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Saccharomyces cerevisiae Tel1 & Tel2 role in DNA double-strand break repairs

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A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science in Chemical Engineering

in

The Cain Department of Chemical Engineering

by
Tommy Nhan Trieu
B.S, University of South Alabama, 2012
December 2015
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NOMENCLATURE

Greek Letters

$\alpha$  
Alpha

$\mu$  
Micro

Abbreviations

DNA  
Deoxyribonucleic Acid

DSB  
Double Strand Break

DDR  
DNA Damage Response

qPCR  
Quantitative Polymerase Chain Reaction

HR  
Homologous Recombination

NHEJ  
Non-homologous end joining

G1  
Gap 1

S  
Synthesis

G2  
Gap 2

M  
Mitosis

MAT  
Mating Type

HML  
Hidden Mating Type Left

HMR  
Hidden Mating Type Right

HO  
Homothallic

PIKK  
Phosphatidylinositol 3-kinase-related kinase

ANOVA  
Analysis of variance

ssDNA  
Single Stranded DNA

WT  
Wild Type

kb  
Kilobase

RPM  
Revolutions per minute

YEP  
Yeast Extract Peptone

MEC  
Mitosis Entry Checkpoint

TEL  
TELomere maintenance

RAD  
RADiation sensitive

Dna2  
DNA synthesis defective 2

MRX  
Mre11, Rad50, and Xrs2

Sgs  
Slow Growth Suppressor

Sae  
Sporulation in the Absence of spo Eleven

FRAP  
Fluorescence recovery after photobleaching

ATM  
Ataxia telangiectasia mutated

TRRAP  
Transformation/Transcription Domain-Associated Protein
ABSTRACT

Genomic DNA is constantly in danger of being damaged by endogenous cellular processes and exogenous agents. Eukaryotes have mechanisms, collectively termed the DNA damage response, that detect and repair DNA damage. After a double-stranded break occurs, protein complexes are recruited to the break site to promote resection. Two main protein kinases involved in DNA repair are the Tel1 and Mec1 proteins. Recent studies have found that the Tel2 protein binds to both the Tel1 and Mec1 proteins and a point mutation of TEL2 on the 129th amino acid disrupts this interaction. Since both of the Tel1 and Mec1 proteins are involved in DNA repair, we believe disruption of this interaction might cause defects in the resection process. A quantitative real-time PCR assay was used to analyze single-stranded DNA content on the MAT locus at three specific sites distal to the break. It was observed that cells containing the point mutation S129N on the TEL2 gene resulted in a lower amount of single-stranded DNA content compared to wild-type cells. These findings show that Tel2 might play a role in the resection process.
CHAPTER 1: INTRODUCTION

1.1 DNA damage and types

The information that encodes our genome comes from deoxyribonucleic acid (DNA). These building blocks are constantly being damaged by endogenous events such as replication errors, but also by exogenous agents like toxins, viruses, and mutagenic chemicals [1]. Fortunately, most organisms have the ability to detect these damages and repair them.

The DNA damage response pathway allows for efficient repair of all types of damage, including double-stranded breaks (DSBs) [1]. *Saccharomyces cerevisiae* is a simple eukaryote that is used as a model organism for DNA damage response studies. The ease with which *S. cerevisiae* can be grown and analyzed in the lab rivals that of *Escherichia coli*, but allows the study of complex eukaryotic processes. Many proteins in *S. cerevisiae* involved in DNA repair are similar in function to those found in other organisms [2,4]. This fact can give insight to how a particular gene or protein might function in higher eukaryotes. Most genes, mutant alleles, and other genetic loci are designated by three italicized letters and followed by a number. Generally, the letters abbreviate a phrase that provides some information about a function or process related to that gene and the number refers to the order of discovery or gene naming. Table 1 provides the genetic nomenclature for *ADE2* as a reference for the nomenclature system in *S. cerevisiae*.

<table>
<thead>
<tr>
<th></th>
<th>Adenine synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ADE</em></td>
<td>A dominant allele for an enzyme in adenine biosynthesis</td>
</tr>
<tr>
<td><em>ADE2</em></td>
<td></td>
</tr>
<tr>
<td><em>ade2</em></td>
<td>A recessive allele for an enzyme in adenine biosynthesis</td>
</tr>
<tr>
<td>Ade2p</td>
<td>The protein encoded by <em>ADE2</em></td>
</tr>
<tr>
<td>Ade2 protein</td>
<td></td>
</tr>
<tr>
<td><em>ade2Δ-1</em></td>
<td>A specific complete or partial deletion of <em>ADE2</em></td>
</tr>
<tr>
<td><em>ade2-1</em></td>
<td>A specific allele or mutation</td>
</tr>
</tbody>
</table>

Two main mechanisms in *Saccharomyces cerevisiae* repair DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR) [4]. Figure 1 illustrates the repair process of double-stranded breaks by NHEJ and HR. NHEJ uses microhomologies often found at the single-stranded overhang ends of double-strand breaks to re-ligate the ends [3]. This is done by the end-binding protein Ku and by a specialized DNA ligase [2]. There are primarily two models for how HR repairs a DSB, synthesis-dependent strand annealing (SDSA) and double-strand break repair (DSBR) [2]. Both models are the preferred method of repair over NHEJ since a homologous DNA duplex is used as a template to repair breaks accurately [4].
Figure 1 Illustration NHEJ and HR. In HR, the DNA duplex that sustained the DSB is resected at the ends by exonucleases. A single-stranded extension is generated invades the intact homologous sister chromatid. The invading strand serves as a template for a DNA polymerase that copies the chromatid information across the break. In NHEJ, DSBs are repaired by the end-binding protein Ku and then sealed by a specialized DNA ligase [2].

1.2 DNA repair process: resection

Factors that regulate DNA repair by NHEJ or HR are determined during the early stages of a DNA break [12]. Resection, depicted in Figure 2, is the process of nuclease-driven 5’–3’ nucleolytic degradation initiated at a DSB site, thereby resulting in long 3’ single-
stranded tails [8]. This process creates single-stranded DNA and once this process transpires, NHEJ is strongly inhibited by resection and the repair process is done by HR [13].

As shown in Figure 3, many proteins are involved in the resection process. When a DSB occurs, progression of the cell cycle is halted by DNA damage checkpoints while the break is being repaired. These checkpoints are controlled mainly by two protein kinases belonging to the phosphatidylinositol 3-kinase-related kinase (PIKKs) family: telomere maintenance 1 (Tel1) and mitosis entry checkpoint 1 (Mec1) [4]. In general, PIKKs proteins are involved in cell growth, gene expression, and DNA repair in eukaryotic cells [7] and follow a generic structure shown in Figure 4.

The Tel1 and Mec1 protein kinases phosphorylate downstream effectors that coordinate cell cycle progression with DNA repair [4]. These protein kinases are also known to be involved in resection. Tel1 contributes to resection by activating the Mre11-Rad50-Xrs2 (MRX) complex that generates 3’-ended single-stranded DNA at the ends of the breaks and allows for recruitment of the Mec1 protein [9]. Mec1 in Saccharomyces cerevisiae plays a bigger role in DNA breaks when single-stranded DNA is generated. Mec1’s role in resection is the recruitment of a protein, Radiation sensitive (Rad9), that inhabits the processing of a break and promotion of proteins that are positive regulators in resection [8]. A study has also shown that mutations in the MEC1 gene experience prolongs cell cycle arrest due to resection defects in long-rate resection [8].

Resection is a continuous process that is carried out during DNA repair. The MRX complex and Tel1 are involved in short-range resection by proteins collaborating in the initial end trimming creating short 3’ single-stranded DNA overhangs [10]. These overhangs are then coated with Replication Protein A (RPA) that prevents single-stranded DNA from self-pairing and forming secondary structures. RPA then recruits the Mec1 protein to the break and activates long-range resection with Exonuclease 1 (Exo1) or the helicase Slow Growth Suppressor 1 (Sgs1) [11].

1.3 *Saccharomyces cerevisiae* Tel2

Studies have shown that the Tel1 and Mec1 proteins interact with a protein called telomere maintenance 2 (Tel2) [15,17]. Tel2 is an essential gene in many eukaryotes and its ortholog in other organisms plays roles in DNA damage response [16]. Mutant alleles of the *TEL2 ortholog in Caenorhabditis elegans* (CDC-Like Kinase 2/ Radiation sensitive 5 (*clk-2/rad-5*)) caused sensitivity to DNA damaging agents and defects in DNA damage checkpoints while in *Saccharomyces pombe*, Tel2 is required for replication stress response [14]. However, Tel2 in *S. cerevisiae* appears to have multiple functions.
Figure 3 Regulation of resection at a mitotic DSB. Breaks that occur in G1 phase are mainly repaired by NHEJ due to certain proteins that inhibits resection. HR repair breaks that occurs in S/G2 phase due to the formation of 3’ ended ssDNA that recruits Mec1 protein to breaks [13].

Figure 4 Generic structure of PIKKs where the kinase domain is flanked by FRAP-ATM-TRRAP (FAT) and FAT-C-terminal (FATC) domains that interact with the three-dimensional structure of the PIKKs carboxyl-terminal domain. The N termini carry long arrays of Huntingtin, Elongation factor 3, Alpha-regulatory subunit of protein phosphatase 2A and TOR1 (HEAT) repeats that may aid in protein-protein interaction [7].
The tel2-1 allele encodes for the single amino acid change S129N, serine to asparagine on the 129th amino acid. A recent study has shown that this tel2 mutant allele disrupted a Tel1-Tel2 protein interaction and interfered with the ability of the Tel1 protein to localize to DSBs [15]. This suggested that S. cerevisiae Tel2 functions upstream of the Tel1 pathway during DNA damage response. Another report has revealed that the Mec1 protein co-immunoprecipitates with the Tel2 protein, which implied a Mec1-Tel2 protein interaction [17]. Similar to the Tel1-Tel2 protein interaction, the tel2-1 point mutation disrupted the Mec1-Tel2 protein interaction, resulting in lower Mec1 protein levels. But unlike the Tel1-Tel2 protein interaction, Mec1’s signaling ability to DNA damage is intact and is still able to localize to the damage site [17].

The Tel2 protein in S. cerevisiae is known to interact with the Tel1 and Mec1 proteins, PIKK proteins that are involved in DNA damage repair [15,17]. Some other function, such as the resection process, of Tel1 or Mec1 might be disrupted by the loss of the Tel2 protein interaction. Because the Tel2 protein is known to interact with proteins involved in resection [8,9], it is hypothesized that disruption of this interaction might cause defects in the resection process. Single-stranded DNA generation at DSBs plays a critical role in the repair process and any defects or failures to execute and regulate single-stranded DNA formation can threaten genome integrity [13]. To tell if the Tel2 protein had an effect on the resection process, a quantitative real-time PCR (qPCR) assay was used to analyze single-stranded DNA content at a site-specific DSB on the MAT locus generated by the homothallic switching (HO) endonucleases [22].

Four yeast cell lines were assayed using qPCR to explore the effects of Tel2 on resection. Wild-type cells (yeast strain with wild-type TEL1 and TEL2) and tel1Δ cells (yeast cells that only have a deletion of the TEL1 gene) were compared to see how much of an effect Tel1 had on single-stranded DNA content during resection. Wild-type cells and tel2-1 cells (yeast cells that only contain the tel2 point mutation S129N) were compared to see if Tel2 had an effect on the resection process. Also tel2-1 cells and tel2-1 tel1Δ cells (yeast cells that contain both the tel2 point mutation and tel1 deletion) were compared to determine if Tel1 and Tel2 have similar pathways for the resection process.
CHAPTER 2: MATERIALS AND METHODS

2.1 Media and growth conditions

Cells were grown in YEP medium (1% yeast extract, 2% bacto-peptone) supplemented with 2% glucose (YEPD), 2% sucrose (YEPS), 2% raffinose (YEPR) or 2% raffinose and 2% galactose (YEPRG). For cells that contained a kanamycin resistance gene, G418 at a concentration of 200 µg/mL was added to the culture.

For HO induction, cells were pre-grown for 12 hours in YEPS and then washed with YEPR. Fresh YEPR was added to seed cultures at an OD600 of 0.15 and allowed to double at least twice while incubating at 31°C and 200 RPM. Nocodazole (Adipogen CAT#: 50-464-564) was added at a final concentration of 15 µg/mL to cultures that contained YEPR + 1% DMSO and incubated for 8 hours. Galactose was then added to a final volume of 2% for induction of the HO gene.

2.2 Strain construction

2.2.1 Creation of plasmid with tel2-1

The tel2-1 mutation contained a point mutation on the 129th amino acid of the open reading frame (ORF) of the TEL2 gene where serine was changed to asparagine. The yeast strain EHB13136 contained this mutation and was used to amplify the tel2-1 ORF. A SalI restriction site and a BamHI restriction site were added to the 5’ and 3’ end respectively. Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used to ensure accurate amplification. The tel2-1 fragment was then digested and ligated onto a plasmid, pFA6a-KanMX6, that contained a kanamycin resistance gene.

Since SalI and BamHI on pFA6A-KanMX6 are adjacent to one other, each cut site was digested separately. SalI was digested first and then BamHI. The reason for this order was because digestion with either enzyme will give a one base pair overhang for the next digestion (Figure 5). Based on New England Biolabs’ recommendations, cleavage close to the end of DNA fragments requires a certain number of base pairs at the end to cut efficiently. SalI needs a minimum of two base pairs to cut with an efficiency of 20-50 % while BamHI will cut with one base pair with an efficiency of 0-20 % [19].

\[
\text{CAGGTACGGATCCCCG} \\
\text{GTCCAGCTGCTAGGGGC}
\]

Figure 5 Digestion of SalI and BamHI on pFA6a-KanMX6. Digestion of SalI or BamHI results in a one base overhang.

The digested PCR fragment and vector were ligated together with T4 ligase (Thermo Scientific) for an hour at 22°C. The ligation product was transformed into XL1-Blue Subcloning Cells (Agilent) and plated on Luria Broth + ampicillin (LB + AMP) plates. Four colonies were screened and verified via colony PCR using DreamTaq DNA
Polymerase (Thermo Scientific). Of the four colonies, only one showed a positive band. This colony was incubated overnight in LB + Amp at 37°C. The following day, plasmid was extracted from cells. The concentration of the plasmid was calculated using a Nanovette and was sequenced to check for the tel2-1 fragment. This constructed plasmid was named pMB100.

2.2.2 Silent mutation of 131st amino acid of tel2-1

Two base pairs of the 131st amino acid (leucine) on tel2-1 ORF were modified on pMB100 by changing TTA to CTT. This step was performed by PCR site-directed mutagenesis. In this process, the forward primer contained base-pair mismatches on the middle of the primer. The reverse primer bound at the tail end of the forward primer. Both primers had the 5’ end phosphorylated using T4 PNK and Phusion DNA Polymerase was used for amplification. The PCR product was circularized by ligation using T4 ligase and transformed into XL1-Blue subcloning cells that were plated onto LB + AMP plates. Four colonies were screened and verified via colony PCR with DreamTaq DNA Polymerase. One sample was incubated overnight in LB + AMP media at 37°C. The following day, the plasmid was extracted and the insert was verified using the DreamTaq DNA Polymerase. The newly created plasmid, named pMB101, was sequenced for verification of the mutations.

2.2.3 Introduction of mutation via transformation

Phusion DNA Polymerase was used to amplify the ORF of tel2-1 L131 and the kanamycin resistance gene on pMB101. The entire ORF of the tel2-1 L131 fragment served as a homology region for the 5’ end while 45 bps were added at the 3’ end for homology. This fragment was transformed into yeast cells using a lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/ss-DNA/PEG) protocol [20]. Cells were plated onto YPD + G418 (250 µg/ml) and incubated at 31°C for 2 days.

2.2.4 Screening methods for mutants

Homologous recombination requires a minimum of 20 base pairs for practical efficiency [25]. Since the 5’ region of the fragment used for transformation contained the entire TEL2 ORF, HR is more likely to occur at the 5’ end as opposed to the 3’ end. Due to the way HR works, problems can arise when cells are screened for the mutation. The tel2-1 point mutation is 400 base pairs downstream from the start codon. This leaves 1600 base pairs of the TEL2 gene that can be used for homology. If HR occurred after the point mutation, the KanMX6 marker would be inserted into the genome but the point mutation would be eliminated during the recombination process. This would allow yeast colonies to form on YPD + G418 plates, but negative for the point mutation when screened via sequencing (Figure 6). This process can be costly and time-consuming if multiple colonies are to be screened.
Figure 6 Schematic representation of transformation via homologous recombination. 
*TEL2* gene located on the bottom while PCR fragment used for transformation on top. A) HR that occur after the mutations will uptake only the kanamycin resistance gene, leaving out the mutations. B) HR that occur before the mutations will include the mutations plus the kanamycin resistance gene.

Additional methods were developed to screen for the presence of the point mutation. One method was to amplify the mutated region with a primer set that has base pair mismatches at the 3’ end. Mismatches at the 3’ end resulted in dimmer bands on an agarose gel due to the polymerase being hindered from extending and amplifying. The extension temperature was optimized to the point where the mismatch produced no amplification, which resulted in no bands on the agarose gel. Another screening method was to perform a silent mutation that added a unique cut site close to the region of interest. Two base pairs on the 131st amino acid of *tel2-1* were modified which resulted in the addition of a HindIII cut site. This region was amplified and digested with HindIII and gave two bands on a gel. Samples were then sequenced as a final confirmation for the point mutation. These methods were used to introduce the *tel2-1* mutation into H1058 and H987. Table 2 summarizes the cells used in this study.
Table 2 Yeast strains used in this study with the listed genotype.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>YMB100 (EHB13029/BY4736)</td>
<td>MATa ade2Δ::hisG his3Δ200 met15Δ0 trp1Δ63 ura3Δ0</td>
<td>Anderson, C. M., et al. [15]</td>
</tr>
<tr>
<td>YMB101 (EHB13136)</td>
<td>MATa ade2Δ::hisG his3Δ200 met15Δ0 trp1Δ63 ura3Δ0 tel2-1</td>
<td>Anderson, C. M., et al. [15]</td>
</tr>
<tr>
<td>YMB102 (H1058/JKM179)</td>
<td>MATa hmlΔ::ADE1 hmrΔ::ADE1 ade1 lys5 leu2-3,112 trp1::hisG ura3-52 ho ade3::GAL-HO</td>
<td>Shroff, R., et al. [26]</td>
</tr>
<tr>
<td>YMB103 (H987)</td>
<td>MATa hmlΔ::ADE1 hmrΔ::ADE1 ade1 lys5 leu2-3,112 trp1::hisG ura3-52 ho ade3::GAL-HO tel1Δ::URA3</td>
<td>Shroff, R., et al. [26]</td>
</tr>
<tr>
<td>YMB104</td>
<td>MATa hmlΔ::ADE1 hmrΔ::ADE1 ade1 lys5 leu2-3,112 trp1::hisG ura3-52 ho ade3::GAL-HO tel2-1 L131::KanMX6</td>
<td>This Study</td>
</tr>
<tr>
<td>YMB105</td>
<td>MATa hmlΔ::ADE1 hmrΔ::ADE1 ade1 lys5 leu2-3,112 trp1::hisG ura3-52 ho ade3::GAL-HO tel1Δ::URA3 tel2-1 L131::KanMX6</td>
<td>This Study</td>
</tr>
</tbody>
</table>

2.3 Cell cycle experiments

Yeast cells were pre-grown in YEPS for 12 hours and then washed with YEPR. Fresh YEPR was added to seed cultures at an OD600 of 0.15 and allowed to double at least twice while incubating at 31°C and 200 RPM. To analyze cellular response to G2/M arrest, nocodazole was added at a final concentration of 15 µg/mL to culture that contained YEPR + 1% DMSO. Time samples were taken every two hours, fixed with ethanol, and stained with propidium iodide. Samples were run through a BD Accuri C6.

Figure 7 shows the data of the flow cytometer experiment plotted on a histogram for YMB102. On the histogram, fluorescence intensity is plotted on the x-axis against cell count on the y-axis. As time passed, cells are blocked in G2/M phase because nocodazole treated cells cannot form metaphase spindles [27]. Since cells that are in G2/M phase contained more DNA than cells in G1 phase, they take up more dye and fluoresce more brightly. This results in increased fluorescence intensity of the propidium iodide that was measured on a flow cytometer and a second peak appears on the x-axis. Result for other samples are located in the Appendix.
Figure 7 Histogram of flow cytometer experiment for YMB102. After 6 hours, cells moves toward S phase (first peak) and G2/M phase (second peak).

2.4 DNA extraction

DNA was extracted based on a modified Bust n’ Grab protocol [21]. Changes to the protocol are as listed:

1. Samples were incubated at 37°C with 2.0 µL of Thermo Scientific RNase A/T1 Mix after 3 freeze-thaw cycles to remove RNA.
2. 200 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was used instead of chloroform for the removal of cellular debris.
3. 75 µL of 1x TE Buffer pH 8.0 was added to the phenol:chloroform:isoamyl alcohol mixture and then vortexed briefly before centrifuging for 5 minutes at 4°C and 18,000g to aid in the extraction of the aqueous layer.
4. 0.125 volume of 5M NaCl and 2.25 volume of 100% ice-cold ethanol were added to the aqueous layer.
5. Incubation was increased to 30 minutes at 4°C before centrifugation at 18,000g for 10 minutes at 4°C for better precipitation of DNA.
6. Pellet was washed with 1 mL of 70% ice-cold ethanol and spun down as above.
7. Pellet was dried on a heat block for 5-10 minutes to remove all traces of ethanol.

2.5 Quantitative analysis of HO-induced DSB end resection

A qPCR assay was used to analyze single-stranded DNA [22] content at a site-specific DSB on the MAT locus that was generated by the HO endonuclease [5]. Cells with deletion of the HML and HMR loci were used to prevent repair of the DSB [6]. The
amount of single-stranded DNA at 0.7, 5.7, and 10kb distal to the break was calculated by qPCR and normalized to an amplicon on chromosome XIII.

qPCR was performed on a MiniOpticon Real-Time PCR System. iTaq Universal Probes Supermix was used in combination of IDT PrimeTime qPCR assay probe system. This system uses a hydrolysis probe with a fluorescent reporter at the 5’ end and a quencher of fluorescence at the 3’ end of the probe. The quencher prevents detection of the fluorescence due to its close proximity to the reporter. The exonuclease activity of a Taq polymerase breaks this reporter-quencher proximity. Excitations with a laser will fluoresce the dye that can be detected with a real-time PCR system.

6-carboxyfluorescein (6-FAM) was used as the reporter fluorescent dye while Iowa Black FQ with internal ZEN was used as quenchers. The following program was used for all reactions: polymerase activation and DNA denaturation at [95°C for 3 min], followed by 40 cycles of denaturation and annealing/extension [95°C for 15 sec, 59°C for 30 sec], then finally a melting curve analysis [55°C to 95°C with 5 sec hold].

Experimental conditions and DNA extraction are as described in previous sections. For resection assay, qPCR values were normalized to values on a positive control. A negative control was used to monitor contamination and baseline subtraction was used to eliminate background noise level before significant amplification occurred. Three biological replicates were used in the experiment. Primers sequences are provided in Figure 11 in the appendix.

Table 3 showed how single-stranded DNA as a percentage of total DNA was calculated for a given sample. qPCR signals (threshold cycle) were measured, using the MiniOpticon Real-Time PCR System, for the reference gene and the gene of interest (GOI) for a certain time-point. Signals were then measured again for samples digested with the StyI restriction enzyme. Next, the GOI signal was normalized to the reference gene signal. Calculation of single-stranded DNA as the percentage of the DNA present for each time-point was given by \% ssDNA = 1/(2^\Delta C_q - 1)+0.5)*100, where \Delta C_q is the difference in cycles between digested template and undigested template of a given time [10]. DNA concentrations were initially measured from a Nanodrop and were calibrated by qPCR of an amplicon on a different chromosome.

<table>
<thead>
<tr>
<th>Sample data calculation of single-stranded DNA</th>
<th>Undigested</th>
<th>Digested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Gene</td>
<td>24.04</td>
<td>23.80</td>
</tr>
<tr>
<td>Gene of Interest</td>
<td>21.73</td>
<td>25.89</td>
</tr>
<tr>
<td>C_q, RG Norm</td>
<td>2.31</td>
<td></td>
</tr>
<tr>
<td>C_q, GOI Norm</td>
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</table>
CHAPTER 3: QUANTITATIVE ANALYSIS OF RESECTED ENDS

3.1 Results and discussion

3.1.1 Effects of Tel1 on single-stranded DNA content

Resection is the process by which single-stranded DNA is created from a DSB. qPCR was used to determine the average amount of single-stranded DNA relative to total DNA at various distances distal to the MAT locus [22]. Figure 8 shows a schematic representation of the qPCR method used to monitor HO-induced DSB end resection. During the resection process, exonucleases bind at the ends of a DSB break creating single-stranded DNA. Eventually, these exonucleases will cleave past a restriction site. Since most restriction enzymes cannot cleave single-stranded DNA, qPCR signals between digested and non-digested samples should be comparable if exonucleases cleave past a restriction site (Figure 8B) or different if the resection process is hindered because the restriction enzyme can now cut double-stranded DNA (Figure 8A).

![Figure 8](image.png)

**Figure 8** qPCR assay to monitor HO-induced DSB end resection. Samples were taken (A) before resection occurred and (B) at time intervals after the break and digested with StyI. qPCR signals were compared between digested and undigested samples to determine the single-stranded DNA content.

As described in the materials and methods section, qPCR signals were measured using the MiniOpticon Real-Time PCR System. Samples were taken before induction of the HO gene and at 90 minute time intervals after induction. Signals were measured for undigested samples and samples digested with StyI restriction enzyme. The signals were then normalized to the reference gene signal. Calculation of single-stranded DNA as the percentage of the DNA present for each time-point was given by % ssDNA = 1/(2^(ΔC_q)-1)+0.5)*100, where ΔC_q is the difference in cycles between digested template and undigested template of a given time [10].

The average amount of single-stranded DNA for yeast cells used in the experiment is plotted as bar graphs in Figure 9 and Figure 10. Figure 9 shows results for TEL1 cells with or without the tel2-1 mutation. Figure 10 shows results for tel1Δ cells with or without the tel2-1 mutation. Error bars depicted in the graphs are 95% confidence interval.
meaning that the true mean of the population will fall within the interval. Table 4 contains the reference table used for multiple comparisons where the means were

---

![Figure 9](image9.png)

**Figure 9** Averaged single-stranded DNA content for TEL1 cells used in the experiment. 95% confidence intervals are depicted.

![Figure 10](image10.png)

**Figure 10** Averaged single-stranded DNA content for tel1Δ cells used in the experiment. 95% confidence intervals are depicted.
compared via Welch's t test and p-values are listed in Table 5 and 6. Group 1 refers to wild-type cells, group 2 refers to cells containing only a tel1 deletion, group 3 refers to cells containing only a tel2-1 mutation, and group 4 refers to cells that contain a tel1 deletion plus a tel2-1 mutation.

Table 4 Reference table distinguishing group identifiers for multiple comparisons. WT = Wild-type, DEL = Deletion, and MUT = Mutation

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</tr>
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It is hypothesized that single-stranded DNA that appeared before induction of the HO endonuclease is due to breaks and lesions that occurred naturally during the cell cycle. The zero point population means that of yeast strains studied exhibited no significant difference from one another, as the zero time-point p-values in Table 5 were all above the significance of 0.05. This means that all strains displayed the same amount of single-stranded DNA before the induction of the HO endonuclease. A study has reported that Tel1 contributed to the generation of single-stranded DNA during the resection process [9] and a qPCR assay was used to determine the effect a deletion of TEL1 had on single-stranded DNA content distal to the MAT locus. For this test, cells that contained only a tel1 deletion (Group 3) were compared to wild-type cells (Group 1). From Table 6, both strains exhibited a significant increase in single-stranded DNA content >90 minutes after HO induction. For any given time-point, there was no significant difference between the wild-type strains and strains with only a TEL1 deletion until six hours after induction (Table 5). These pairwise comparisons (Group 1 and Group 3) yielded p-values of 0.117, 0.051, 0.173, and 0.043 for 90, 180, 270, and 360 minutes respectively (Table 5). Consistent with a previous study that analyzed single-stranded DNA formation by Southern blot [9], our test revealed cells lacking only TEL1 showed some reduction in single-stranded DNA formation in response to a single DSB after six hours as the p-value was significant at this time-point.

This finding indicates that Tel1 has a role in DSB end processing, presumably by activating the MRX complex that generates 3’-ended single-stranded DNA at the ends of the breaks [9]. Less single-stranded DNA means reduced RPA as single-stranded DNA is often coated with RPA, which, prevents formation of secondary structures and protects single-stranded DNA from degradation [11]. This could also affect Mec1 as RPA-coated single-stranded DNA serves to recruit the Mec1 kinase to a break under DNA-damaging conditions [12].
Table 5 Pairwise (A-B) comparisons of group means for data from this work. P-values under a significance of 0.05 are highlighted in red.

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Table 6 Simultaneous multiple comparisons using Welch’s t-test and holding familywise error rate (FWER) to 0.05 via the Šidák correction. P-values of significantly different population means are highlighted in red.

<table>
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3.1.2 Effects of tel2 mutant cells on resection

Figure 9 demonstrates that cells containing only the tel2-1 mutation affected resection in a way that the average amount of single-stranded DNA distal to the MAT locus over time
was comparably less than those of the wild-type cell, as the average single-stranded DNA content >90 minutes after HO induction was less for the mutants. Pairwise comparisons (Group 1 and Group 2) yielded p-values of 1.86E-07, 9.61E-07, 7.90E-05, and 7.24E-06 for 90, 180, 270, and 360 minutes respectively (Table 5). Also at 90 minutes after induction, single-stranded DNA content for cells containing only the tel2-1 mutation was higher compared to wild-type cells (Figure 9). Reasoning for this might be due to degradation of single-stranded DNA. A report has shown that tel1Δ cells reduced RPA binding to a DSB and caused a decrease in the formation of single-stranded DNA [24]. Lower average and degradation of single-stranded DNA seen in tel2 mutant cells might be due to a side effect of the instability in the Tel1 or Mec1 proteins caused by the loss of the Tel2 protein interaction.

Because studies have shown that the tel2-1 mutation disrupts the Tel1-Tel2 protein interaction [15], cells were assayed to determine if Tel1 had any effect on resection with the tel2-1 mutation in place. The tel2-1 mutation is known to interfere with Tel1 pathway in DNA damage signaling and results from cells with only a tel2-1 mutation were expected to be similar to cells with only a tel1 deletion. Surprisingly, as shown in Figure 10 for time-points greater than 90 minutes, cells with only a tel2-1 mutation averaged much less single-stranded DNA content distal to the MAT locus than cells that contained only a tel1 deletion. P-values (1.05E-06, 5.77E-05, 9.88E-04, and 6.64E-04 for 90, 180, 270, and 360 minutes respectively) from Group 2 and Group 3 for all time-points after induction were under the significance level of 0.05 (Table 5). This implies that Tel2 might have a more involved role in the resection process independent of Tel1. It was tested to see if Tel2 involvement in resection was due to Tel1. This effect was decoupled when comparison of p-values for Group 3 and 4 in Table 5 showed there was a significant difference in single-stranded DNA content between cells that contained only a tel1 deletion and cells that contained a tel2-1 mutation plus a tel1 deletion. This result suggests that Tel2 effects on resection are independent of any effect that Tel1 might have on the resection process, suggesting that they act in two different pathways.

Comparison of single-stranded DNA content before and after induction of the HO gene revealed an important finding. P-values for cells with only a tel2-1 mutation were 0.029, 0.145, 0.182, and 0.358 for 90, 180, 270, and 360 minutes respectively (Table 6). For cells with a tel2-1 mutation plus a tel1 deletion, p-values yielded 0.333, 0.087, 0.437, and 0.011 for 90, 180, 270, and 360 minutes respectively (Table 6). All time-points after induction were above the significance level (Table 6). This showed that tel2-1 mutant cells halted the resection process, as there was no change in single-stranded DNA content over time.
CHAPTER 4: CONCLUSION

4.1 Future work

Various mutants of Tel2 have yet to be explored to see what effect the Tel2 protein might have among other PIKKs. A recent study isolated a new mutant of Tel2 with a more severe phenotype, tel2-13, comprised of eight amino acid changes [18]. They found that this mutant enhanced rapamycin lethality due to decreased levels of target of rapamycin complex 1 (TORC1), which is essential for viability [18]. Other assays to determine the rate of resection might give more quantitative information of Tel2 on DNA repair. There are also many other proteins that are involved in the resection process. By comparing the effects of tel2 mutants to those proteins that are known to be involved in resection, such as EXO1 or Fun30, light may be shed on Tel2’s role in resection [23].

4.2 Conclusion

The Tel1 and Mec1 proteins’ kinases have been shown to be involved in the resection process [8,9], and since the Tel2 protein is known to interact with these proteins [15,17] it is believed disruption of this interaction might cause defects in the resection process. It was tested to see if this interaction affected the resection process using a qPCR assay that measured single-stranded DNA formation [22] and this work showed that Tel2 might have a role in the resection process.

Results showed a slight decrease in single-stranded DNA content for cells with only a tel1 deletion after six hours compared to wild-type cells, indicating that Tel1 has a role in DSB end processing. Cells that contained only the tel2-1 mutation resulted in lowered amounts of single-stranded DNA content compared to wild-type cells and cells with only a tel1 deletion, suggesting that Tel2 might have an involved role in the resection process. Tel1’s effects on resection were decoupled from Tel2’s when there was a significant difference in single-stranded DNA content between cells that contained only a tel1 deletion and cells that contained a tel2-1 mutation plus a tel1 deletion, implying that they act in two different pathways. Comparison of single-stranded DNA content before and after induction of the HO gene revealed that tel2-1 mutant cells halted the resection process, as there was no change in single-stranded DNA content over time.

Much can still be learned about Tel2’s interaction with PIKKs and this work shed some light on the topic. During the early stages of a DSB repair, nucleases bind to the ends of a break to promote nucleolytic degradation of DNA ends to create 3’-ended single-stranded DNA. This process, known as resection, is a crucial step in determining the repair pathway of a break, as HR is the preferred model for repair. Overall, it has been shown that Tel2 might have a role in the resection process. Cells that contained the tel2-1 mutation resulted in lowered amounts of single-stranded DNA content and the results could mean that there are defects to execute and control single-stranded DNA generation at DSBs.
REFERENCES


## APPENDIX

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Figure 11 PCR primers for qPCR assay.
Figure 12 Resolved trimmed data for single-stranded DNA content.
Figure 13 Histograms of flow cytometer experiments for A) YMB103 B) YMB104 and C) YMB105 up to 8 hours with 2-hour time interval.
VITA

Tommy Nhan Trieu is a native of Mobile, Alabama. After completing his schoolwork at Baker High School in Mobile in 2008, Tommy entered the University of Alabama in Tuscaloosa, Alabama for one year. He transferred to the University of South Alabama in 2012 and received his bachelor’s degree with a major in chemical engineering and minor in chemistry. He plans to receive his master’s degree in December 2015.