STABILIZATION OF THE ENDS OF CHROMOSOMES IN YEAST YKU70 MUTANTS: ROLE OF TELOMERASE COMPLEX SUBUNITS AND TELOMERE-ASSOCIATED PROTEINS

by

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CHAPTER I

INTRODUCTION

The nucleus of eukaryotic cells contains hereditary material in the form of deoxyribonucleic acid (DNA) that guides the development of the organism. Information is encoded in DNA through the use of four nitrogenous bases: adenine, guanine, cytosine, and thymine. Attached to each base is a sugar-phosphate backbone, creating a nucleotide. These nucleotides are joined together at the sugar-phosphate backbone creating a DNA strand. The DNA forms double-stranded molecules that are strengthened through hydrogen bonds between two strands at the nitrogenous bases. Hydrogen bonds occur between two complementary bases (adenine to thymine and cytosine to guanine), creating a molecule with anti-parallel complementary strands. The two DNA strands form into a helical structure that may exist as a linear polymer or a circular form.

Cellular DNA is constantly exposed to damage from environmental and chemical sources, forming lesions and breaks within the strands. Ionizing radiation, chemicals, free radicals, and endonucleases are all damaging agents (1). Each different agent will induce a different set of of lesions in DNA, such as damaged bases, altered sugars, cross-linking, single-strand breaks, or double-strand breaks (DSBs) (2).

Restriction endonucleases are enzymes that catalyze the hydrolysis of phosphodiester bonds in the backbone of DNA at specific sequences, creating a DSB. Enzyme recognition sites are usually palindromic, and range from 4 to 8 bp (3). Four main types of restriction endonucleases exist that are categorized based on structure,

recognition sequence, and position of cleavage near the recognition sequence (3, 4). Type II enzymes are the largest class and the most well-studied. These enzymes produce DSBs with either single-stranded DNA overhangs (3' or 5') or blunt DNA ends. Single-stranded DNA overhangs can be created by two mechanisms. Recognition from the major groove leaves a 5' overhang, while recognition from the minor groove leaves a 3' overhang or blunt ends (4, 5).

EcoRI is a type II restriction enzyme that recognizes the sequence G^AATTC. The up arrow indicates the location of the strand cleavage, in this case between the guanine and adenine. Expression of EcoRI can be tied to a galactose-regulated promoter in yeast cells to study cellular responses to and repair of DSBs *in vivo* (6-8). EcoRI reportedly only produces DSBs, unlike chemicals or radiation, which generate many other types of DNA damage. DSBs are the most detrimental of known DNA lesions. Their presence can cause mutations, loss of cell function, and death if unrepaired or improperly repaired (2-14).

Eukaryotic cells can repair DSBs with either of two highly conserved and independent pathways: nonhomologous DNA end joining (NHEJ) or homologous recombination (HR) (14-15). NHEJ is less accurate than HR and is primarily used in humans, but is the secondary pathway in yeast. Errors are derived from not using a homologous chromosome as a template. Instead, three protein complexes, Ku (sometimes called Yku in yeast cells), Mrx, and DNA Ligase IV bind to the DSB to initiate repair, as seen in Figure 1. The Ku complex, composed of Yku70p and Yku80p, binds to the damaged ends of DNA, protecting them from degradation by nucleases. The lowercase p after Yku70 and Yku80 indicates that the proteins are being referenced. Mrx

tethers the two DNA ends together, using Mre11p, Rad 50p, and Xrs2p proteins. Once together, DNA Ligase IV, comprised of Dnl4p, Lif1p, and Nej1p, covalently links the phosphate backbones together, repairing the DNA strands. It is likely that other proteins, not yet identified, are also involved in this pathway.

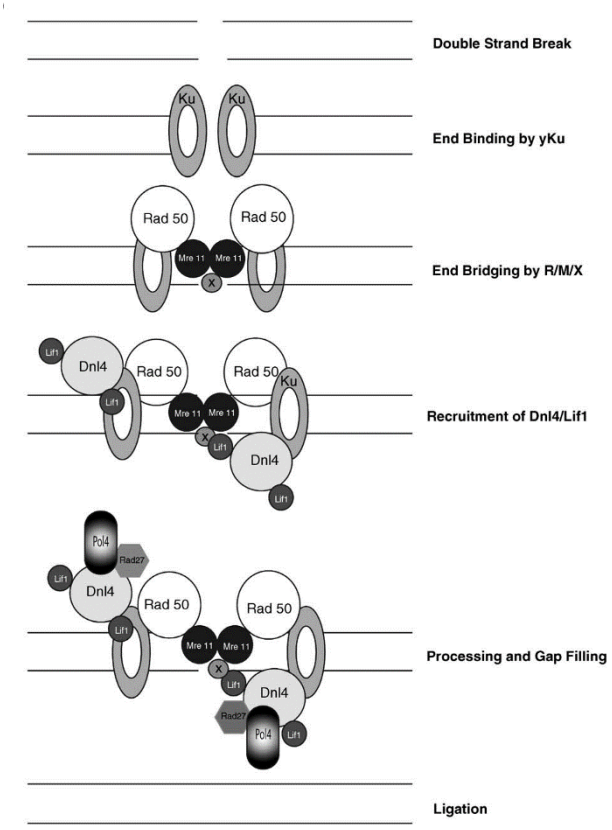

Figure **1**: **Schematic of nonhomologous end-joining repair pathway in *Saccharomyces cerevisiae* (budding yeast)** (3).

Homologous recombination uses homologous chromosomes (chromosomes with similar sequences) and multiple protein complexes to repair DSBs. This method is more time and energy intensive, but is nearly error free (16, 17). Several proteins are unique to HR including, Rad51p, Rad52p, Rad54p, Rad55p, Rad57p, and Rad59p, as seen in Figure 2 (16). Initiation of HR occurs when Mrx and other proteins such as, Exo1p, Sae2p, Sgs1p, and Dna2p create long 3' single-stranded overhangs on both sides of the

DNA break site. Single-stranded DNA (ssDNA) binding protein complex Rpa and the Rad51p, Rad52p, Rad54p, Rad55p, Rad57p and Rad59p proteins are then used to search for homologous regions, strand invasion, and information exchange. Once exchange is complete, DNA replication, branch migration, nucleosome assembly, and resolution create a repaired DNA double helix. HR efficiency is affected by several proteins from other processes, including DNA damage checkpoint response, nucleosome remodeling, sister chromatid cohesion, chromatin reassembly, and nuclease end-processing proteins (16, 17).

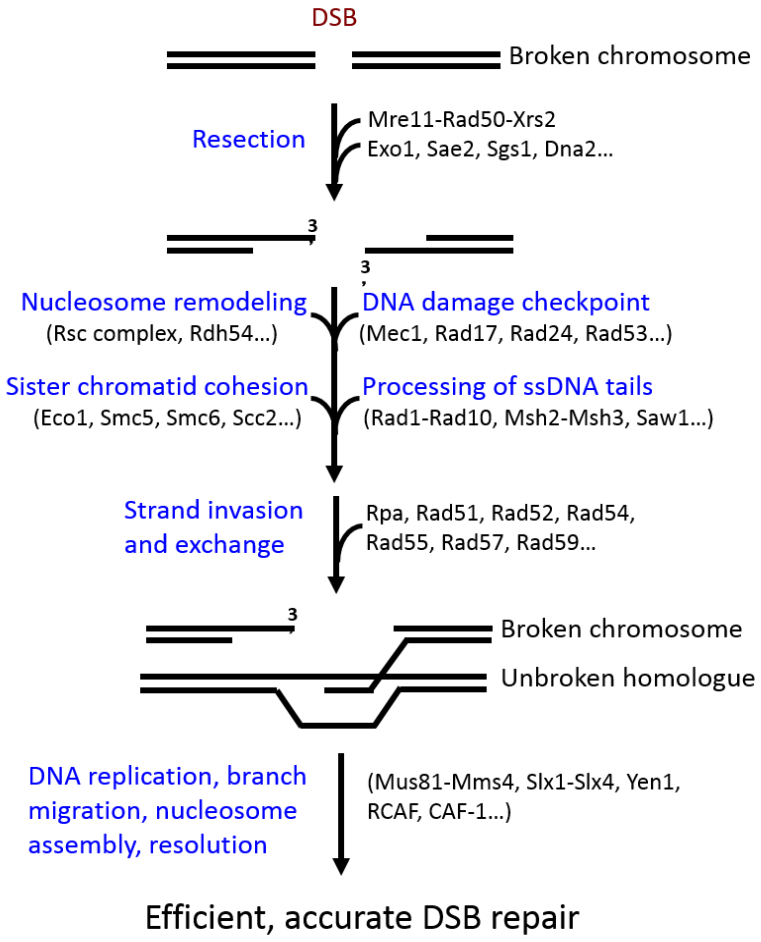

Figure **2**: **The homologous recombination repair pathway in *Saccharomyces cerevisiae* (budding yeast)** (18).

The Ku complex is required for NHEJ, and can influence the efficiency of HR, though it is not directly involved in the latter pathway. This well conserved complex is formed from two proteins, Ku70 and Ku80 (called Yku70p andYku80p in yeast cells). Yku70p and Yku80p form a dimeric, ring-shaped structure that possesses high affinity for the ends of linear double-stranded DNA, such as DSBs or chromosome ends. They are responsible for the stabilization of chromosomes and maintenance of genome integrity (19-22).

A major function of the Ku complex is the rejoining of broken ends at DSB sites within chromosomes. NHEJ is initiated by binding of the Ku complex to the DSB site. Once bound to the linear ends, Ku will protect the vulnerable ends from degradation or modification by nucleases or other types of enzymes and initiate recruitment of associated protein complexes for repair. As the initiating factor of NHEJ, Ku will coordinate repair pathway selection. This is important during G_1 phase in wild type cells to suppress recombinational repair and activate NHEJ (23-28). Ku also has limited intrinsic dRP/AP lyase activity, which may be used to edit damaged bases on the ends of DNA during the repair process (29, 30). Other activities assigned to Ku are deubiquitinase, DNA helicase, and ATPase activities, but these functions are not fully verified (31-34).

When bound to chromosome ends, Ku forms strong associations with telomeric DNA. In budding yeast (*Saccharomyces cerevisiae*) Ku protects the exposed ends from nuclease degradation, aberrant recombination, and end-to-end fusions; Ku also recruits he telomerase DNA polymerase complex, and tethers the chromosome ends to nuclear pore complexes (32-35).

Knocking out either *YKU70* or *YKU80*, produces yeast mutants that display several phenotypes. Assays can measure different outcomes such as the specific efficiency of DSB repair by the NHEJ pathway, strain-dependent sensitivity to DNA damaging agents, and modified rates of DNA recombination (31, 35, 36-41). Growth rates of *yku* mutants at 30°C or below is similar to wildtype cells, but telomeres are shorter than normal, though stable. The mutants are characterized by the presence of long single-stranded tails at the ends of telomere DNA, reduced telomere tethering, and reduced transcriptional silencing (19, 20, 31, 42). Growth at 37°C causes the mutants to undergo a DNA damage-induced cell cycle checkpoint response resulting in arrest in G_2 phase and loss of viability. Elevated temperature leads to unstable protein cap structures and telomeres shorten at a rate of approximately 10-15 bp per generation (43). The cell cycle checkpoint arrest is dependent upon a suite of DNA damage checkpoint response genes including, *RAD9*, *RAD24*, *RAD53*, *MEC1*, and *CHK1* (43, 44). Higher growth temperatures also increase the amount of single-stranded telomeric DNA, possibly the primary trigger for the cell cycle checkpoint response (44-46). Deletion of *EXO1*, a 5' to 3'exonuclease, can suppress the checkpoint response and loss of viability in *yku* mutants. Evidence suggest that Exo1p gains access to and degrades chromosome ends whenever the telomere cap structure is disturbed (47-48). Other genes with limited alleviation of the checkpoint response when inactivated in *yku70* mutants are *RIF1*, a negative regulator of DNA synthesis by telomerase, and *MAD2*, a spindle checkpoint gene (45-47).

The core telomerase complex of *S. cerevisiae* is composed of three proteins, Est1p, Est2p, and Est3p, and one RNA component encoded by *TLC1*, as seen in Figure 3. Est2p is the catalytic subunit with RNA-dependent DNA polymerase activity, using

TLC1 RNA as a guide to synthesize longer telomeres (48-51). When *EST2* or *TLC1* RNA is overexpressed in *yku* mutants, better survival at 37°C is seen, denoting rescue of the temperature sensitivity (43, 52, 53). Rescue has also been seen with *EST1* overexpression, but the literature is conflicting on the effectiveness of the rescue by this subunit (52-53). Another gene that a past study has identified to partially suppress the killing of *yku* mutants when co-inactivated with *YKU70* is *RTT103* creating *yku70 rtt103* double mutants, *RTT103* is a gene with an unclear role in DNA repair and transcription termination (54).

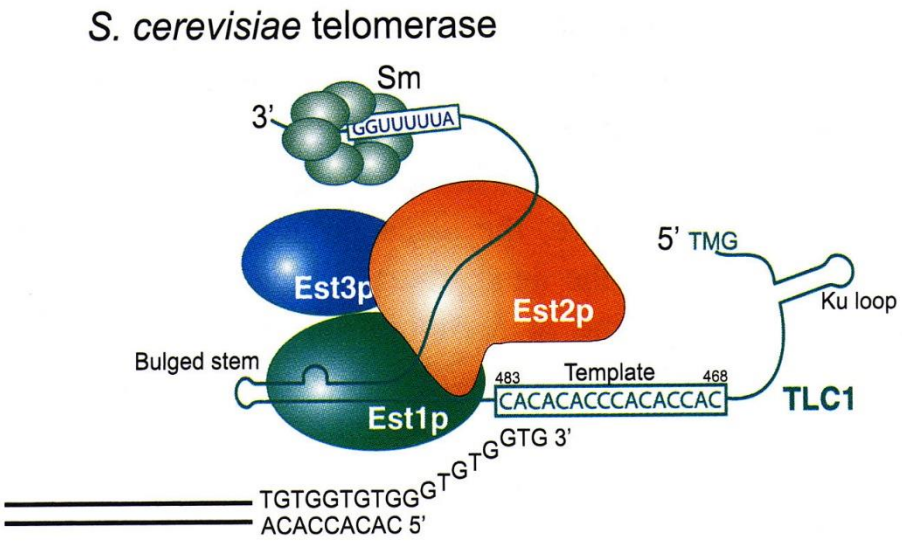

Figure **3**: **Cartoon representation of the Ku complex at a telomere end with associated protein and RNA components** (55).

Two previous studies by Askree *et al.* and Gatbonton *et al.* screened diploid yeast deletion libraries to identify mutants with abnormal telomere lengths (56, 57). Askree *et al.* identified six mutants possessing very long telomeres compared to normal cells. Gatbonton *et al*. classified seven mutants to have exceptionally long telomeres. Among

these two studies, both identified r*if1, rif2,* and p*if1* as mutants with long telomeres. In addition to e*lg1, htl1,* and *rps17A* were identified by Askree *et al*., while Gatbonton observed that r*ad27, srb8, ard1,* and r*sa1,* mutants had long telomeres.

Srb8p protein encode for a subunit of RNA polymerase II mediator complex. This protein has been shown to have involvement in transcriptional regulation due to its association with RNA polymerase II holoenzyme, it has also be implied in glucose repression (58, 59, 60). Ard1 protein is a subunit of the N-terminal acetyltransferase NatA. *ARD1* is involved in telomere silencing, cell cycle progression, heat-shock resistance, mating differencation, and sporulation. Also, of note levels of the protein increase during DNA replication stress events (61, 62, 63). Rad27p is a 5' to 3' exonuclease, a flap endonuclease required for processing and maturation of Okazaki fragments and base excision repair. Rad27p is normally found in the nucleus but during hypoxia it relocates to the cytosol (64, 65, 66, 67). *RSA1* encode a protein involved in the assembly of the large ribosomal subunit in yeast (60S) (68).

Rsc1 is a subunit of the RSC chromatin remodeling complex, effecting DNA structure. It also is required for the activation of genes required for late mid to late sporulation. During the yeast genome replication event a paralog of *RSC1* was created called Rsc2. (69, 70, 71). Hsp82 is a redundant chaperone of Hsp90 that is required for pheromone signaling, mitochondrial preprotein delivery, and negative regulation of HsFlp. Hsp82p has been show to promote binding of telomerase to DNA, nucleotide addition, also during times of DNA replication stress protein levels increase. The paralog of HSP82 is HSC82 (63, 69,72-75). RAD9 is a DNA damage checkpoint protein that is

required for cell cycle arrest and activation of post replication checkpoints, including RAD59. RAD9 is also the target of hyperphosphorylation by MEC1 and TEL (76-79).

ELG1 is required for maintenance of genome stability, it has also been linked to the HR pathway and checkpoint response through Rad24p and CTF18p. Elg1p is homologous with sliding clamp loading proteins, specifically replication factor C, the literature states it loads an alternative sliding clamp for the checkpoint response (80, 81). Htl1p is also part of the RSC complex, it coordinates with protein kinase C to regulate G_2/M phase transition (82). Rps17Ap is the ribosomal protein 51 of the small (40s) subunit and has a paralog, Pps17Bp (83).

Adenine (Ade) is a purine base in DNA, RNA and an important cellular messenger in *Saccharomyces cerevisiae* and other organisms. Biosynthesis of Ade in in *S. cerevisiae* is a *de novo* synthesis pathway composed of twelve step process to create adenine monophosphate (AMP) (Figure 4). The Ade synthesis pathway can also be used to create guanine by deviation at the tenth step to create guanine monophosphate, utilizing a different modification to inosine monophosphate (IMP) to create the different purine base. Two enzymes in Ade pathway can be disrupted to create strains that are unable to synthetize Ade *de novo* or from salvaging other components. The most common genes that are disrupted are *ADE1* and *ADE2.* These genes are involved in regulation of the pathway and, when absent are responsible for creating a buildup of the red pigment P-ribosylaminoimidazole (AIR), giving *ade1* and *ade2* mutants a red coloration. The disruption of the pathway occurs after the oxidation of AIR (Figure 4) (84, 85). This red coloration can be used to screen or assay mutant yeast cells since wildtype cells are white or cream colored compared to the red or pink color of *ade1* or

ade2 mutant cells (85). This selectable marker can be powerful due to this visual attribute. For example, chromosome loss has been monitored by creating diploid strains with wildtype *ADE2* on one copy of chromosome XV and *ade2* on the other homologous chromosome (86). Cells that have spontaneously lost the chromosome containing wildtype *ADE2* go from an *ADE2/ade2* heterozygous genotype to being only *ade2.* Such cells grow up to form red or pink colonies on the plates, providing an easy way to measure chromosome loss. *ADE2* genes have also been engineered into the telomerase of some yeast chromosomes and used to monitor change in the transcription and stability in telomeric repeat sequences (87).

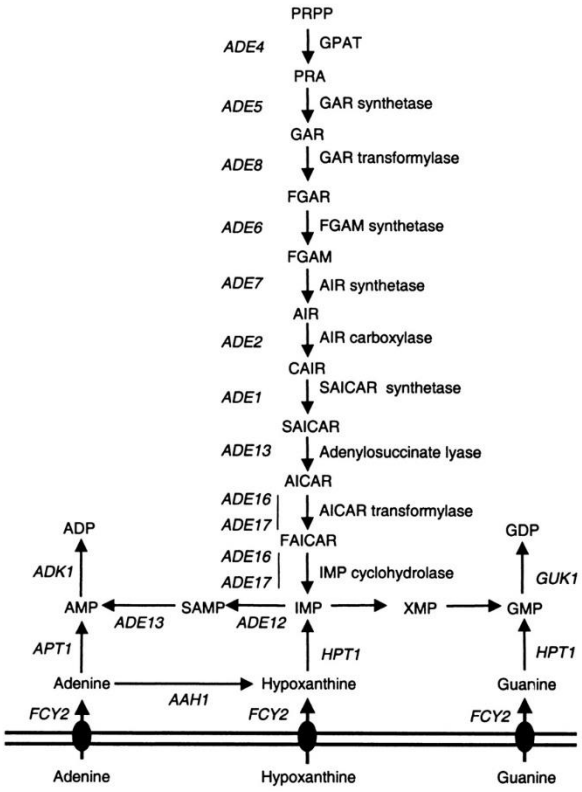

Figure **4**: **The full purine biosynthesis pathway with all required enzymes and intermediates** (88).

The mechanism by which overexpressed levels of Est2p DNA polymerase stabilize telomeres and rescue the telomere instability of *yku70* and *yku80* mutants at elevated temperatures is unknown. This study was conducted to characterize the mechanism of action of *EST2* overexpression and to identify other genes affecting telomere stability and survival in *yku70* and *yku80* mutants.

CHAPTER II

MATERIALS AND METHODS

Materials

General Reagents

Agarose, dithiothreitol (DTT), and 5-flurooorotic acid (5-FOA) were purchased from Gold Biotechnology (St. Louis, MO). Ethylenediaminetetraacetic acid (EDTA) and sodium hydroxide (NaOH) were from EMD Chemicals, Inc. (Darmstadt, Germany). Dimethyl sulfoxide (DMSO), lithium acetate (LiAc), and polyethylene glycol (PEG 4000) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS) was purchased from Mallinckrodt-Baker, Inc. (Paris, KY). The sonicated salmon sperm carrier DNA was obtained from Stratagene (La Jolla, CA). Ethidium bromide (EtBr) was obtained from Shelton Scientific, Inc. (Shelton, CT). Sodium chloride was purchased from Fisher Scientific (Fair Lawn, NJ).

Enzymes and PCR Reagents

RNase A was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Restriction enzymes, dNTPs and Taq DNA polymerase were purchased from New England Biolabs (Beverly, MA). ExTaq DNA polymerase was obtained from Takara (Madison, WI). Ku70 test primers were created by Integrated DNA Technologies (IDT) (Coralville, IO).

Bacteriological and Yeast Media

All amino acids, nitrogenous bases, and ampicillin (Amp) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). D-(+)-glucose, soy peptone, and yeast nitrogen base dropout media were made by Amresco (Solon, OH). Bacto yeast extract was created by Becton, Dickinson, and Company (Sparks, MD). Ammonium sulfate and Tris base were obtained from VWR International (West Chester, PA). Agar, molecular biology grade was obtained from Teknova (Hollister, CA).

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 bases without amino acids, 0.5% ammonium sulfate, 2% D-(+)-glucose, 2% agar, and all essential amino acids minus amino acids used for plasmid selection. Synthetic media containing 2% galactose was used to express plasmid-encoded genes with the galactose promoter. Selection for cells that had lost the *URA3* marker on a plasmid due to spontaneous plasmid loss was done by the addition of 5-FOA to synthetic media.

E. coli cells were grown using LB $_+$ ampicillin (amp) broth (1% bacto tryptone, 0.5% yeast extract, 0.5% NaCl, 0.01% Amp) or for sustained growth on LB + Amp plates (LB broth + Amp + 1.5% agar).

Table 1. **Strain List.** A complete list of *S. cerevisiae* strains used for this project identified by Lewis lab designation and genotype.

Strain	Genotype	Source
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	89
VL6α	*MATα ura3-52 trp1(Δ63) lys2-801 his3-Δ200 met14 ade2-101*	90
VL6-48	VL6α *Δleu2::G418^r*	90
BWG1-7a	*MATα leu2-3, 112, ura3-52, his4-519, ade1-100 (γ^R)*	91
YLKL475	VL6-48(s) *leu2*::*G418^r* Δ*trp1*-Δ1	92
YLKL477	*MATα ura3*-52 *leu2*-Δ1 *his3*-Δ200 *lys2*-801 *ade2*-101	93
YLKL566	*MATα Δade2::hisG Δhis3*-200 *leu2*-Δ0 *lys2*-Δ0 *met15*-Δ0 *trp1*-Δ63 *ura3*-Δ0	89
YLKL568	*MATα his3*-Δ1 *leu2*-Δ0 *lys2*-Δ0 *ura3*-Δ0	89
YLKL593	BY4742, *yku70Δ::HIS3*	92
YLKL808	*MATα ade2-1 can1-100 leu2-3,112 his3-11,15 ura3-1 trp1-1 RAD50$^+$*	94
YLKL868	BY4742, *sir2*Δ::*HygB yku70*Δ::*URA3*	95
YLKL869	BY4742, *rif1*Δ::*HygB yku70*Δ::*URA3*	95
YLKL870	BY4742, *rif2*Δ::*HygB yku70*Δ::*URA3*	95
YLKL871	BY4742, *sir4*Δ::*G418^r yku70*Δ::*URA3*	95
YLKL982	BY4742, *exol*Δ::*G418^r yku70*Δ::*URA3*	95
YLKL983	BY4742, *mms4*Δ::*G418^r yku70*Δ::*URA3*	95
YLKL984	BY4742, *rad1*Δ::*G418^r yku70*Δ::*URA3*	95
YLKL985	BY4742, *rad10*Δ::*G418^r yku70*Δ::*URA3*	95
YLKL986	BY4742, *mus81*Δ::*G418^r yku70*Δ::*URA3*	95
YLKL987	BY4742, *rad17*Δ::*G418^r yku70*Δ::*URA3*	95
YLKL988	BY4742, *rad24*Δ::*G418^r yku70*Δ::*URA3*	95
YLKL991	BY4742, *tlcl*Δ::*HygB^r yku70*Δ::*HIS3* + pLKL83Y (*ADH1p*::*TLC1 URA3*)	95
YLKL1555	*rsc1::G418^r yku70*Δ::*URA3*	This Study
YLKL1549	*rad27::G418^r yku70*Δ::*URA3*	This Study
YLKL1552	*htl1::G418^r yku70*Δ::*URA3*	This Study
YLKL1554	*hsp82::G418^r yku70*Δ::*URA3*	This Study
YLKL1550	*elg1::G418^r yku70*Δ::*URA3*	This Study
YLKL1551	*ard1::G418^r yku70*Δ::*URA3*	This Study
YLKL1553	*rad9::G418^r yku70*Δ::*URA3*	This Study

Table 2. **Plasmid List.** A complete list of plasmids isolated from *E. coli* and transformed into *S. cerevisiae* strains.

Plasmid	**Genotype**	**Source**
pGEM4z S-H/URA	*yku70*::*URA3* – gene disruption plasmid, Amp^r	96
pLKL64Y	*ADH1p*::*TLC1 LEU2* 2μ	97
pLKL74Y	pRS424-*GAL1p*::*TLC1* 2μ	97
pRF4-6NL	*GAL1p*::*TRP1* 2μ	98
pRF4-6NL+RIF1	*GAL1p*::*RIF1 TRP1* 2μ	98
pRF4-6NL+RIF2	*GAL1p*::*RIF2 TRP1* 2μ	98
pRF4-6NL+SIR3	*GAL1p*::*SIR3 TRP1* 2μ	98
pRF4-6NL+SIR4	*GAL1p*::*SIR4 TRP1* 2μ	98
pRS425	Vector *LEU2* 2μ	99
PRS426	Vector *URA3* 2μ	100
pVL715	*ADH1p::EST2 URA3* 2μ	100
pVL735	*ADH1p*::*EST2*-D530A *URA3* 2μ	100
pVL743	*ADH1p*::*EST2*-D670A *URA3* 2μ	100
pVL744	*ADH1p*::*EST2*-D671A *URA3* 2μ	100
pVL784	*ADH1p*::*EST1 LEU2* 2μ	100
pVL799	*TLC1p LEU2* 2μ	100
pVL999	*ADH1p*::*EST2 LEU2* 2μ	100

Methods

Gel Electrophoresis

Gel electrophoresis was performed in a Life Technologies Horizon 11-14 gel rig using 0.7 – 1.5% agarose gels in 1X TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) running buffer at voltages of ~120 -140 V. Ethidium bromide (0.5 μg/ml) was used to stain agarose gels and images were captured on either a Kodak IS440 CF imaging system with Kodak 1D imaging software or an Alpha Innotech Red Imaging system.

DNA Transformations

Yeast strains were transformed using a modified rapid lithium acetate/DMSO transformation method described by Tripp *et al.* (101). Cells were spun down and the growth media was removed. Carrier DNA (50 – 300 ng) and plasmid DNA (5-10 uL) 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 $1/10^{th}$ of DMSO was added to the mixture. This mixture was incubated for 30 min at 30°C, then at 42°C for 15 min (or 7 min for *yku70* mutants, which are temperature sensitive). The mixture was spun down, the supernatant discarded, the pellet suspended in 200 uL dH_2O and cells were plated to selective media at 30°C for colony formation.

Chromosomal DNA Purification

An optimized protocol was designed by the Lewis lab as follows. Three mL of yeast cells were pelleted, 300 uL 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 min and transferred to a water/ice bath for 5 min. 150 uL 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 21000 x g for 10 min to pellet protein and cell debris. The supernatant (containing the nucleic acid component) was mixed with 500 uL isopropanol and mixed for 30 s before being spun at 21000 x g for 2 min. The supernatant was removed and the pellet was washed with 500 uL 70% ethanol, then incubated at room temperature for 1 min before the ethanol was removed. The remaining ethanol was removed through the use of a speedvac for 10 – 15 min until dry. The pellet was suspended in 50 uL TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA), and 1 uL of 2 mg/mL RNase A was added to degrade RNA contaminates. This mixture was incubated at 37°C for 10 min. The pellet was allowed to dissolve at 4 °C then stored at -20°C.

Polymerase Chain Reaction

PCR cycles were performed using 94 °C for 30 s, annealing temperatures from 51 °C – 53 °C for 40 s, and an extension temperature of 72 °C for 120 s. PCR was conducted in an Applied Biosystems 2720 Thermal Cycler for 32 cycles.

Dilution Pronging Survival Assay

Cells were harvested into sterile ddH_2O, diluted to 1/40, then sonicated for 8 s at 2-3 watts using a Sonics Vibracell Ultrasonic Processor or 20% amplitude using a Sonics Vibracell VCX130 (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 1 X 10^7 – 4 X 10^7 cells per 220 µl. These cells were serially diluted 5-fold, 6 times across the columns of the plate. The cells were then pronged onto either YPDA or selective

synthetic plates depending on the requirements of the assay. For induction of *RIF1, RIF2, SIR3* and, *SIR3* in *yku70* mutants, cells were pronged to both synthetic plates containing glucose (control) and plates with galactose (expression). 5-FOA (1.0 g/L) was used to select for plasmids loss in Ura^- cells, to determine if cells could survive without *TLC1* RNA. Pronged cells were incubated at 30 °C as a permissive control temperature and also incubated at 37 °C or 38 °C based on the requirements of the assay. Cells were allowed to grow for 2 or 3 days and images were taken of the plates using a Canon Powershot G3 digital camera and saved as JPEG files.

Yeast Double Knockout Strain Construction

Yeast strains YLKL1553 (*rad9::G418^r yku70::URA3),* YLKL1549 (*rad27::G418^r yku70::URA3),* YLKL1552 *(htl1::G418^r yku70::URA3),* YLKL1554 *(hsp82::G418^r yku70::URA3),* YLKL1550 *(elg1::G418^r yku70::URA3),* YLKL1551 *(ard1::G418^r yku70::URA3)* and YLKL1555 (*rsc1::G418^r yku70::URA3)* were constructed by transforming single mutant cells from the single deletion yeast strain library with HindIII + EcoRI digested *YKU70* deletion plasmid pGEM4Z S-H/URA (see Table 2). Cells were spread to glucose minus uracil plates for colony growth and streaked to fresh glucose minus uracil plates for further purification. Three to four isolates were patched to fresh glucose minus uracil plates for use in chromosomal DNA isolation. PCR confirmation was used to verify the *yku70* deletion in all strains.

CHAPTER III

RESULTS AND DISCUSSION

Previous work in the Lewis lab regarding *YKU70* was completed by Brian Wasko and Cory Holland (95, 102). They focused on components that rescue the temperature sensitivity and death of *yku70* mutants at 37°C. Brian Wasko focused on overexpression of *TLC1,* the RNA component of the telomerase complex, while Cory Holland investigated overexpression of *EST2,* the polymerase component of telomerase. When y*ku70* and y*ku80* yeast strains have telomere instability, this can be visualized as slow growth at temperatures above 30°C with death occurring at 37°C. The previous work showed that high intracellular levels of *TLC1* RNA or Est2p alleviate the temperature sensitivity and death at 37°C. When overexpressed, the 5'-to-3' exonuclease Exo1p exacerbated the death of *yku70* mutants at 37°C, but when *TLC1* or *EST2* was overexpressed, Exo1p-induced lethality was suppressed.

The mechanism by which high levels of Est2p or *TLC1* RNA can stabilize telomeres and block degradation in *yku* cells is unknown. Possible mechanisms include activation of DNA repair pathways, increased DNA synthesis by telomerase to make telomeres longer, physical stabilization of the telomere cap, or a combination of these mechanisms. To determine if DNA repair by HR was involved, Brian Wasko created yeast double mutant strains with both *YKU70* and *RAD52* inactivated. *RAD52* is an essential gene in the HR pathway. If rescue of *yku70* at 37°C required DNA repair by HR, then *yku70 rad52* cells would not be rescued by overexpression of *EST2* or *TLC1*. Survival of the double mutants was found to be increased by *EST2* and *TLC1* (103), indicating that HR was not involved. Cory Holland performed similar experiments. He

created double mutants with *yku70* by knocking out genes associated with tethering of telomeres to nuclear pores (*sir2*, *sir3*, *sir4*, *mlp1*, *mlp2*) and genes known to regulate telomerase enzyme activity (*rif1* and *rif2*). In each double mutant (e.g., *yku70 sir2* cells), overexpression of *EST2* or *TLC1* still rescued the inviability of cells at 37°C. These results indicated that the proteins produced from these genes were not required for telomere stabilization in *yku70* cells. Finally, these studies also revealed that lethality and rescue was similar in *yku70* single mutant, *yku80* single mutant, and *yku70 yku80* double mutants, indicating that one subunit did not retain function in the absence of the other.

Previous work in this lab and others has shown that destabilization of the telomere cap at 37°C makes telomere DNA susceptible to degradation by Exo1, a 5' to 3' exonuclease. Inactivation of the *EXO1* gene in *yku70* cells, creating *yku70 exo1* double mutants, suppresses the killing at 37°C. The potential role of other nucleases in the degradation of telomeres in *yku70* mutants is unclear (43, 51, 95). *RAD1* and *RAD10* encode nuclease subunits that have been shown to be involved in processing of broken DNA ends (103-105). *MMS4* and *MUS81* have been shown to encode subunits for a nuclease complex that acts on DNA ends and on recombinational repair pathway intermediate structures (106, 107). In order to expand on previous work and determine if these nuclease genes affect telomere stability, each nuclease was tested to see its role in killing of *yku70* mutants at 37°C using dilution pronging survival assays.

Dilution pronging survival assays involved harvesting cells into water, followed by dilution and sonication to separate the cells. Using a hemocytometer, cells were counted to create dilutions. Aliquots were then added to a 96-well microtiter plate, where they were diluted 5-fold, 6 times, and then pronged to selective media plates (as seen in

Figure 5). Plates were incubated at 30°C or 37°C for 3-4 days to ensure complete growth. New experiments were initiated in the current project to improve quantification through more precise temperature control to ensure the killing of *yku70* mutants. In particular, 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 Petri dishes to ensure that the internal temperature was constantly maintained at 37°C.

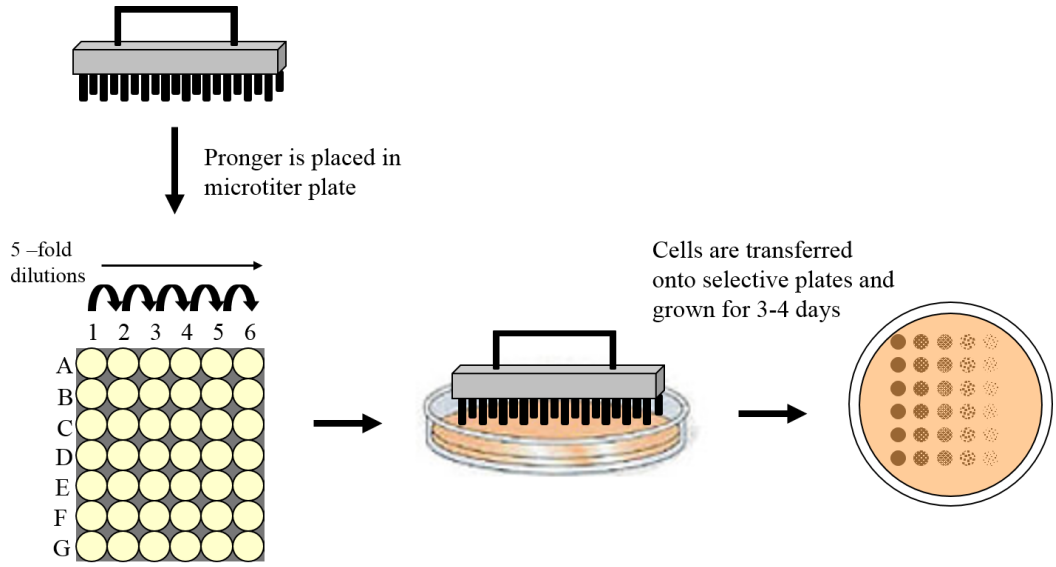

Figure 5. **Illustration of serial dilution pronging assay procedure.** Dilutions were made across the columns. The pronging instrument was placed into the microtiter plate and then onto the surface of a Petri dish, transferring cells to selective media plates, where they were grown for 3-4 days to form colonies.

The impact of inactivating RAD1, RAD10, MMS4, and MUS81 in *yku70* mutants, creating double mutants, was tested using strains created by Cory Holland (95). Double knockout strains were transformed with either pRS435 (a *LEU2* vector) or pVL999 (*ADH1p::EST2 LEU2)* (Figures 6 and 7, respectively). These experiments showed that the inactivation of *exo1* in *yku70* mutants prevented the killing effect at 37°C, i.e., *exo1 yku70* cells showed the same growth as the wildtype cells, with similar colony sizes and colony numbers (Figure 6, row 3). The *yku70* mutants with mutations in

other nucleases, *rad1, rad10, mms4,* and *mus81,* showed levels of killing identical to single *yku70* mutants (Figure 6, compare rows 4-7 to row 2). This level of killing was comparable with the *yku70* single mutant and implies that the tested nucleases do not significantly degrade the telomere ends when the cap becomes destabilized at 37°C.

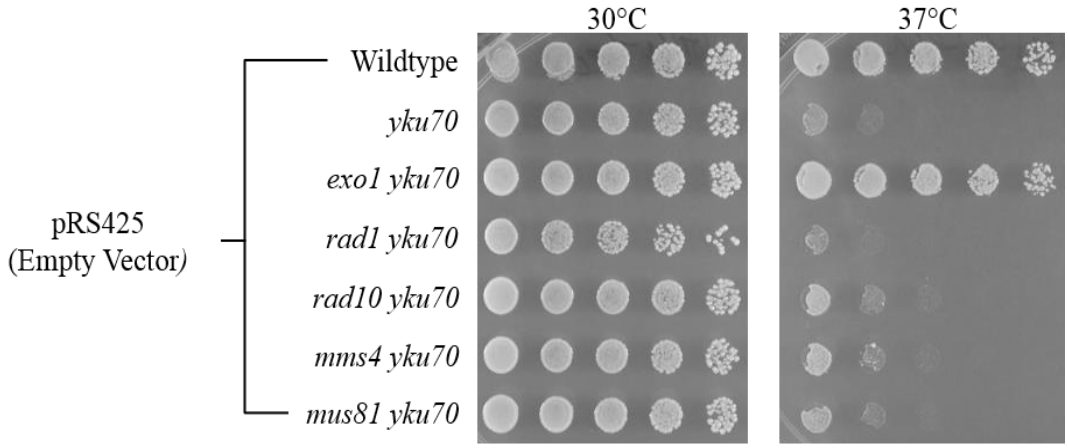

Figure 6. **Pronging survival assays with vector controls in *yku70* mutants and *yku70* nuclease double mutants.** Deletion of *RAD1, RAD10, MUS4,* or *MMS81* nuclease genes does not rescue *yku70* mutants from death at 37°C. Deletion of *EXO1* does rescue the *yku70* mutants, as seen by growth being identical to wildtype.

The same double mutants (*exo1, rad1, rad10, mms4* and *mus8* combined with *yku70*) were transformed with a plasmid containing *EST2* under the control of the *ADH1* promoter and grown at 30°C and 37°C (Figure. 7). This experiment showed rescue by *EST2* was not dependent on any of the four tested nuclease genes (*RAD1, RAD10, MMS4,* and *MUS81) (*Figure 7, compare the strong growth in rows 5-8 with the killing seen in row 2). If any of the tested nuclease double mutants showed killing similar to the *yku70* single mutant, it would imply that the nuclease assisted in *EST2* rescue of temperature

induced killing, but this was not shown in any other tested nucleases showing they are not required for rescue.

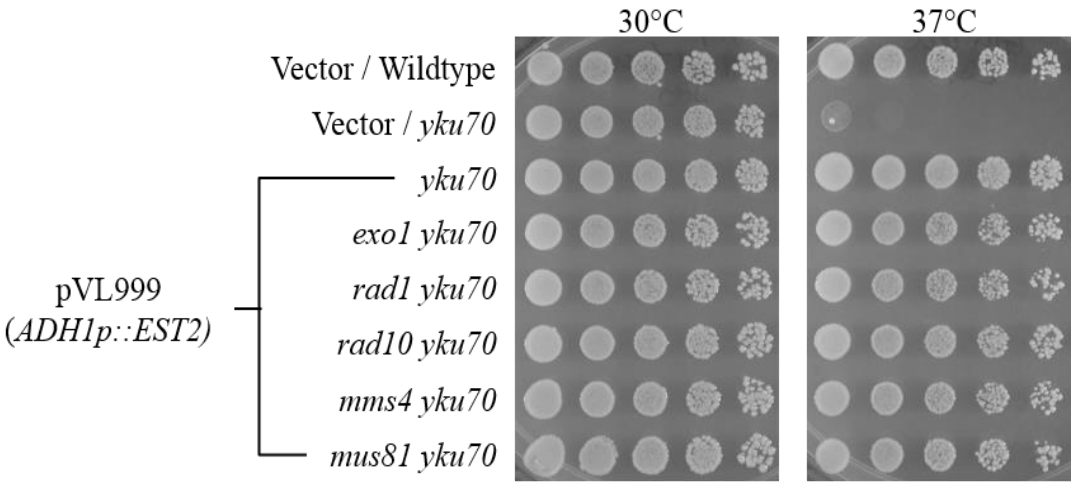

Figure 7. **Pronging survival assay with overexpression of *EST2* in *yku70* single mutants and *yku70* nuclease double mutants.** Overexpression of *EST2* in nuclease double mutants rescues survival at 37°C.

When shifted to 37°C, *yku70* mutant cells arrest growth at a cell cycle checkpoint, in G_2 phase. This arrest is known to be dependent on DNA damage checkpoint genes like *RAD17, RAD24* and *RAD9* (43, 44). The dependence of rescue by *EST2* on two checkpoint genes, *RAD17 and RAD24,* was tested next. Single mutants (*yku70*) and double mutants (*rad17 yku70* and *rad24 yku70*) were transformed with empty vector (pRS425) or an *EST2* overexpression plasmid (pVL999) as before and growth was tested at 30°C and 37°C. The results from this experiment showed overexpression of *EST2* rescued temperature sensitive killing in both the *rad17* and *rad24* double mutants (Figure 8, compare row 1 vs. 2 and 3 vs. 4). The amount of growth in the *EST2* overexpressed

double mutants at elevated temperature indicates that the tested checkpoint genes (*RAD17* and *RAD24)* are not required for the rescue of *yku70* cells.

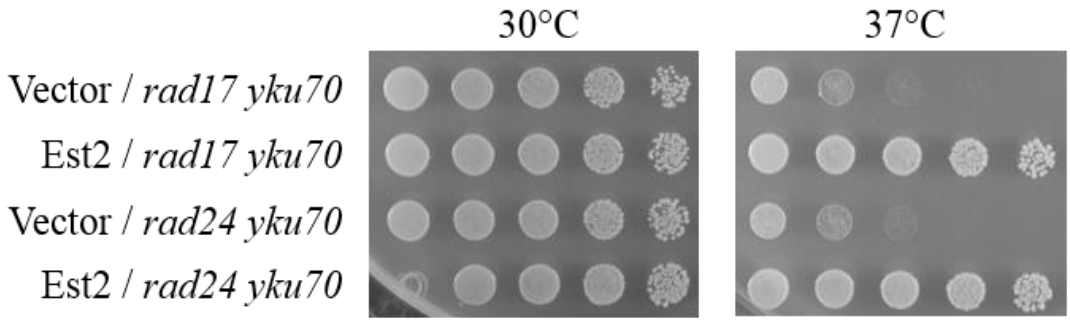

Figure 8. **Pronging survival assays with overexpression of *EST2* in *yku70* checkpoint double mutants.** Overexpression of *EST2* rescues both double mutants at elevated temperatures.

Active and functional telomerase complex requires the Est2p (polymerase), Est1p, and Est3p proteins and the template RNA *TLC1*. In order to determine if rescue by *EST2* is dependent on an active and functional telomerase complex, an experiment was designed to remove *TLC1* RNA from the complex. Yeast strain YLKL991 was ideal for this experiment due to both *YKU70* and *TLC1* being disabled on the chromosome, but cells remain viable due to the presence of the plasmid pLKL83Y *(URA3 ADH1p::TLC1*) expressing *TLC1* RNA constitutively. *tlc1* cells that do not contain a plasmid with the wildtype *TLC1* gene cannot make a functional telomerase enzyme. Without telomerase, the cells experience progressive telomere shortening and can grow for only 60-70 cell cycles before stopping growth and eventually dying. The *URA3* marker present in pLKL83Y allows the use of 5-FOA to select for cells that have lost the plasmid, or selecting for cells that have switched their genotype from *yku70 TLC1*$^{+}$ to *yku70 tlc1*$^{-}$ due to loss of pLKL83Y (Figure 9). 5-FOA is a chemical analog of an intermediate in the

uracil biosynthesis pathway called orotodine monophosphate. When cells are exposed to 5-FOA and the Ura3p enzyme is active, the 5-FOA enters the uracil synthesis pathway and is acted upon by Ura3p, creating a buildup of a toxic byproduct, 5-flurouracil (5-FU), leading to cellular death (Figure 10). Two isolates of YLKL991 were tested as biological duplicates to ensure verifiable and repeatable results. The two isolates of LKL991 were transformed with pRS425 and pVL999. These plasmids used a *LEU2* metabolic marker.

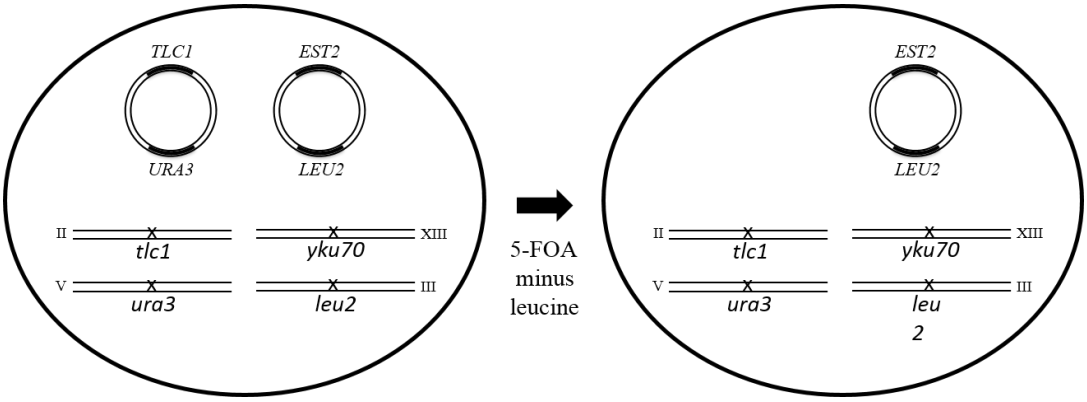

Figure 9. **Illustration of *yku70 tlc1* mutants containing a *TLC1* plasmid before and after exposure to 5-FOA.** The cells start with two plasmids for expression of *EST2* (pVL999) and *TLC1* (pLKL83Y) to maintain viability. After exposure to 5-FOA, the fraction of cells that have lost the *URA3* plasmid survive and form colonies, changing the genotype from *yku70* to *yku70 tlc1*.

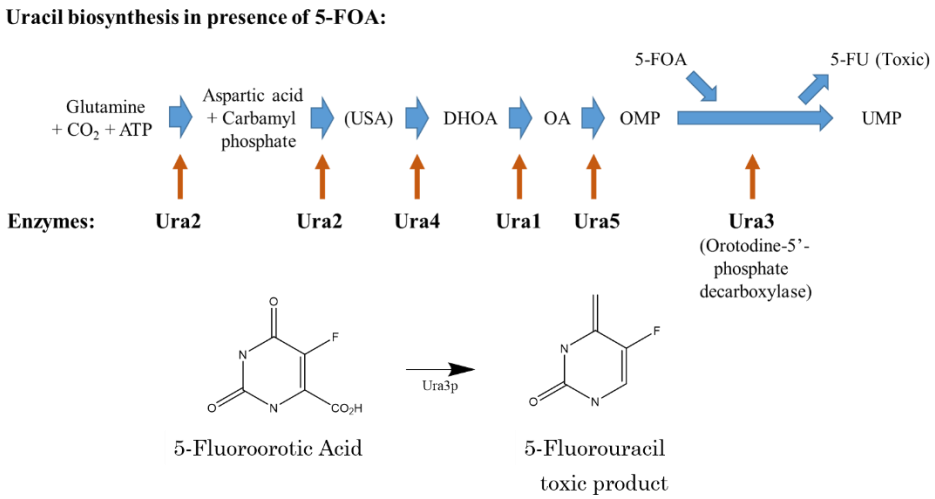

Figure 10. **The uracil biosynthesis pathway in the presence of 5-FOA.** When cells are exposed to 5-FOA, it is shuttled into the uracil biosynthesis pathway, and acted on by Ura3p creating 5-FU, a toxic product. Cells without Ura3p are not killed by the accumulation of 5-FU.

Cells were grown on both glucose minus Leu (without 5-FOA) and glucose minus Leu media with 5-FOA. The minus leucine plates were used to maintain selection pressure on the *EST2* overexpressing plasmid, so that all cells that grew contained the plasmid. There was no selective pressure on the *TLC1* plasmid, allowing the cells to lose the plasmid without consequences (Figure 11). On the Glu-Leu plates the cells are expressing both *TLC1* and *EST2*, but when grown on 5-FOA only the cells that have spontaneously lost the *TLC1* plasmid survive, leaving cells with only the *EST2* overexpression plasmid.

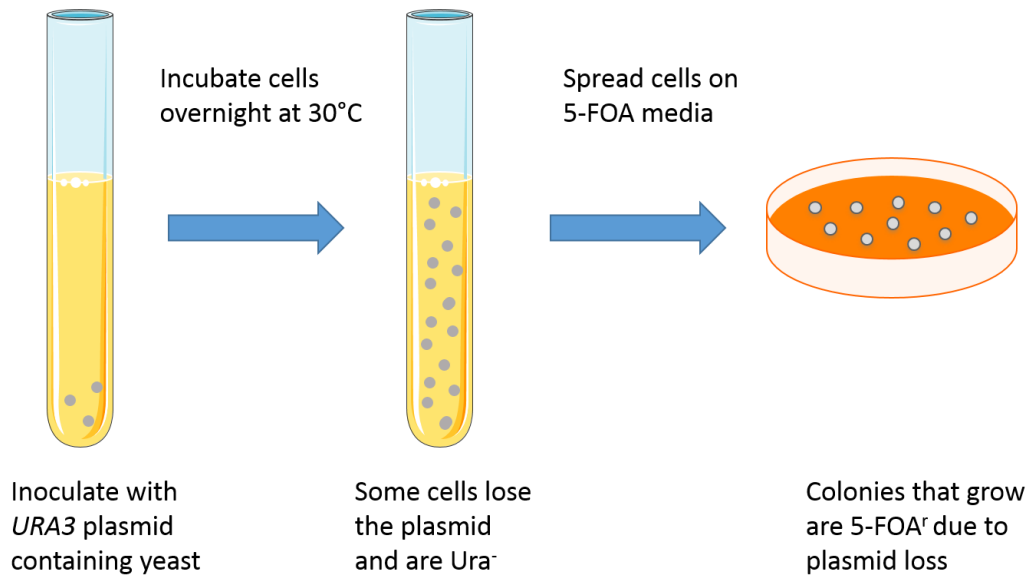

Figure 11. **Illustration of *URA3* plasmid loss leading to growth on 5-FOA media.** Yeast cells containing a *URA3* plasmid will spontaneously lose the plasmid when no selective pressure is applied. When these cells are plated to 5-FOA media, the cells that have lost the *URA3* plasmid will form colonies, while cells with the plasmid will die.

Cells that have spontaneously lost the *TLC1* plasmid became *ura3*$^{-}$ and therefore form colonies on 5-FOA plates. Eukaryotic cells spontaneously lose plasmids and chromosomes at frequencies of approximately one in a thousand to one in ten thousand cells. Thus, a typical dense culture of YLKL991 yeast cells that has 1 X 10^{8} cells per mL will have many cells that have lost a plasmid (Figure 11). Such cells have changed genotype from *yku70* to *yku70 tlc1*. This change in genotype allows monitoring of cellular growth when cells are devoid of a working *TLC1* gene. When cells were pronged to a Glu-Leu plate without 5-FOA, single mutant *yku70* cells with the vector died at 37°C but survived at the more permissive 30°C and RT. In contrast, the cells overexpressing *EST2* survived at all tested temperatures (Figure 12a, row 1 vs. 2). Survival of cells overexpressing *EST2* and *TLC1* together at 37°C was strong and similar to that seen in cells overexpressing *EST2* alone (compare row 4 vs. row 2 in Figure 12 part a). Increased

survival at 37°C was also seen in cells containing the *TLC1* plasmid that did not also have the *EST2* overexpressing plasmid (Figure12a, row 3). Colony numbers were moderately reduced relative to cells overexpressing only *EST2* (Figure 12a, compare row 3 vs. row 2 at 37°C), indicating that survival was higher when *EST2* was overexpressed. Growth on 5-FOA shows the same trend in the controls (*yku70* single mutants transformed with empty vector and the pVL999 *EST2* plasmid) as seen on Glu-Leu media. *EST2* overexpression rescued *yku70* mutants at all temperatures, and cells transformed with the empty vector showed death at 37°C, but not at RT and 30°C, as expected (Figure 12a and 12b, compare rows 1 and 2). In *yku70 tlc1* cells, *EST2* overexpression did not rescue at 37°C or, unexpectedly, at even 30°C and RT (Figure 12b, row 4). The vector controls experienced death at all temperatures as well (Figure 12b, row 3). Normally, *tlc1* mutant cells grow for 60-70 generations and then cease growth due to senescence (49, 50, 109). The implication from rows 3-6 in Figure 12b suggests that *tlc1 yku70* mutants undergo senescence at a much faster rate than *tlc1* single mutants. It can be estimated that these mutants are undergoing less than 20 generations (cell cycles) of growth, since it takes approximately 20 generations for colony formation and no colonies are visible. This suggests that without *TLC1* RNA being produced in the *yku70* cells, they enter senescence rapidly, due to extremely short telomeres due to the additional mutation in *yku70*.

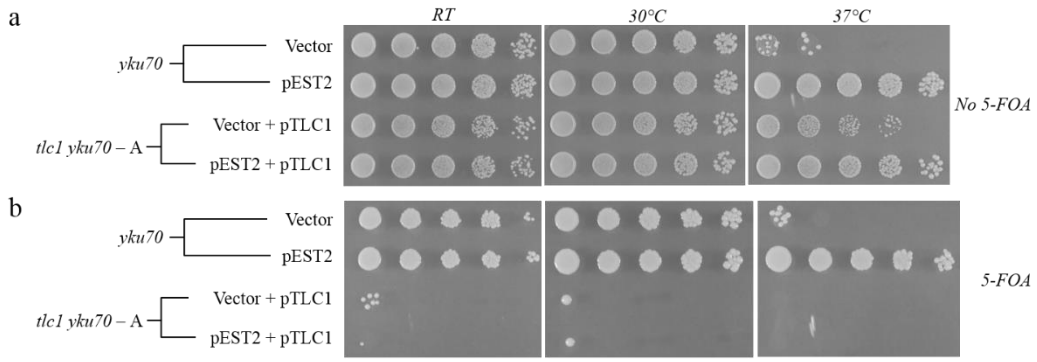

Figure 12. **Overexpression of *EST2* in *yku70 tlc1* cells with and without the *TLC1* gene plasmid pLKL83Y.** When grown on Glu-Leu with overexpression of both *EST2* and *TLC1*, all cells survived at 37°C. On 5-FOA media, cells that did not express *TLC1* died before forming any colonies.

Figure 13 gives an illustrated model of chromosome ends in *yku70* and *yku70 tlc1* cells, with the telomere ends in red and the chromosome in blue. This model was created based on the data obtained in Figure 12 and gives an explanation for why the cells go into senescence sooner than expected. Part **a** shows the wildtype telomere, which is long. In part **b**, a *yku70* mutant is shown at 30°C. The telomere is shorter then wildtype, but still stable. When the temperature is elevated to 37°C the telomere cap structure is compromised, allowing nucleases such as Exo1p more access to the telomere ends, leading to shorter telomeres over time. Part **c** is derived from the data from Figure 12. When *TLC1* RNA is removed entirely from the cell the telomeres rapidly become extremely short, causing the cell to enter senescence. This telomere shortening is not alleviated by *EST2* overexpression and happens at all temperatures. While the cell death was not unexpected, the speed of death was unexpected. Without the *TLC1* template

RNA, the cell is unable to synthesize telomere repeats, preventing elongation of the telomere regions.

Figure 13. **Illustration of proposed rapid telomere shortening leading to senescence.** Part **a** shows wildtype telomeres in red and chromosome in blue. Part **b** depicts *yku70* mutants at 30°C and 37°C. The telomeres are shorter than normal at 30°C, but when the temperature is raised to 37°C the length decreases over time. Part **c** shows *yku70* mutants when *EST2* and *TLC1* are expressed. With both products, telomeres are normal, but when *TLC1* is removed with 5-FOA the telomeres shorten rapidly, causing the cells to quickly enter senescence.

Est2p is the catalytically active polymerase unit of the telomerase complex. It is responsible for synthesizing the telomere repeats during S phase. When overexpressed in *yku70* single mutants, it mitigates telomere degradation and rescues cells from death at elevated temperatures. There are at least two different possible mechanisms for *EST2*-based rescue. The first involves higher levels of Est2p leading to greater synthesis of telomere repeats, creating longer telomeres, counteracting the degradation from nucleases. In the second mechanism, Est2p binds with other telomere cap associated proteins, and telomere DNA or RNA associated proteins, forming a physical structure that prevents exonucleases (such as Exo1p) from shortening telomere ends. Both proposed mechanisms are viable pathways for *EST2*-based rescue.

Cech *et al.* have created several catalytically inactive mutants of *EST2*, including

est2-D530A, *est2-D670A*, and *est2-D671A*. These mutant genes have been shown to produce proteins that are stable *in vivo* but do not possess detectable polymerase activity (110). Previous experiments in this lab by Brian Wasko suggested that overexpression of Est2-D530Ap and Est2-D670Ap could rescue *yku70* cells, but Est2-D671Ap was never tested (55). Plasmids encoding these catalytically inactive mutants were transformed into *yku70* strain YLKL652 using the plasmids pVL735 (*ADH1p::est2-D530A*), pVL743 (*ADH1p::est2-D670A*), and pVL744 (*ADH1p::est2-D671A*). A control vector called pRS426 was also transformed into the cells. After transformation, cells were pronged at 30°C and 37°C onto plates without uracil. The results from this experiment showed that the *yku70* mutants died at 37°C and *EST2* overexpression rescued them, as expected (Figure 14, rows 1 and 2). Overexpression of each of the catalytically deficient proteins also rescued the temperature-induced death of *yku70* mutants as efficiently as overexpression of wildtype Est2p protein (Figure 14, compare rows 3-5 to row 2). The implication from catalytically inactive enzymes rescuing as well as the wildtype enzyme is that the polymerase function of Est2p is not required for the rescue of temperature-based death. These results support the structure-based mechanism, not the synthesis-based one. If synthesis was the main mechanism of rescue, the catalytically inactive mutants would not have rescued as well as the wildtype gene since they could not elongate the telomere repeat sequences. These results support the structural cap-based

protection of telomere ends from degradation, since the polymerase function of Est2p was not required for rescue.

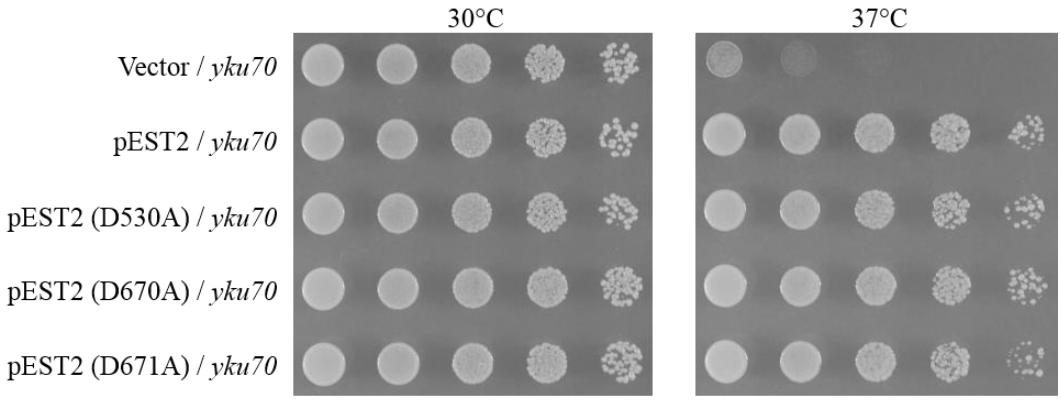

Figure 14. **Rescue of *yku70* mutants by overexpression of catalytically inactive Est2p proteins.** *yku70* cells die at 37°C, but were rescued when *EST2* was overexpressed. Plasmids capable of overexpressing three proteins without polymerase activity were transformed into yeast cells and the transformante was grown at 30°C and 37°C.

Yeast telomerase contains three protein subunits (Est1p, Est2p, and Est3p) and one RNA subunit (*TLC1*). The precise function of Est1p is unknown. Est2p is the catalytically active subunit, showing RNA-dependent DNA polymerase activity. Est2p uses *TLC1* RNA as a template for telomere repeat synthesis extending the telomere (48, 49). Past studies have indicated that overexpression of two of the protein subunits of telomerase (Est1p, Est2p) or the *TLC1* RNA can rescue the death of *yku70* cells at 37°C (43, 51-53). However, previous work in this lab by graduate student Brian Wasko was not able to reproduce rescue of *yku70* cells by the *EST1* gene using the same plasmid pVL784 (*ADH1p::EST1*) employed by Nugent *et al.* (51). The only difference between Nugent *et al*. and Brian Wasko's experiment was that Nugent *et al.* used *yku80* cells and Brian Wasko used *yku70* mutant cells. In order to resolve this discrepancy between the literature and previous results in the Lewis lab, both mutant backgrounds (*yku80* and *yku70*) were transformed with a vector (pRS435), an *EST1* overexpression plasmid

pVL784 (*ADH1p::EST1*), and an *EST2* overexpression plasmid pVL999 (*ADH1p::EST2*). Cells were grown on Glu-Leu plates, harvested into water, counted and pronged to new Glu-Leu plates and grown at 30°C, 37°C, and 38°C (Figure 15). Vector controls in both *yku70* and *yku80* strains grew as expected, exhibiting survival at 30°C and death at the elevated temperatures of 37°C and 38°C (Figure 15a and b, row 1). Rescue was seen when the polymerase gene *EST2* was overexpressed in both *yku70* and *yku80* mutants at all temperatures (Figure 15a and b, row 2). Interestingly, when *EST1* was overexpressed in either *yku70* or *yku80* single mutants, rescue was not seen, as growth was similar to the vector control (Figure 15a and b, compare row 1 and row 3). This result supports Brian Wasko's conclusion (102), in that *EST1* does not rescue either *yku70* or *yku80* mutant strains from temperature-induced cellular death. Only *EST2* and *TLC1* overexpression rescued these mutants from the death response at elevated temperatures.

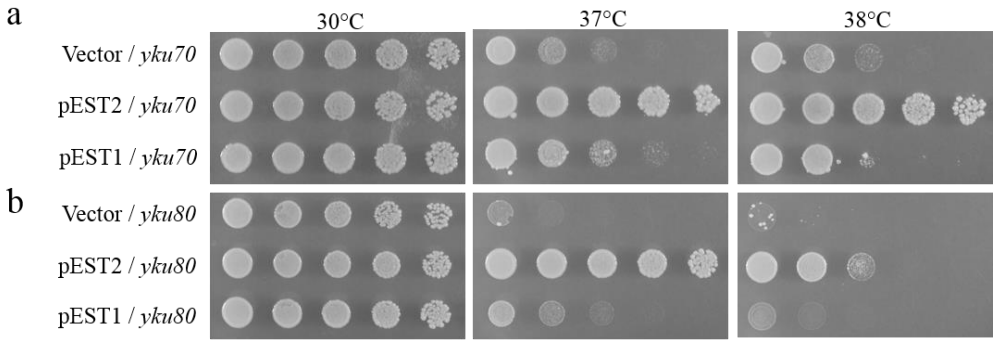

Figure 15. **Survival of *yku70* and *yku80* mutants overexpressing *EST2* or *EST1*.**

As mentioned previously, wildtype telomerase is composed of three protein subunits (Est1p, Est2p, and Est3p) and the RNA component encoded by the *TLC1* gene. Other proteins are associated with the ends of chromosomes too, including Yku70p, Yku80p, Rif1p, Rif2p, Rap1p, Sir2p, Sir3p, Sir4p, Cdc13p, and several other proteins (Figure 16). Some of these proteins are part of the telomere cap complex which protects the telomeres from degradation by exonucleases such as Exo1. These nucleases create long single-stranded DNA regions. Rif1 and Rif2 (Rap1-interacting factors 1 and 2) are regulators of telomere length and are recruited by Rap1. They negatively regulate the synthesis of telomere DNA repeats, and are part of chromatin structure at telomere ends (43, 111). Sir2, Sir3, and Sir4 are important for tethering the ends of chromosomes to the nuclear pores and silencing transcription near the telomeres (42, 43, 112). Cdc13 is a protein known to associate with the ends of chromosomes and with the telomerase complex, helping to recruit and regulate telomerase activity (113).

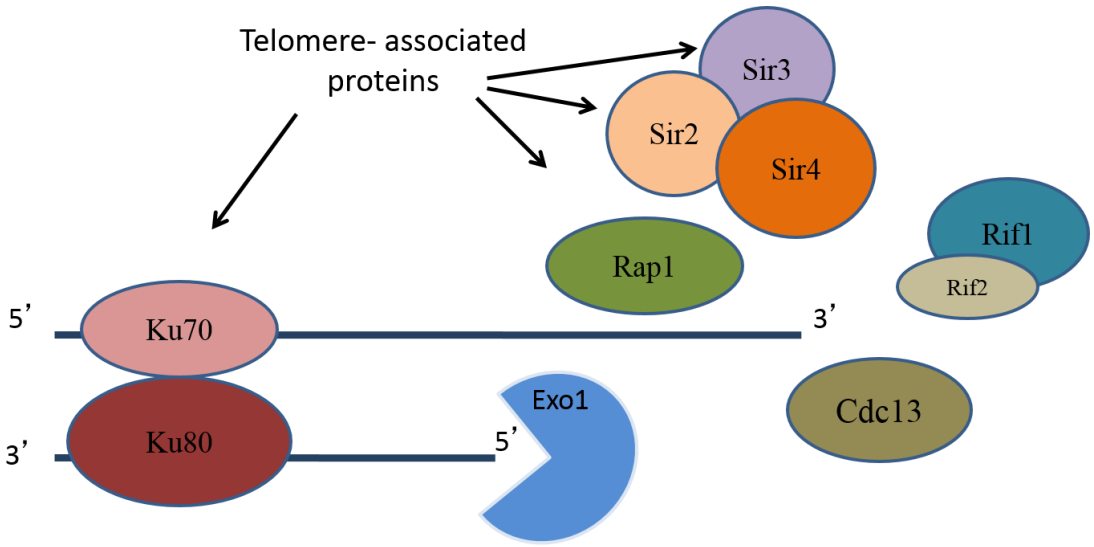

Figure 16. **Illustration of telomere ends and several known telomere binding proteins.**

Some telomere-associated components (Est2p and *TLC1* RNA) are able to rescue *yku70* mutants from temperature induced death when overexpressed. The possibility that other telomere proteins might also be able to stabilize chromosome ends in *yku70* strains has been little explored. To address this, plasmids capable of overexpressing other telomere binding proteins were transformed into yeast strain YLK652 (*yku70).* The plasmids contained genes for *RIF1, RIF2, SIR3*, and *SIR4* under control of the strong *GAL1* galactose promoter and also contained *TRP1* as a selectable marker. This allowed the expression of the proteins to be controlled, so they were only expressed in the presence of the sugar galactose. After plasmids were transformed into *yku70* cells and spread to glucose plates without tryptophan (Trp), colonies were patched to galactose media without Trp, grown for 2 days at 30°C, and pronged to 30°C and 37°C on galactose plates without Trp. Harvesting from galactose plates ensured the cells were overexpressing the telomere binding proteins prior to exposure to high temperature and would not experience any lag time in expression. The vector control cells grew as

expected, displaying excellent growth at 30°C and death at 37°C (Figure 17, row 1). This was in contrast to the *EST2* overexpressing cells that showed rescue at 37°C (Figure 17, row 2). Overexpression of *RIF1*, *RIF2*, *SIR3*, or *SIR4* did not affect growth at 30°C, but also did not rescue the killing of the cells at 37°C (Figure 17, rows 3-6). At 37°C, three of the overexpressed proteins showed slightly worse growth than the vector-containing cells (Figure 17, compare row 1 to rows 4-6). The results from this experiment show that overexpression of *RIF1*, *RIF2*, *SIR3* or *SIR4* did not stabilize telomeres nor rescue *yku70* mutants at elevated temperatures.

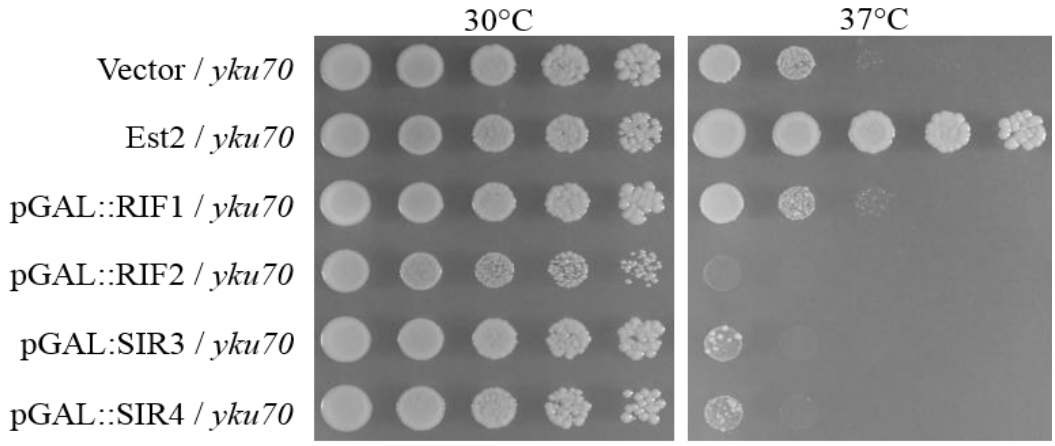

Figure 17. **Overexpression of *RIF1, RIF2, SIR3,* or *SIR4* does not suppress killing of *yku70* cells at 37°C.**

Using a library of 4,852 haploid yeast strains with a single mutation in each strain, Askree *et al.* and Gatbonton *et al.* assessed telomere lengths in the cells in two different studies (56, 57). In their experiments, chromosomal DNA was prepared from each mutant in the libraries and was then digested with XhoI. This digestion removed the telomere end region from each chromosome, creating an end fragment whose length was dependent on how long the telomeres were. The digestion products were analyzed by gel electrophoresis and Southern blots to identify telomere DNA lengths (depicted

schematically in Figure 18). Using the blots to visually identify telomere lengths, the genes were categorized based on their size relative to average telomere lengths (56,57).

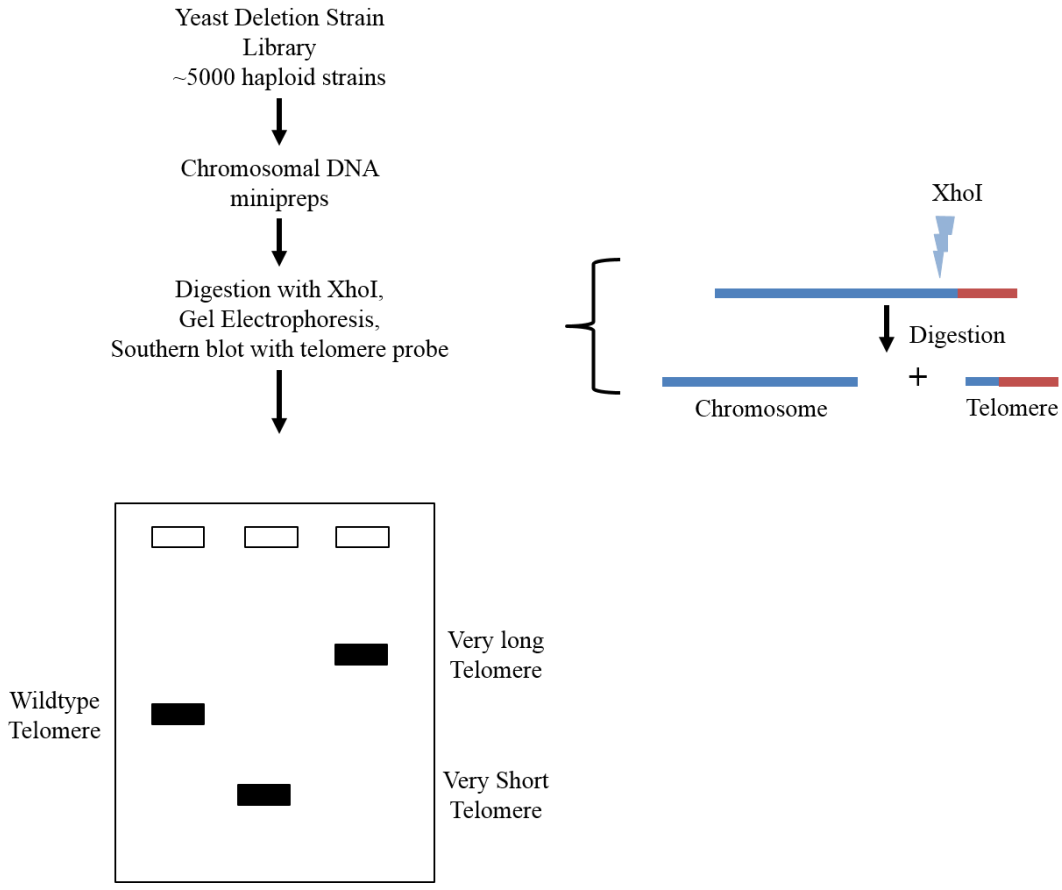

Figure 18. **Workflow of Askree *et al.* and Gatbonton *et al.* telomere length screens.** Using a yeast deletion strain library of 4852 mutants, chromosomal DNA was isolated and digested with XhoI restriction enzyme. XhoI has a cut site near the ends of telomeres that separates the telomere end from the chromosome. Following digestion with XhoI, samples were run on agarose gels to visualize and characterize the telomere lengths for each mutant.

Using the lists generated from Askree *et al.* and Gatbonton *et al.*, mutants of interest to this project were determined based on extreme telomere length variation. Telomeres that were identified as extremely long or extremely short in the Askree and Gatbonton lists are shown in Table 3, which tabulates the mutants of interest by telomere length and function.

Table 3. **Table of yeast deletion library mutants with extremely long or extremely short telomeres identified by Askree *et al.* and Gatbonton *et al.*.**

Mutant	Phenotype	Function
ard1	Very long telomere	N-terminal acetyltransferase complex
elg1	Very long telomere	RFC complex
htl1	Very long telomere	RSC complex interacting protein
pif1	Very long telomere	DNA helicase
rad27	Very long telomere	Flap-endonuclease
rif1	Very long telomere	Telomere binding protein
rif2	Very long telomere	Telomere binding protein
rps17a	Very long telomere	Ribosomal protein S17A
rsa1	Very long telomere	Ribosomal large subunit assembly
srb8	Very long telomere	Mediator complex component
mrc1	Very short telomere	DNA damage response
tel1	Very short telomere	PIK homologue

Using the list of genes, mutants with extremely long or short telomeres were identified and selected to have *YKU70* disrupted within them, creating double mutant yeast strains. *yku70* strains normally have shorter than normal telomeres that become shorter over time when exposed to elevated temperatures. Several of the mutants identified in these studies possess extremely long telomeres. Creating double mutants would allow the investigation of the impacts of genes known to affect telomeres on survival of *yku* cells. The genes chosen for further study were *elg1, htl1, rad27, ard1, srb8, tel1,* and *mrc1*. Three other genes (*rsc1, hsp82,* and *rad9)* were also selected to make new *yku70* double mutants. These 3 genes were chosen based on analyses of data within the Saccharomyces Genome Database, which revealed that the protein products of these genes also influence telomeric chromatin structure or telomerase activity. Mutants such as *rif1, rif2* and *pif1* from Table 3 were not selected for additional study due to the fact that double mutants with *yku70* have already been created and analyzed in the Lewis lab (114). Very short telomere mutants (*mrc1* and *tel1)* were selected to test the effects of mutants with extremely short telomeres, in contrast to the other mutants that had long telomeres. Tests were performed to see if these double mutants experience the same death at 37°C that *yku70* single mutants experience.

In order to make double mutants, haploid strains from a *MATα* yeast deletion library were obtained from Open BioSystems. Cells of the single mutants were transformed with EcoRI and HindIII-digested pGEM4Z S-H/URA plasmid to knock out *YKU70* (Figure 19). pGEM4Z S-H/URA is a *YKU70* gene deletion plasmid used to disrupt the *YKU70* gene in wildtype cells by inserting *URA3* into the gene (96). This was accomplished by flanking the *URA3* gene with ~300 bp corresponding to the 5' and 3'

ends of the *YKU70* gene. The construct is surrounded by cut sites for EcoRI and HindIII. Following digestion with these enzymes, the fragment from the plasmid was transformed into cells. A representative digestion gel is shown in Figure 20. Once transformed, the DNA fragment associated with homologous sequences in the genome in the nucleus and inserted the *URA3* gene into the chromosome, primarily into the *YKU70* gene (Figure 20). This recombination event occurs in only a small fraction of cells, but can be selected for using plates deficient in uracil: only cells that have incorporated the fragment into the chromosome gain the Ura^+ genotype. The short telomere mutants (*mrc1* and *tel1)* were transformed and incubated, but no colonies grew after 4 days. The transformation was repeated, and again yielded no colonies after 4 days. The results imply that, when combined with *yku70,* these mutations form a synthetically lethal gene suite. These results suggest that combining drastically shorter telomeres than normal with an unstable telomere cap structure from the *yku70* mutation creates cells that are inviable at normal growth temperatures (30°C)

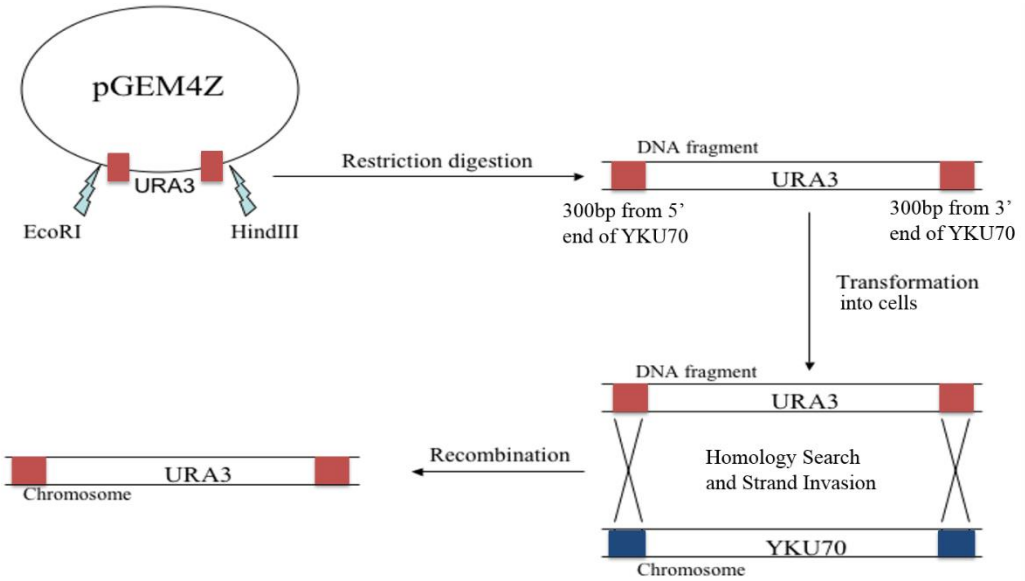

Figure 19. **Illustration of pGEM4Z S-H/URA disruption process.** The pGEM4Z S-H/URA plasmid has the *URA3* gene flanked on either side by the 5' and 3' coding regions of the *YKU70* gene. This insert is between an EcoRI and HindIII restriction enzyme cut site. Once digested and transformed into yeast cells, the flanking regions will undergo genetic exchange with homologous sequences in the chromosome, inserting the construct into the *YKU70* gene, disrupting the gene, creating cells with a *yku70*$^{-}$ genotype.

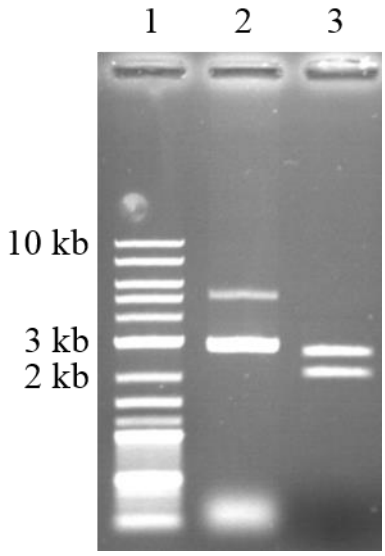

Figure 20. **Verification gel of pGEM4Z S-H/URA plasmid DNA digested with EcoRI and HindIII.** Lane 1 is 2-log DNA ladder. Lane 2 is uncut pGEM4Z S-H/URA, showing two bands at ~ 5 kb and ~3 kb, corresponding to nicked open circular and supercoiled plasmid. Lane 3 is pGEM4Z S-H/URA digested with BamHI and HindIII, creating two bands at ~3 kb and ~2 kb corresponding to removal of the *URA3* gene from the plasmid.

Verification of the successful construction of *yku70* mutants is traditionally accomplished by testing growth of the yeast cells at 37°C. Since *yku70* mutants have a temperature sensitivity phenotype, this characteristic can be used to screen yeast colonies. After identifying Ura^+ transformants that grew poorly at 37°C on streak plates, PCR was used to verify correct insertion of *URA3* into the *YKU70* gene.

Using primers that flank the wildtype *YKU70* gene on the 5' and 3' ends called Ku70a and Ku70b, it was possible to copy the entire gene using purified chromosomal DNA as a template. When run on an agarose gel, different sized bands appeared depending on the insertion, ~2.1 kb for wildtype and ~3 kb for the disrupted gene. The specific size of the wildtype *YKU70* band is 2223 bp, but when the *URA3* gene is inserted

it raises the band size to 2758 bp. Figures 21 – 23 show PCR analyses of DNA from Ura$^+$ colonies obtained after transformations of *elg1, hsp82*, and *rad9* mutants, respectively. The specific sizes of the PCR bands allowed positive identification of isolates that had the *YKU70* gene correctly knocked out.

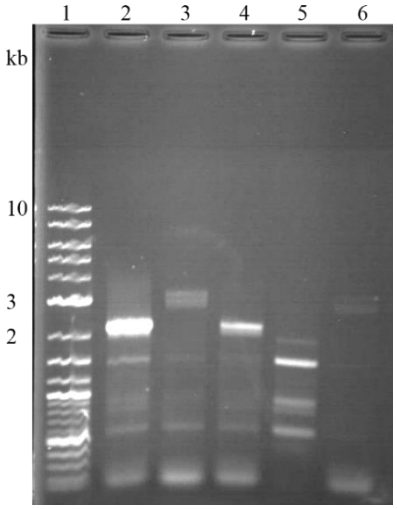

Figure 21. **Verification gel showing PCR confirmation of *elg1 yku70* double mutants.** Lane 1 is 2-Log DNA ladder. Lane 2 is a positive control for wildtype *YKU70* and had the corresponding major band at ~2 kb. Lanes 3 – 6 are isolates 1-4 of potential *elg1 yku70* strains. Lanes 3 and 6 have bands at ~3 kb, indicating successful insertion of *URA3* into *YKU70*, verifying that these isolates are double mutants.

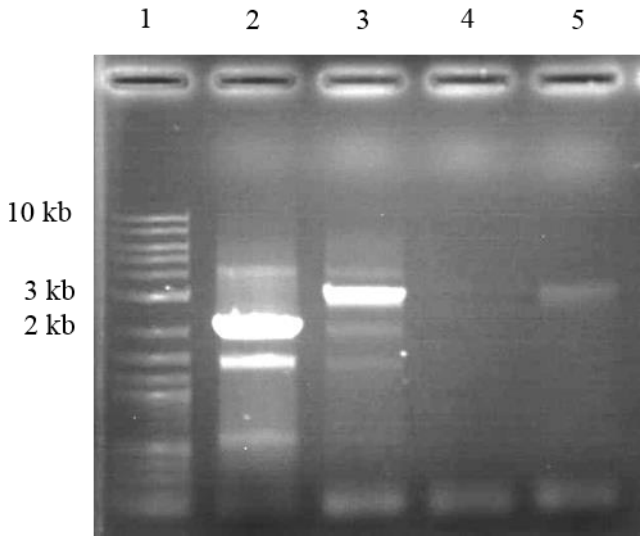

Figure 22. **Verification gel showing PCR confirmation of *hsp82 yku70* double mutants.** Lane 1 is 2-Log DNA ladder. Lane 2 is a positive control for wildtype *YKU70* and had the corresponding major band at ~2 kb. Lanes 3 – 5 are isolates 1-3 of potential *hsp82 yku70* strains. Lanes 3 and 5 have bands at ~3 kb, indicating successful insertion of *URA3* into *YKU70*, verifying that these isolates are double mutants.

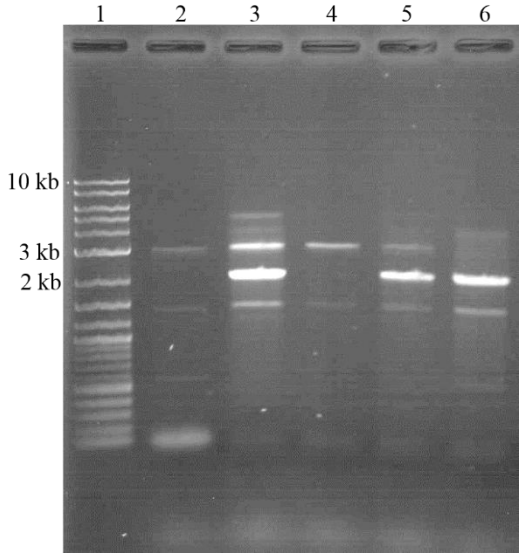

Figure 23. **Verification gel showing PCR confirmation of *rad9 yku70* double mutants.** Lane 1 is 2-Log DNA ladder. Lane 6 is a positive control for wildtype *YKU70* and had the corresponding major band at ~2 kb. Lanes 2 – 5 are isolates 1-4 of potential *rad9 yku70* strains. Lanes 2 and 4 have bands at ~3 kb, indicating successful insertion of

Once two isolates were verified using PCR to ascertain the disrupted *yku70* gene, the cells were transformed with pVL799 (*TLC1* overexpression), pVL999 (*EST2* overexpression) and pRS425 (*LEU2* vector). This was done to determine the effect the double mutations would have on rescue by either *EST2* or *TLC1* RNA. Once transformed with the plasmid DNA, cells were plated to glucose minus leucine plates to maintain selective pressure for the plasmids, and grown at 30°C for 3 – 4 days. Several strains were not transformed successfully. The very short telomere mutants (*mrc1* and *tel1*) were not transformed due to the apparent synthetically lethal phenotype of combining those selected genes with *yku70* (described above). Also, *srb8 yku70* strains were not successfully created due to this strain's high degree of flocculation, preventing cell cultures from achieving adequate densities for transformations. The remaining strains were transformed successfully with each plasmid. Colonies from the transformations were patched to glucose minus leucine plates and grown for 2 days before being harvesting into water for pronging assays.

Strains transformed with pVL799 (*TLC1* overexpression plasmid) were pronged to glucose minus leucine plates at both 30°C and 37°C and allowed to grow for 3 days. At 30°C all 4 double mutant strains grew normally (Figure 24). At 37°C reduced growth is expected from an *ard1* strain, since that strain background is phenotypically recognized to have increased heat sensitivity, leading to reduced growth (SaccharomycesGenomeDatabase). The poor growth of *ard1 yku70* cells at 37°C was not rescued by overexpression of *TLC1* (Figure 24, row 2).The other strains in Figure 24 (*elg1 yku70*, *htl1 yku70* and *rad27 yku70*) showed strong killing at 37°C in vector containing cells (Figure 24, rows 3, 5 and 7). The three strains showed strong rescue of

growth in the cells overexpressing *TLC1* (Figure 24, rows 4, 6 and 8). Thus, when *TLC1* RNA was overexpressed in these cells they showed growth comparable to 30°C, indicating these genes are not required for *TLC1* RNA-based rescue.

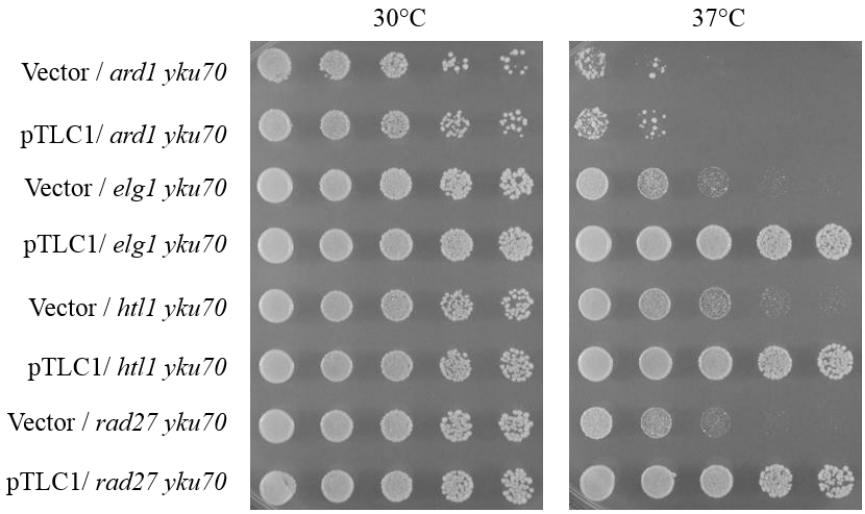

Figure 24. **Pronging survival assays with overexpression of *TLC1* in *yku70* telomere length effecting double mutants.**

Strains transformed with pVL999 (*EST2* overexpression plasmid) were pronged to glucose minus leucine plates at both 30°C and 37°C and allowed to grow for 3 days. Results were similar to those seen with *TLC1* overexpression. *ard1 yku70* cells were not rescued by *EST2* overexpression, but growth at 37°C was restored in the other three double mutants (Figure 25). When *EST2* was overexpressed in these cells they showed growth comparable to that seen at 30°C, indicating that the genes are not required for *EST2*-based rescue.

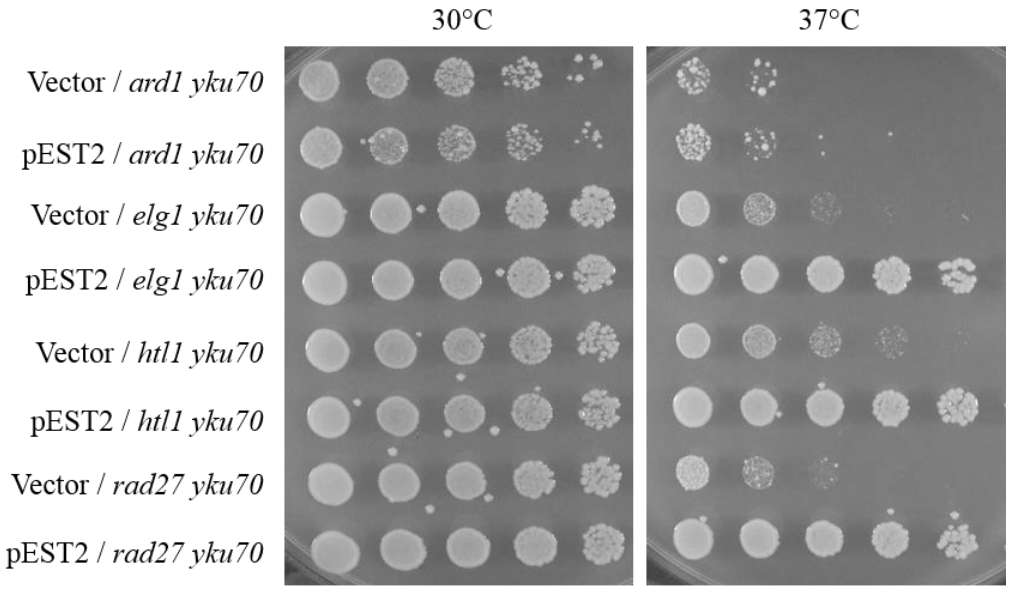

Figure 25. **Pronging survival assays with overexpression of *EST2* in *yku70* telomere length effecting double mutants.**

Mutations in the Ade biosynthesis pathway, specifically *ade1* or *ade2* mutations, lead to increased levels of the red pigmented molecule AIR. AIR gives the normally white to cream colored yeast colonies a pink to red coloration. This red coloration can be used to screen or assay for mutant yeast cells that go from, e.g., $ADE2^{+}$ to $ade2^{-}$. Some *ade2* yeast strains have a strong red color, but for unknown reasons some strain backgrounds produce only a pink color, making visual identification of the colonies difficult. During the course of this thesis project, several experiments were performed in an attempt to improve the growth properties of a *yku70* mutant strain call YLKL593, which is a derivative of the published strain VL6α (90). While varying the concentration of various nutrients, an interesting observation was made. Normal yeast growth media contains 5 g/L of ammonium sulfate as a nitrogen source. Additional ammonium sulfate in the growth media was able to amplify the coloration of the weakly red *ade* mutants to a stronger hue, increasing its ability to be visually recognized. The mechanism for color

amplification is unknown, but upon subsequent testing of other strains, it was found to be a reproducible effect (Figures 26 and 27). Increasing the concentration of the nitrogen source in the plates from 5 g/L to 10 g/L produced stronger red colony color in several strains tested, including YLKL593, VL6α, YPH102, and W1588-4c. Three strains, BY4704, BY4705, and BWG1-7a showed only slight improvement. The improved ability to detect *ade*$^{-}$ cells arising from this discovery will make genetic scans easier in the lab in the future.

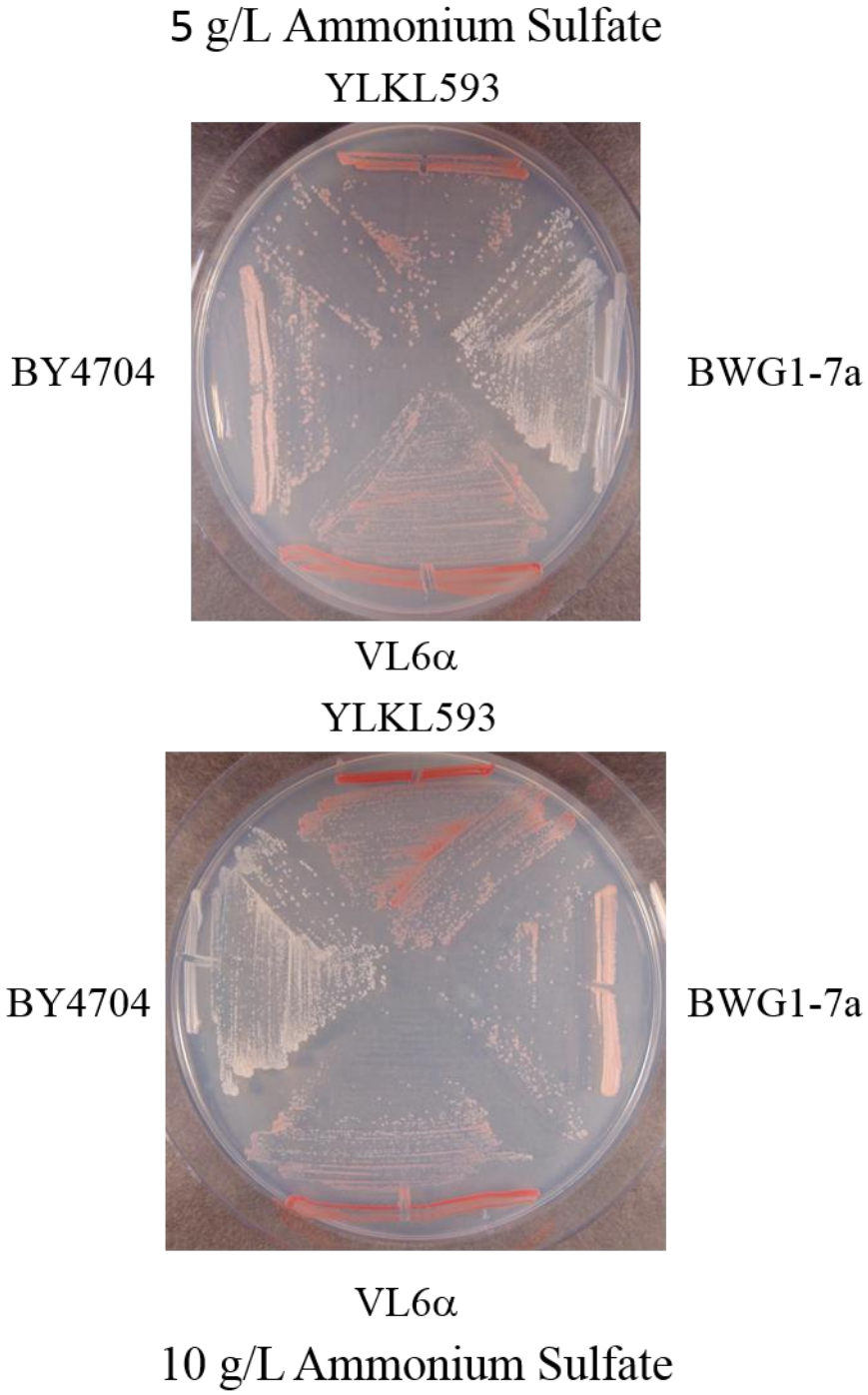

Figure 26. **Colony color amplification in yeast strains YLKL593, BWG1-7a, VL6α. BY4704**

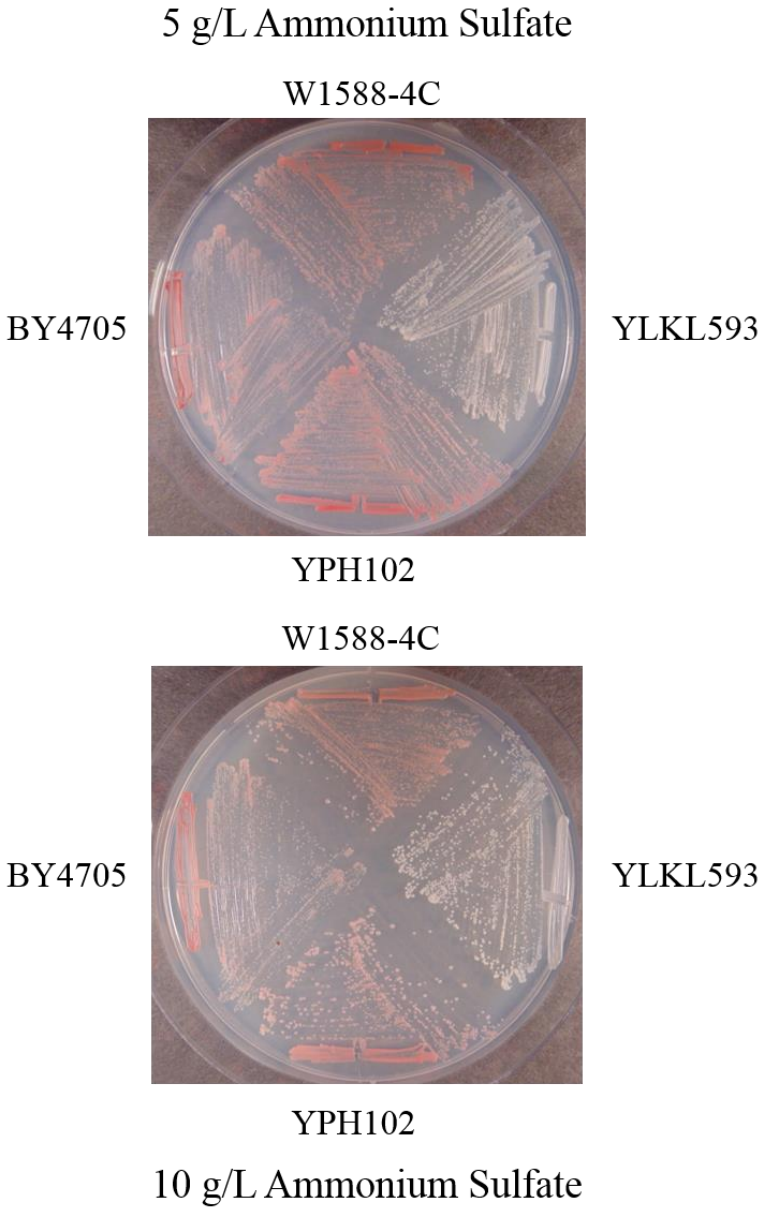

Figure 27. **Colony color amplification in yeast strains W1588-4C, BY4705, YLKL593, YPH102**

CHAPTER IV

SUMMARY AND CONCLUSIONS

The primary purpose of this study was to characterize the mechanism of action of *EST2* overexpression and secondarily identify genes affecting telomere stability and survival in *yku70* and *yku80* mutants. .

This work expanded on previous work in the lab by verifying and investigating the role of nucleases, checkpoint response genes, and genes known to affect telomere stability and structure on survival of *yku* cells at 37°C. None of the nuclease or checkpoint genes tested showed signs of affecting *EST2*-based rescue.

When *yku70 tlc1* cells containing *EST2* and *TLC1* overexpression plasmids were grown in the presence of 5-FOA to select for cells that lost the TLC1 plasmid, creating cells that are deficient in both *yku70* and *tlc1,* the cells died rapidly and could not be rescued by overexpression of *EST2*. Yku70p and *TLC1* RNA, there is dramatic shortening of the telomeres, sending the cells into senescence, causing them to stop dividing and die.

This study also gave evidence for a model involving structural cap–based protection of telomere ends from degradation. Catalytically inactive Est2p was overexpressed in *yku70* cells and they still rescued the *yku70* cells from death at 37°C. This implies that the protection of telomere ends does not require the polymerase functionality of Est2p; it needs to be physically present, likely complexed with other telomerase subunits and telomere-binding proteins, in order to rescue. Several genes shown to create longer telomeres when inactivated were tested in double knockouts with

yku70 (ard1, elg1, htl1, rad27, rad9, hsp82, rsc1). None of tested genes affect rescue by *EST2*.

This study also tested overexpression of other proteins that were part of telomerase (EST1p) or known to affect telomere structure in telomere regions (RIF1p, RIF2p, SIR3p, and SIRp). The testing of *EST1* confirmed that it could not rescue *yku* cells from death as *EST2*. Results from this study showed that *EST1* did not rescue either *yku70* or *yku80* cells from death. Overexpression of RIF1p, RIF2p, SIR3p, and SIR4p also did not rescue *yku* cells from death.

This study also determined that changing the amount of an additive to media to increase the utility of yeast strains with *ade1* or *ade2* mutations that do not strongly express the red pigmentation from a buildup of AIR. Increasing the concentration of ammonium sulfate in media from 5 g/L to 10 g/L caused yeast strains that are normally light pink to darken, improving ease of visual identification.

In summary, this project has identified several genes that are not part of *EST2*-based rescue of cellular death of *yku* mutants at elevated temperatures. It has also clarified what telomere-associated proteins can rescue *yku70* cells from death.

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