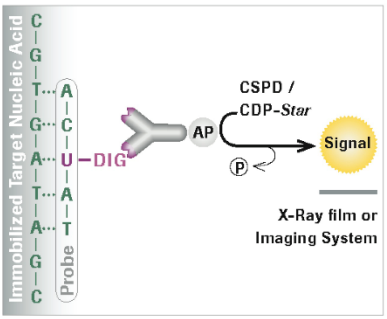

Figure 37. Overview of chemiluminescent detection of DIG labeled nucleic acids. Immobilized nucleic acids are hybridized to a complementary DIG-labeled probe. Anti-digoxigenin antibodies coupled to alkaline phosphatase (AP) react with a chemiluminescent substrate to produce a signal for detection (Roche Diagnostics).

The Southern blots confirmed that signal could be seen using either transfer method. The capillary transferred Southern blot showed a better signal due to the presence of more DNA, but it also had a few band artifacts (Figure 38A). Despite poor transfer of larger bands and faint signal with the Trans-Blot Turbo method, the shorter telomere bands were uninterrupted and could be identified more clearly (Figure 38B). The lowest bands in the XhoI-digested DNA are the most commonly used fragments to determine telomere lengths in this type of analysis. The telomeres in *yku70* cells are shorter than wildtype (compare the lowest band in lane 3 to the lowest band in lane 1 in part B).

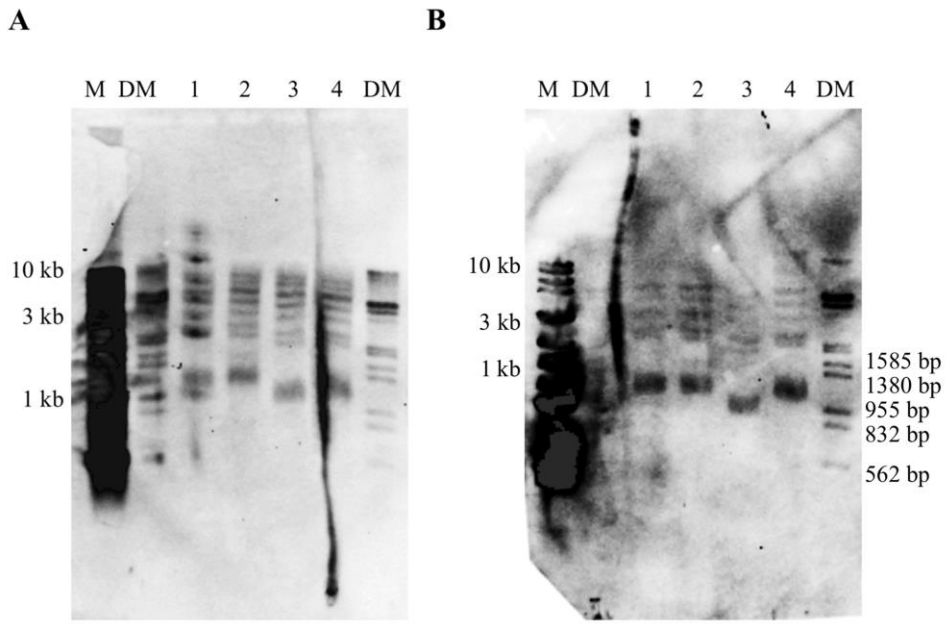

Figure 38. A side-by-side comparison of two Southern blots. Both Southern blots were treated identically except for the transfer method: capillary transfer (A) and Trans-Blot Turbo transfer method (B). Lane 3, wildtype cells; lane 2, *sgs1* mutant; lane 3, *yku70* cells; lane 4, *sgs1 yku70* double mutant.

The distance traveled by the lowest bands in each sample were measured and compared to the bands of known size within the DIG-labeled standards (Figure 39). Graphical analysis allowed calculation of the band sizes, and they were 1,194 bp, 1,202 bp, 1,003 bp, and 1103 bp for wildtype, *sgs1*, *yku70*, and *sgs1 yku70*, respectively. The sizes of the telomere fragments in WT cells are consistent with previous data from the Lewis laboratory, adding confidence to these initial results (4). The results indicate that *sgs1 yku70* double mutants have shorter telomeres than wildtype or *sgs1* cells, but longer telomeres than *yku70* single mutants. These initial experiments demonstrate that the method has room for improvement in transfer of larger bands, which may be increased by longer transfer times at a higher voltage and resolution needs to be improved also. Both images together provide a preliminary indication that inactivation of *SGS1* partially

alleviates the short telomere phenotype of *yku70* mutants when cells are grown at 30°C and could be influencing the temperature sensitivity and survival of *yku70* cells, in part, by increasing telomere lengths, though it may also affect degradation by Exo1.

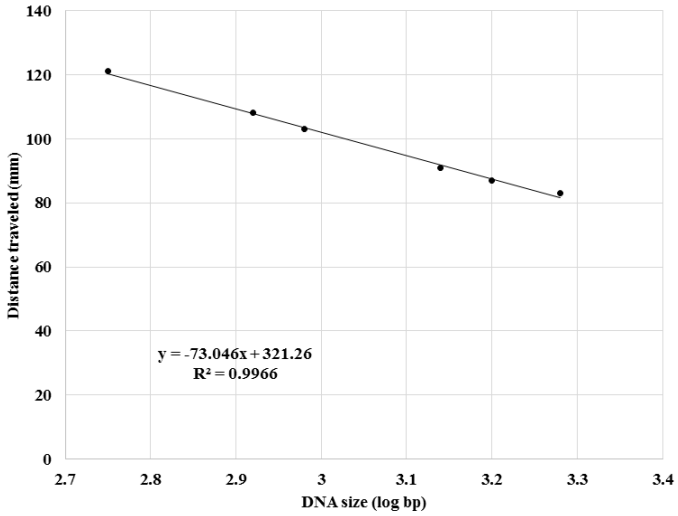

Figure 39. Plot of distance traveled on gel vs. Size of DNA fragment. Statistical analysis of the DIG standard DNA generated a formula that could be used to calculate the size of the telomeres from the distances traveled.

Table 5. List of *yku* deletion mutants and their temperature sensitivities at 37°C. Effect of co-inactivation of genes affecting DNA repair, telomere length, telomere stability, chromatin remodeling, or DNA damage and spindle checkpoints on survival of *yku70* cells at 37°C.

Double mutant	Suppression of *yku* 37°C ts phenotype	Reference
Major HR and NHEJ components		
yku70 rad50	No	36, 58, 69
yku70 mre11	No	36, 58, 69
yku70 rad52	No	36, 58, 69
yku70 dnl4	No	36, 58, 69
yku70 yku80	No	36, 58, 69
DSB repair-associated proteins		
yku70 sgs1	**Yes**	This work
yku70 exo1	**Yes**	36, 58, 69
yku70 rad1	No	36, 58, 69
yku70 rad10	No	36, 58, 69
yku70 mms4	No	36, 58, 69
yku70 mus81	No	36, 58, 69
yku70 sae2	No	36, 58, 69
yku70 srs2	No	36, 58, 69
Telomere and telomere-associated proteins		
yku70 rif1[a]	**Yes**	36, 58, 69
yku70 rif2[a]	No	36, 58, 69
yku70 sir2	No	36, 58, 69
yku70 sir3	No	36, 58, 69
yku70 sir4	No	36, 58, 69
yku70 mlp1	No	36, 58, 69
yku70 mlp2	No	36, 58, 69
yku70 mlp1 mlp2	No	36, 58, 69
yku70 pif1[a]	No	36, 46, 58, 69
DNA damage checkpoint-associated proteins		
yku70 rad9	**Yes**	36
yku70 chk1	**Yes**	36, this work
yku70 mec1	**Yes**	36
yku70 mec3[b]	No/ Yes	36, this work
yku70 rad17	No	36, 46, 57
yku70 rad24	No	36, 46, 58, 69
yku70 ddc1	No	36, this work
yku70 dun1	No	36, this work

Table 5 Continued.

Spindle checkpoint proteins		
yku70 mad2	**Yes**	36, this work
yku70 bub2	No	36
Chromatin assembly proteins		
yku70 rsc1	**Yes**	This work
yku70 rsc2	**Yes**	This work
Very Long Telomere (VLT) mutants[c]		
yku70 rsa1	**Yes**	This work
yku70 srb8	**Yes**	This work
yku70 htl1[d]	No/Yes	57
yku70 rps17a	No	This work
yku70 ard1	No	57
yku70 elg1	No	57
yku70 rad27	No	57
Other		
yku70 cgi121	**Yes**	59, this work

[a] *pif1*, *rif1*, and *rif2* mutants are also VLT mutants.
[b] In this study *yku70 mec3* cells grew at 37°C. However, a previous study by Maringele *et al*. reported that these double mutants did not grow at 37°C (36).
[c] Askree *et al.* and Gatbonton *et al.* identified 10 genes that caused cells to have very long telomeres when inactivated (49, 50). These genes include *ARD1, ELG1*, *HTL1*, *PIF1, RAD27, RIF1, RIF2, RPS17A, RSA1*, and *SRB8.*
[d] *htl1 yku70* mutants consistently exhibited slightly increased survival at 37°C relative to *yku70* mutants (57).

CHAPTER IV

SUMMARY AND CONCLUSIONS

Many genes have been knocked out in *yku* strains in an effort to identify DNA metabolic pathways responsible for the telomere instability and death of these mutants at high temperatures. *yku* double mutants created and tested in the current project and in previous studies are listed in Table 5. Overall, this study identified 5 new genes that rescue *yku70* cells at 37^{o}C when inactivated (*RSA1*, *SRB8*, *RSC1*, *RSC2*, and *SGS1*). Suppression by 4 other genes was confirmed in this work (*RAD9*, *CHK1*, *MAD2*, and *CGI121*).

Among three VLT genes tested in this project, two of them suppressed the ts phenotype of *yku70* cells. These genes were *SRB8* and *RSA1*, which are involved in the RNA polymerase II mediator complex and ribosomal assembly, respectively. It is not clear how defects in these processes might reduce Exo1 degradation and shortening of telomeres in *yku70* cells. The fact that these mutants have very long telomeres, though the mechanism is unknown, may help to compensate for the shortening process that occurs at 37^{o}C. It is possible that these mutants will still die at 37^{o}C, but the process takes much longer. A single pronging therefore may not involve a long enough time of growth for the shortening telomeres to reach a critical short length that leads to cell death. In future experiments, colonies grown at 37^{o}C could be picked and restreaked again and again to 37^{o}C to see if they eventually die.

T-loops and G-quadruplex structures are believed to exist in the telomere regions. Inactivation of the chromatin assembly genes *RSC1* and *RSC2* might cause changes in these structures and the nucleosomes bound to them that affect access by Exo1. The mechanism by which inactivation of DNA damage checkpoint response genes (*CHK1, DDC1, DUN1*, and *MEC3*) or spindle checkpoint genes (*MAD2*) influences survival of *yku70* mutants at 37ºC is not well understood. In particular, it is unclear how inactivation of some checkpoint genes can suppress killing of *yku70* cells, but not others. Future work will also need to be done to address the contradictory results with *MEC3*.

C*GI121* inactivation rescued survival of *yku70* cells at 37ºC and it has been shown to influence the amount of ssDNA at the telomere ends and add stability to the telomere regions. However, little is known about the function of *CGI121* within the KEOPS kinase complex or its role in affecting the overall stability of telomere ends and no specific substrate for this kinase has been identified. Survival of *sgs1 yku70* mutants was most interesting, as they did not grow in a way that had been seen before. *sgs1 yku70* cells had high frequencies of paps, suggesting there may be an epigenetic factor associated with survival at 37ºC. Hardy *et al*. have shown that *sgs1* cell cultures develop suppressors at high frequency, and this may be a related process (68). Sgs1 protein forms a complex with the Dna2 nuclease/helicase and influences DSB repair and telomere replication, but its precise role at the telomeres remains unclear.

Increased cellular levels of the catalytic subunit of telomerase, Est2, had previously been shown to rescue growth of *yku70* cells at elevated temperatures. Many of the double mutants tested in the current experiments increased survival at 37ºC so well that effects of *EST2* or *TLC1* overexpression could not be measured in our pronging

assays. Two strains produced results that had not been seen before: supraphysiological levels of Est2 in *rsc2 yku70* cells was inhibitory to growth at both 30ºC and 37ºC. Also, *sgs1 yku70* cells showed no difference in growth at 30ºC and 37ºC with *EST2* overexpression. This result indicates that some part of the telomere stabilization process is dependent on the presence of Sgs1.

Overexpression of *TLC1* RNA in each of the double mutants gave similar results to those seen with *EST2* overexpression. Our laboratory has previously observed that only overexpression of Est2 protein or *TLC1* RNA can rescue *yku70* cells, i.e., the other telomerase subunits Est1 and Est3 do not work (36, 46). The nearly identical effects seen with *TLC1* RNA and Est2 in the current study suggest that they may stabilize telomeres by the same mechanism. This mechanism is likely to involve stabilization and protection of the telomeres rather than DNA replication to increase telomere lengths (36, 57, 58).

Southern blot analysis demonstrated that inactivation of *SGS1* partially alleviates the short telomere phenotype of *yku70* mutants when cells are grown at 30°C and could be influencing the temperature sensitivity and survival of *yku70* cells, in part, by increasing telomere lengths. *yku70* mutants lacking *sgs1* were partially rescued at 37°C, were not influenced by telomerase overexpression, and possessed longer telomeres than *yku70* single mutant cells. These results and work by Huang *et al.* showing that *SGS1* is required for telomere elongation in the absence of telomerase suggests that *SGS1* may be involved in a telomerase-independent pathway for the rescue of telomere lengths (64).

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