VALIDATING THE ROLE OF PCL6 IN THE CELL CYCLE IN YEAST (*SACCHAROMYCES CEREVISIAE)*

BY

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A THESIS

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DEDICATION

To my daughter, Maliah Corrine Edwards, I thank you for blessing me like you did. You came into my life at what seemed to be the wrong time but you were in fact, right on time! You gave me a drive I never knew I possessed, and served as a constant reminder that what God has for me, is for me! On the nights you sensed I had to be up late doing work and reading articles, thank you for allowing me that time with little to no fuss. On days you knew mommy was overwhelmed, thank you for embracing me and cuddling me a little harder that night. You are my everything and more. I hope that once you become of age you will understand that my hard work and dedication was for you. The early mornings and late nights were for you. Everything that I have done, am doing and will do is for you. I am eternally grateful for you and this journey we are embarking on. You are my princess forever and I love you with everything in me.

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ABSTRACT

The cell cycle consists of a set of processes that result in the duplication of cellular constituents. Cyclin dependent kinases (Cdks) ensure successful progression of cells through the cell cycle. Cdk activation requires cyclin binding and phosphorylation of its appropriate substrate to tightly regulate cell cycle progression. There are six known Cdks in yeast *(Saccharomyces cerevisiae);* Cdc28, Kin28, Srb10, Ctk1, Bur1 and Pho85. Cdc28 is the only essential Cdk due to its crucial role in cell cycle progression. The remaining Cdks contribute to cell cycle progression through regulation of gene expression and cellular metabolism. The cyclin partners for Pho85 includes Pcl6. Knowledge about Pcl6 is limited. The goal of this project is explore and validate the role of Pcl6 in yeast cell biology. Based on literature review, we hypothesized that Pcl6 is regulated by components of the Nucleotide Excision Repair (NER) complex in yeast. The NER pathway in yeast is known to repair endogenous DNA damage in yeast cells. Our results confirm our hypothesis. Components of the NER pathway regulate the stability of Pcl6 in our NER mutant strains. Our studies also explore the sensitivity of exogenous DNA damage on the viability of the NER mutants. Our results show cell sensitivity to exogenous DNA damage by 4-Nitroquinoline 1-Oxide, Hydroxyurea, Hydrogen Peroxide, Nocodazole and Methyl-Methane Sulfonate. Future studies will determine the effects of endogenous and exogenous DNA damage on Pcl6 protein level.

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LIST OF ABBREVIATIONS

AP: Apurinic/Apyrimidinic endonuclease
ATP: Adenosine Triphosphate
BER: Base Excision Repair
CDK: Cyclin Dependent Kinases
CDP: Cyclobutane Pyrimidine Dimers
DNA: Deoxyribonucleic Acid
dNTP: Deoxyribonucleotide Triphosphate
G1/G2: Growth Phase
HA: Hemagglutinin
HR: Homologous Recombination
HU: Hydroxyurea
M: Mitotic Phase
MMS: Methyl-Methane Sulfate
NEF: Nucleotide Excision Factor
NER: Nucleotide Excision Repair
O.D: Optical Density
PIC: Protease Inhibitor Cocktail
RNR: Ribonucleotide Reductase
S: Synthesis Phase
TAP-tag: Tandem Affinity Purification
TOP1: Topoisomerase 1
UBL: ubiquitin-like
UV: Ultraviolet
YEPD: Yeast Extract Peptone Dextrose
YNB: Yeast Nitrogen Base
4HAQO: 4-Hydroxyaminoquinoline 1-oxide
4NQO: 4Nitro-Quinoline 1-Oxide
6-4PP: 6-4 Photoproducts
8OHdG: 8-Hydroxyguanosine

CHAPTER 1:
INTRODUCTION & BACKGROUND

Yeast Cell Cycle

The cell cycle is a regulatory system that controls the progression and ultimate fate of the cells through a series of checkpoints. The cell cycles most basic function is to duplicate its DNA and distribute the copies successfully onto the daughter cells (Alberts et al.,2002). This cell cycle is responsible for regulating cell proliferation, replication, differentiation and growth. There are four phases of the cell cycle; Growth 1 phase (G1), Synthesis phase (S phase), Growth 2 phase (G2) and Mitotic phase (M phase). Each phase plays a crucial role in the progression of the cell ranging from synthesis and DNA replication to cytokinesis (Barnum, O'Connell, 2014). G1 phase is responsible for the cells physically growing larger and replicating the cells organelles. Synthesis phase is pertinent to DNA replication as well as the microtubule structure. G2 phase allows for the cells to continue to grow and the preparation for the mitotic phase. Within the mitotic phase the cells undergo additional stages that include prophase, metaphase, anaphase and telophase. Afterwards, cytokinesis occurs resulting in 2 diploid cells which will continue to oscillate throughout the cell cycle (Barnum, O'Connell, 2014). Cell cycle events are highly conserved in yeast and mammalian cells. Most of the cell cycle genes and events are conserved in both yeast and humans (Brul, 2015). Although the cellular events are conserved, unlike mammalian cells, yeast grow via budding. As the cell grows to a certain size and has fulfilled majority of its events within the cycle, the diploid cell will begin to bud (Herskowitz, 1988). Upon the comple-

tion of the new diploid cell budding from the parent cell, it may either continue to duplicate or undergo sporulation. Sporulation is a process in which there are four new gamete cells (Herskowitz, 1988). For these four new haploid cells to be produced, there needs to be an appropriate environment for cell growth and sporulation. The process of sporulation first includes a deprived environment of nutrients, which more specifically will starve the yeast of a nitrogen source. As a result of this stressful environment, the cells exit mitosis to enter meiosis. The structure and function of these new haploid cells are constructed in both meiosis I and II(Neiman, 2005). Sporulation is advantageous because it allows researchers to select for specific mating types to generate various strains with diverse genotypes.

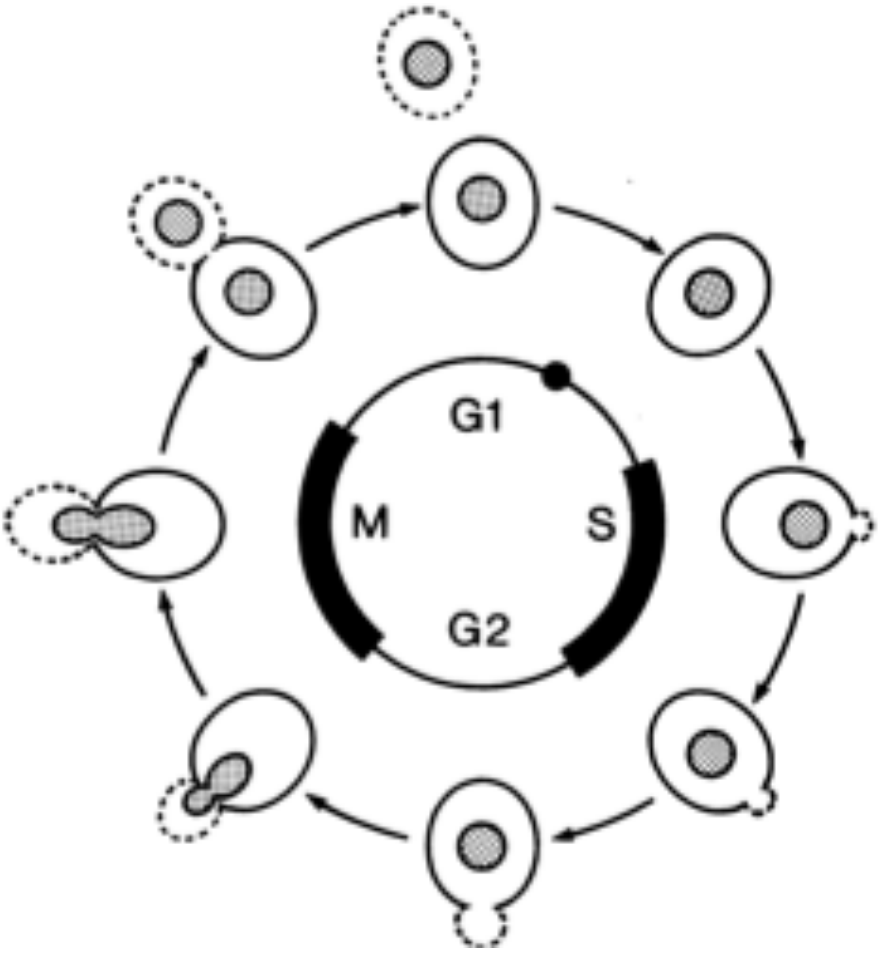


Figure 1. ***Saccharomyces cerevisiae*** **mitotic cell cycle (Herskowitz, 1988)**
This image illustrates the four phases of the yeast cell cycle and their approximate proportion to their length of time spent in each phase. The cell cycle is recognized by its four phases; G1 (growth phase), S (synthesis phase), G2 (growth phase), and M (mitotic phase). The parent cell is represented by the bolded line, and the new cell is represented with the hashed line. The shaded domain is representation of the nucleus.

CDKs and Cyclins

Cells contain proteins, which are critical in cellular processes including the Cell Cycle. The Cell Cycle is tightly regulated by Checkpoints, Cyclins and Cyclin Dependent Kinases (CDKs). The functions of these CDK's are highly conserved in both mammalian cells and yeast cells, allowing a greater insight and control of the cells system and mechanisms (Malumbres, Barbacid, 2009). The confirmation of the regulatory mechanisms being universal in all eukaryotic cells was first discovered in yeast. (Sanchez & Dynlacht, 2005). CDKs contain a serine-threonine specific catalytic core and they partner with regulatory subunits known as cyclins, which control kinase activity and substrate specificity (Lim et al., 2013). These kinases are responsible in both the growth and progression of cells through the cell cycle. There are three major phases of the cell cycle. Recognized as G1, S-phase and G2, with the CDK/Cyclin complex triggering each transition into the next (Morgan 2007). The binding of CDKs to their appropriate cyclin partners form an active complex, which allows the cell to progress into its next phase (Alberts et al., 2002). Each CDK/cyclin complex, once activated, is responsible for its unique role in the cells progression. CDKs remain constant throughout the cell cycle. There are six known CDKs in yeast.. Cdc28, Pho85, Kin28, Srb10, Ctk1 and Bur1. These CDKs are recognized for either having multiple cyclins or single cyclins that respond to transcriptional events. Cdc28 and Pho85 are the two CDKs that contain a multitude of cyclins necessary for its activation. The remaining four CDKs only need its one respective cyclin to help aide in regulation (Malumbres, 2014).

Cdc28 is the CDK essential for the start and initial commitment to the progression of the Cell Cycle. It is abbreviated for cell division control, as it is integral for cell division. When

bound to its various Cyclins, it regulates the cellular events and its ultimate fate of cell division via budding. Its cyclin partners varies throughout the different phases of the cell cycle (Andrews, Measday, 1998). In G1, Cdc28 may be bound to Cln1, Cln2, or Cln3 to regulate the commitment to the Cell Cycle. During S phase Cdc28 may be bound to Clb5 or Clb6 to aide in DNA replication. Clb1 and Clb2 help to regulate the cell cycle in preparation for the mitotic phase. Lastly, Clb3 and Clb4 are present in the M phase regulating spindle formation and cell division. As studies have shown, Cdc28 and its cyclin family are necessary for not only the commitment to the Cell Cycle but its cellular events throughout its different phases. Unlike Cdc28, Pho85 is the non-essential CDK in yeast that also has multiple cyclin partners.

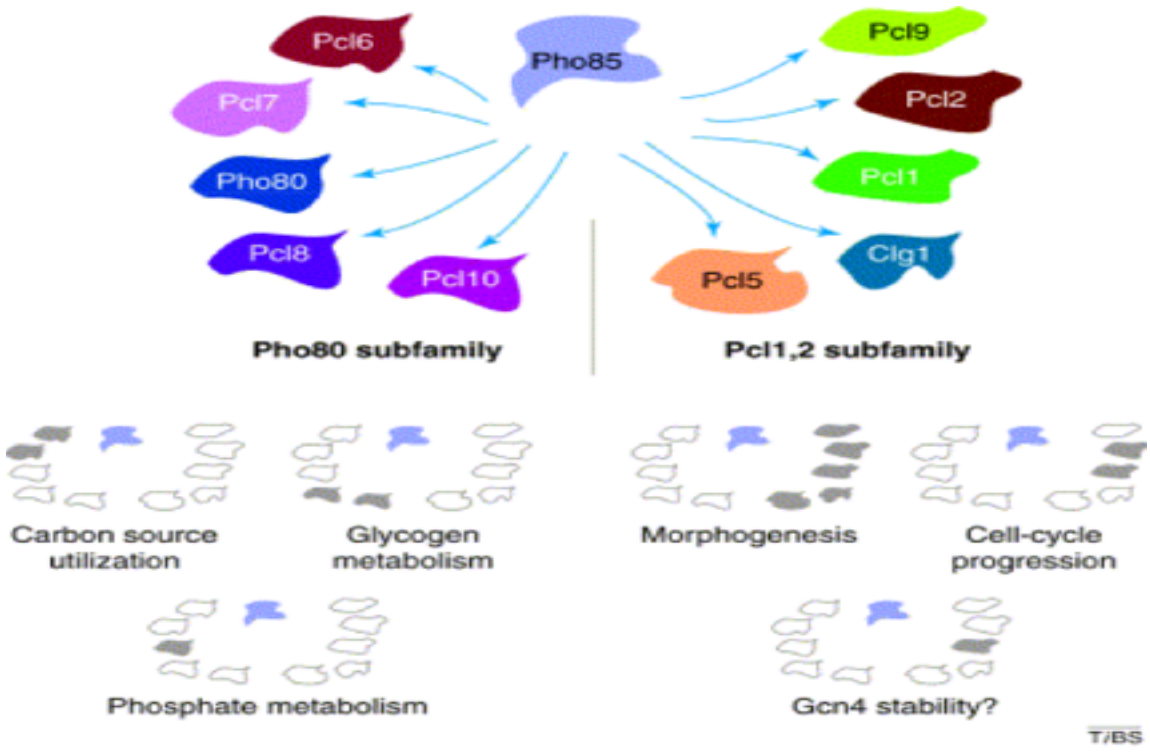


Figure 2. Pho85 cyclins and their functions (Carroll, O'Shea, 2002)
Pho85 is the non-essential CDK withs diverse functions when bound to its appropriate cyclin. This image shows the regulatory functions of each complex when either bound to Pho80 cyclins or Pcl1/2 cyclins. Pho80 Cyclins regulate Carbon source utilization, Glycogen metabolism and Phosphate metabolism. Pcl1/2 cyclins regulate morphogenesis, Cell Cycle progression and Gcn4 stability.

Pho85 and its cyclin partners

Pho85 is a non-essential CDK in yeast that function in cell metabolism and cell division. Pho85 plays a pivotal role in multiple regulatory processes including cell survival in stressful environments, cell morphogenesis as well as the cell cycle (Carroll, O'Shea, 2002). Its cyclin partners are categorized into two subfamilies known as Pho80 and Pcl1/2. Pho80 subfamily consist of Pho80, Pcl6, Pcl7, Pcl8, and Pcl10. Pcl1/2 subfamily includes Pcl1, Pcl2, Pcl5, Pcl9 and Clg1 (Measday et al., 1997). Pho80 Cyclins are known to regulate Carbon source utilization, Glycogen metabolism and Phosphate metabolism. Whereas Pcl1/2 cyclins help to regulate morphogenesis, Cell Cycle progression and Gcn4 stability. Our knowledge about some of the Pho85 cyclins (Pcl6/Pcl7) is limited. Literature shows that Pcl6 is stabilized by Elongin C (Elc1), a component of the Nucleotide Excision Repair (NER) pathway (Hyman et al., 2002).

CDK5

CDK5 is the mammalian homolog of Pho85. CDK5 forms active complexes with p35 (D.H. et al., 1999). This neuron-specific regulator plays a role in neuronal migration and neurite outgrowth. More specifically when p35 interacts with CDK5, it regulates a kinase referred to as Pak1, which influences the cytoskeleton (D.H. et a;., 1999). Both Pho85 and its homolog CDK5 have been shown to regulate the cells cytoskeleton. Data shows that CDK5 can be activated by Pho85 cyclins, Pho80 and Pcl2. Additionally, when Pho85 associated with activators of CDK5 (p35, p25), it can successfully form an active complex. This data confirms the 56% identity and 72% similarity between the two CDK's (D.H. et al., 1999).

Pcl6/Pcl7

Literature shows that there are ten Pho85 cyclin partners. These cyclins are categorized into two subfamilies; Pho80 cyclins and Pcl1/2 cyclins. Our knowledge about the regulation of Pcl6 and Pcl7 is limited. Research shows these cyclins to be relatively similar over a large region of the cyclin box, with 64% identity in over 186 amino acid residues (Measday et al., 1997). Other studies suggest similar structure and function for Pcl6 and Pcl7. The literature shows both cyclins expressed constantly throughout the cell cycle (Andrews and Measly, 1998). Pcl6 and Pcl7 are categorized as Pho80 cyclins (Wang et al., 2001). Pcl6 has been shown to be stabilized by Elc1 (Hyman et al., 2002). The stability of Pfcl6 in wild-type cells remained constant for at least 30 minutes. However after only 4 minutes, the stability of Pcl6 in *elc1* mutant cells decreased significantly (Hyman et al., 2002). It has been reported that the Pho85/Pcl7 complex results in protein kinase activity and PCL7 expression is shown throughout all phases but heavily in S phase (Wang et al., 2001). There have been studies done to better understand the role of Pcl6 and Pcl7 in glycogen metabolism. As data suggest, with the deletion of both cyclins, there becomes an accumulation of glycogen which further indicates their role in regulation of metabolism (Wang et al., 2001).

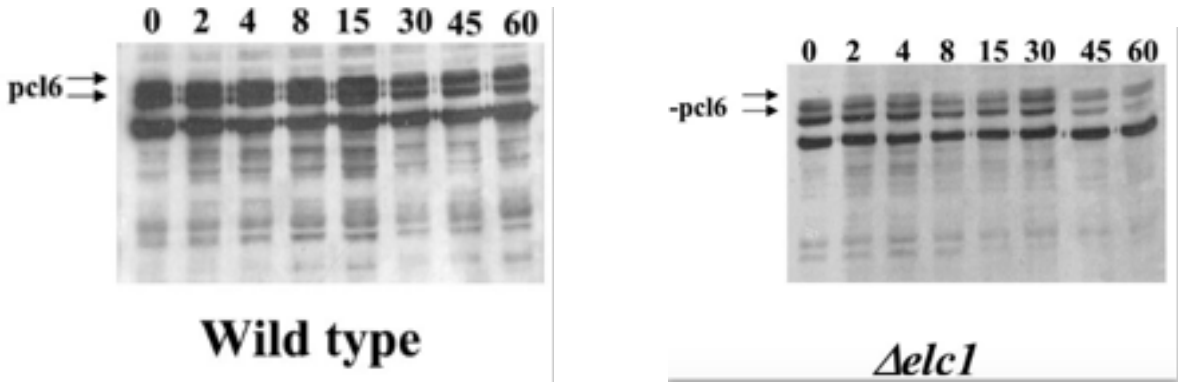


Figure. 3 Deletion of *elc1* results in rapid degradation of HA-Pcl6 *(Hyman et al.,2002)*
This is a Western Blot analysis showing the stability of HA-Pcl6 in wild-type and in elc1 mutant cells. Cells were grown to 0.7 O.D. Next the cells were treated with Cyclohexamide and harvested at indicated time points. Crude extract was obtained and 25µg of protein mass was loaded on a polyacrylamide gel and the protein levels were detected via Western Blot analysis. Results show that Pcl6 remained stable for 30 minutes, however the stability of Pcl6 decreased in *elc1* mutant after only 4 minutes.

Preliminary Data

Genotype	Pcl7-TAP 1/2 life (hrs.)	Pcl7-TAP 1/2 life (hrs.)
WT	8.4 ± 1.2	0.8 ± 0.11
Elc1	2.3 ± 0.5 *	0.5 ± 0.06*

$p < 0.05$ compared to wild-type

Table 1. Elc1 stabilizes Pcl6-TAP and Pcl7-TAP
Similar methods were done as in Hyman et al. literature to regenerate the results. This is a Western Blot analysis showing the stability of Pcl6-TAP and Pcl7-TAP in wild-type and in *elc1* mutant cells. Cells were grown to 0.7 O.D. Next the cells were treated with Cyclohexamide and harvested at indicated time points. Crude extract was obtained and 25µg of protein mass was loaded on a polyacrylamide gel and the protein levels were detected via Western Blot analysis. Results show that Pcl6 remained stable for 8.4 hours, however the stability of Pcl6 decreased in *elc1* mutant after only 2.3 hours. This data validates Elc1 in stabilizes Pcl6 and Pcl7.

DNA damage and Checkpoints in Yeast

DNA damage is an event that is inevitable throughout cellular processes. DNA damage can be attributed to a host of cellular events including mutations, stalled replication or even single/double strand breaks. The cells respond to such DNA damaging agents by inducing instability and cell arrest, due to the interrupted processes of replication and transcription (Borges, 2008). There are mechanisms at hand to rid the cell of DNA damage accumulation so that the cell may continue to work efficiently (Hoeijmaker et al., 2001). DNA damage can be induced by endogenous or exogenous damaging agents. Endogenous DNA damage occurs within the cell during normal cellular processes, which alter cellular metabolic processes through hydrolysis, oxidation, alkylation and mismatch basepairs (Hakem, 2008). Exogenous DNA damage can be induced by environmental factors such as UV irradiation, ionizing irradiation and a host of chemical agents (Hakem, 2008).

There are two mechanisms responsible for repair this DNA damage; Base Excision Repair (BER) and Nucleotide Excision Repair (NER). Base excision repair is the response to minor damage within the DNA. The DNA bases have been modified and altered due to deamination or alkylation. The cells damaged DNA is repaired with the help of enzymes Apurinic/Apyrimidinic (AP) endonuclease, DNA polymerase and DNA ligase (Samanthi, 2017). While BER is only capable of repairing minor DNA damage, NER can repair DNA damage up to 30 base pairs in length (Samanthi, 2017). NER has a crucial role in eliminating bulky adducts which furthermore results in a distortion of the DNA helix (Laat et al., 1999). The most common lesions repaired by the NER pathway are cyclobutane pyrimidine dimers (CDP's) and (6-4) photoproducts (6-4PP's).

This sort of DNA damage creates lesions as a result of UV irradiation or chemicals that mimic its effect. These CDPs can be formed by two adjacent pyrimidines bases. Evidence of these lesions are shown with high proportions of p53 mutations in photocarcinogenesis detected at bipyrimidine sites (Thiers, 2007).

The NER pathway contains two sub pathways; Global Genome Repair (GG Repair) and Transcription Coupled Repair (TC Repair). Global genome repair is responsible for repairing DNA damage in the entire genome, whereas transcription coupled repair responds to damage within the transcription region of DNA (Boiteux et al., 2013).

Nucleotide Excision Repair Pathway in Yeast

Nucleotide excision repair in yeast responds to both endogenous and exogenous DNA damage, by cleaving the damaged DNA lesions (Prakash, 2000). The genes involved in the repair pathway can be categorized into two cellular processes; the initial incision and repair reaction. The first class of genes responsible for the initial incision include genes RAD1, RAD2, RAD3, RAD4, RAD10, RAD14, and RAD25. The remainder four genes necessary for the efficacy of repair are RAD7, RAD16, RAD23 and MMS19 (Gudzer et al., 1996). Mutations to the initial incision genes may result in extreme sensitivity to UV light along with other DNA damaging agents (Reed et al., 1998). The remaining NER factors result in only a moderate level of sensitivity to UV light and chemical agents that mimc its affect (Strauss and Wilson, 1990). These factors make up three different complexes within the NER pathway; NEF4, NEF2 and Ela1.

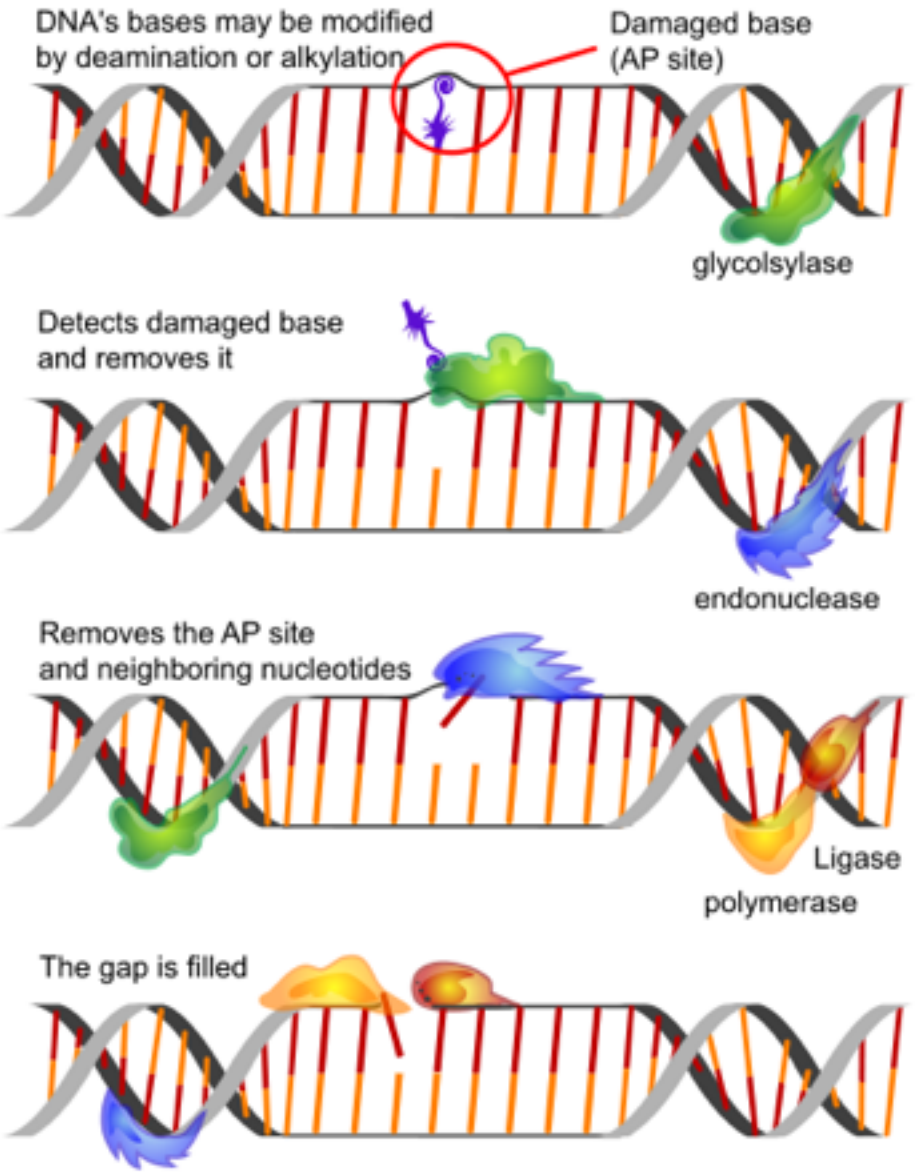


Figure 4. Base Excision Repair (Difference Between, (Samanthi, 2017)
The BER pathway, as illustrated in this image, may repair DNA damage in a series of steps. When DNA damage is detected by a DNA glycosylase, it creates an Apurinic/Apyrimidinic (AP) site. The endonuclease enzyme then makes an incision to remove the damaged fragment via a phosphodiesterase. The DNA polymerase and ligase fill the gap and seal the strand. These enzymes are necessary for the assembly of DNA and joining of the DNA strand.

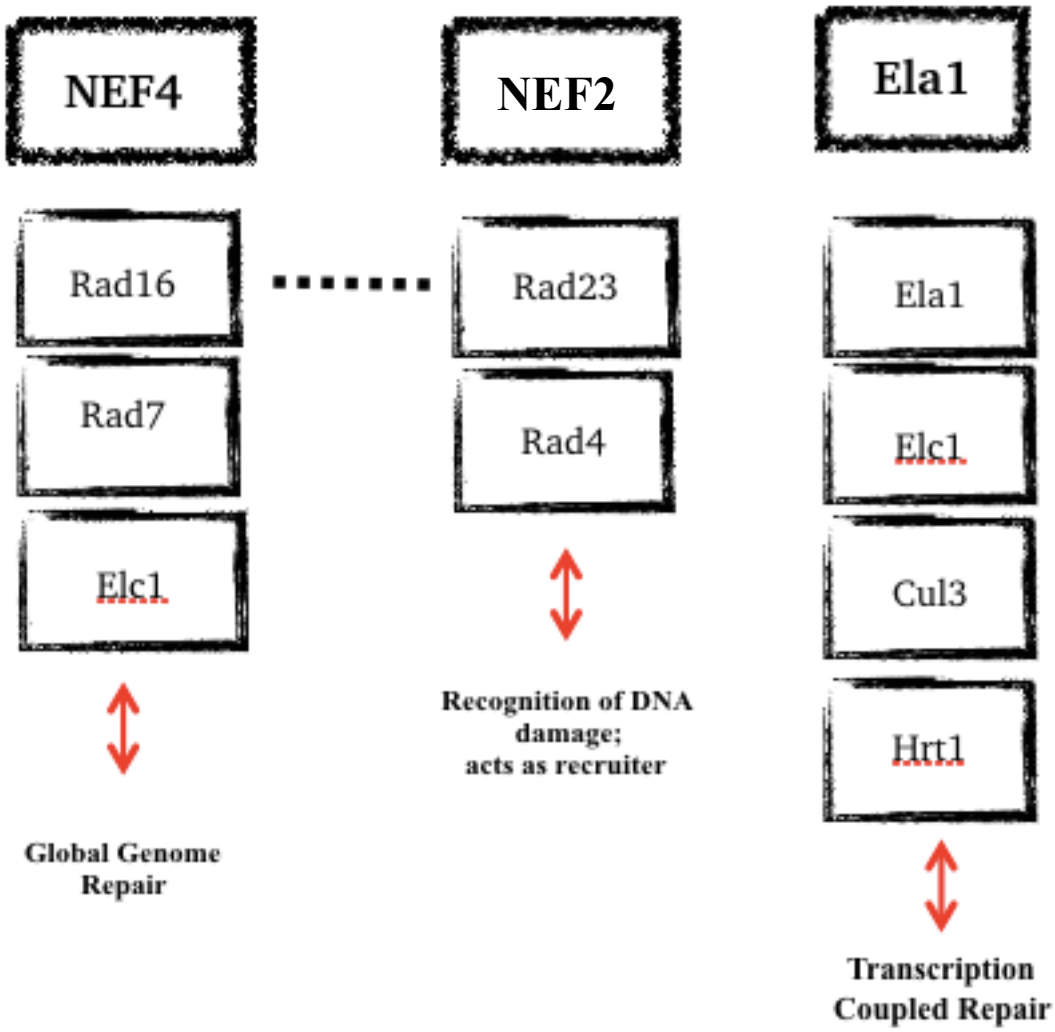


Figure 5. Nucleotide Excision Repair pathway
The NER pathway is composed of three complexes; NEF4/NEF2/Ela1. Each contain different components necessary for DNA damage repair and recovery. NEF4 is responsible for Global Genome repair. NEF2 signals the repair, acting as the recruiter. The literature suggest a possible interaction between NEF4 and NEF2 (represented by the hash line). Ela1 is responsible for Transcription Coupled repair.

NEF2/NEF4

Nucleotide excision factors (NEFs) are needed for DNA damage signaling and repair in the NER pathway. Each factor is composed of proteins that interact with one another to repair the damage DNA. NEF4 is the complex that aides in DNA damage repair in both transcribed and untranscribed regions of the cell. It contains Rad16, Rad7, and Elc1. NEF2 is the signaling complex, composed of Rad23 and Rad4. As these complexes are important for DNA damage repair, studies have shown connections to various disorders. Literature suggest a level of homology between RAD7 and RAD16 with Xeoderma Pigmentosum complementation group C (XPC) in human cells, because the phenotypes closely resemble one another (Verhage et al., 1994). XPC is an inherited condition of extreme sensitivity to UV light that affects areas of the body visible to the sun or damaged eye sight (Lehman et al., 2011). This research further indicates that the function of these yeast genes in question (RAD7/RAD16), may remain consistent in the XPC pathway (Verhage et al., 1994). NEF2 is solely responsible for signaling the DNA damage that has accumulated in the cell, serving as a recruiter. This complex is composed of Rad23 and Rad4. Together Rad23 and Rad4 bind promotors of damaged DNA to repress their transcription through an incision reaction to the UV damaged DNA (Guzder et al., 1998).

Furthermore, the NEF4 complex is responsible for the repair of the entire genome. It is composed of Rad16, Rad7 and Elc1. These core factors bind to the damaged DNA in an ATP dependent manner, as well as remodel the chromatin to allow NER machinery to complete its role (Ramsey et al., 2004). Additional research suggest that there is an interaction between both the NEF4 and NEF2 complexes (Ramsey et al., 2004). The literature shows that Rad23 is a non es-

sential factor and Rad4 interacts with NEF4 factor Rad7 via two-hybrid analysis (Ramsey et al., 2004). Data also confirms that Rad23 interacts with a 26S proteasome, and this ubiquitin-like (UBL) domain is necessary to target proteins for degradation (Ramsey et al., 2004). Furthermore, studies by Lommel et al., (2002), show that Rad23 enhances Rad4 stability, ultimately protecting it from degrading upon UV light damage.

NEF4 complex is known to enhance the role of NEF2 (Ramsey et al., 2004). Rad16 functions as a RING domain supporting NEF4 as an ubiquitin ligase, also referred to as an E3. These RING domains interact with ubiquitin conjugating enzymes known as E2 (Lorick et al., 1999). The assembly of order is unclear in which these factors bind to the damaged DNA. It also remains unclear the role of Elc1 as an NEF4 factor. Literature suggest that Elc1 helps the efficiency of NER turnover amongst the NEF4 complex. Furthermore, It has been shown that Elc1 is essential in the Ela1 complex (Ribar et al., 2006). This Elongin C is responsible for the degradation and ubiquitination of RNA Polymerase II following DNA damage (Reed et al., 1998).

This physical interaction of Rad16-Rad7-Elc1 (NEF4) regulates the levels of Rad4 (Ribar et al., 2006). The role of Rad4 is not clearly understood.

NEF	Components	Function
NEF1	Rad1, Rad10, Rad14	DNA endonucleases, DNA damage recognition
NEF2	Rad4, Rad23	DNA damage binding, Tethering of NEF1 with NEF3
NEF3	Rad2, Rad3, Ssl2, Ssl1,Tfb1, Tfb2, Tfb4	DNA endonuclease, DNA helicase
NEF4	Rad7, Rad16, Elc1	DNA dependent ATPase, DNA damage recognition
RPA	Rfa1, Rfa2, Rfa3	DNA damage recognition

Table 2. Nucleotide Excision Repair Factors in Yeast (Prakash et al., 2000)
This table represents the different repair factors in Yeast, and their role in DNA damage repair.

Elongin C

Elongin C is a highly conserved protein found in multiple protein complexes including human, rat, fly, worm and yeast cells (Hyman et al., 2002). There are three components that make up an elongin complex in mammalian cells referred to as A, B, and C. Elongin BC complex acts as a positive regulator of Elongin A, which is a crucial role in RNA polymerase II (Aso et al., 1997). Elongin BC complex are components of von Hippel Lindau (VHL), which is a tumor suppressor gene (Hyman et al., 2002). This VHL complex shares similarities with that of the ubiquitin-ligase (SCF) complex in yeast, serving as an E3 ligase (Lisztwan et al., 1999). Elongin B and C stabilizes the VHL protein to allow the binding to other ubiquitin ligase molecules Cul-2 and Rbx1 preventing the degradation of VHL (Kamura et al. 1999). Although there are no reports of Elongin B in *Saccharomyces cerevisiae* in yeast, the Elongin A and Elongin C remain.

The level of homology of Elc1 between mammalian and yeast cells is 41% identity and 71% similarities (Botuyan et al., 1999). This was further confirmed through the binding of VHL protein to that of Ela1 (Botuyan et al., 1999).

In a yeast cell model, Ela1 has a role in ubiquitination of RNA polymerase II during DNA damage response and repair (Ribar et al., 2006). Elc1 is present in two complexes in the NER. When Elc1 associates with Rad7-Rad16, it completes the NEF4 complex. This complex is necessary for Global Genome repair not specific to transcribed regions (Ramsey et al., 2004). Elc1 is present in another NER sub-complex with Ela1, Cul3 and Hrt1. These proteins are required for ubiquitination and degradation in DNA damaged yeast cells (Ribar et al., 2007). Upon this DNA

damage accumulation and distortion, Ela1 complex will repair and remove DNA lesions that have been created.

DNA damaging chemicals

There are several chemical agents that can be used to induce exogenous DNA damage. Exogenous DNA damage is the accumulation of DNA damage as a result of environmental factors, stress factors, and chemical agents (Hakem, 2008). In this study we assessed the cell viability and sensitivity to various drugs.

4-Nitroquinoline 1-Oxide (4NQO)

4NQO is a carcinogen that mimics the biological effects of UV light. This reaction is due to the binding of macromolecules that create lesions upstream from the genes that typically are responsible for regulating drug responses. Research shows that its reactive oxygen species may be a result of the DNA damage that has occurred within the cell, which then will attempt to repair the cell from 4NQO and its metabolites (Yaeno et al., 2006). Further studies show that once 4NQO is metabolized into 4-hydroxyaminoquinoline 1-oxide (4HAQO), it will produce 8-hydroxyguanosine (8OHdG) (Yaeno et al., 2006). It has been shown that the base pairing of 8OHdG with both adenine and cytosine during the process of replication will result in mutations of G:C and T:A. Furthermore these mutations were found to be present in 4NQO induced tumors (Ide et al., 2001).

Hydroxyurea (HU)

Hydroxyurea serves as a carcinogen with a dual role as a chemotherapeutic drug that interferes with DNA synthesis. Cells treated with Hydroxyurea will disrupt replication and lead to stalled replication forks and double strand breaks (Petermann et al., 2010). When the replication forks have been stalled, it will then inhibit ribonucleotide reductase (RNR). Ribonucletide reductase are enzymes that help aide in the formation of deoxynuclosidetriphosphates (dNTP). Yeast RNR's fall under class 1 of 3 which are made up of two dimeric subunits referred to as R1 and R2. These dimeric subunits require oxygen to maintain a stable tyrosyl radical. HU targets these R1 and R2 subunits by allowing the tyrosyl radical transferred to a cysteine residue in R1, generating the thiyl radical that is needed for substrate activation (Singh & Xu, 2016). Singh and Xu also found that when RNR has inhibited DNA synthesis via HU, the cells that are proliferating have undergone cell arrest in S phase. This occurs due to a decrease in dNTPs, essential for DNA replication and synthesis by DNA polymerase. The cytotoxicity of HU at various concentrations generate oxidative stress which decreases cell viability.

Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide may be used for a wide range of applications but is heavily used in biological assessments. It is an oxidizing agent, further recognized as an Reactive Oxygen Species (ROS), that can be used to induce DNA damage. Cells treated with H_2O_2 can induce DNA lesions due to cytotoxic events through environmental stress, oxidative stress or UV irradiation. These DNA lesions result in either single or double strand breaks (Driessens et al., 2009). Additional studies state that when Hydrogen Peroxide induces DNA lesions, it alters 8-oxogua-

nine (8-oxoG). This lesion traps DNA Topoisomerase I (TOP1) in cleavage complexes, disrupting its purpose of relieving chromosomal tension (Daroui et al., 2003).

Nocodazole (No)

Nocodazole is an anti-mitotic drug that interferes with the structure and function of microtubules. Microtubules are necessary for the cytoskeleton of the cell. It has been shown that yeast cells treated with Nocodazole are subjected to apoptosis.(Endo et al., 2010). The regulators of apoptosis (caspases) are conserved in yeast and mammalian cells. Caspases are a family of endoproteasomes that act as enzymes to control cell death to maintain homeostasis throughout the cell (Mcilwain et al., 2013). Literature also suggest that introducing Nocodazole to cells may trigger a series of responses resulting in endothelial barrier dysfunction (Smuriva et al., 2008). This finding has further validated the lethality of the interaction that Nocodazole may impede on cells.

Methyl-Methane Sulfonate (MMS)

Methyl-Methane Sulfonate is a chemical agent that is used to induce the effects of DNA damage as an alkylating agent and carcinogen. When MMS is introduced to the cells the DNA strand is altered from guanine to 7-methylguanine and adenine to 3-methyladenine (Lundin et al., 2005). When yeast cells are treated with MMS their sensitivity increases due to the disruption of the homologous recombination (HR) pathway, which leads to double strand breaks (Lundin et

al., 2005). Also, data suggest that when cells are treated with MMS, N^7-methylguanine accounts for 80% of the lesions and N^3-methyladenine account for 10% (Conn & Conconi, 2008).

Preliminary Data

Preliminary data influenced our research interest to investigate the NER complex response to DNA damaged cause by exogenous DNA damaging agents. **(Table 1)**. We hypothesize that the NER mutant strains will be sensitive to the exogenous DNA damaging agents tested. These strains were generated in the lab and have yet to be assessed for their sensitivity to exogenous DNA damaging agents. There are two main specific aims for this study:

Specific Aims:

1) Validate the genotype of the NER mutant strains generated for the studies.

2) Assess cell sensitivity of the NER mutant strains to selected exogenous DNA damaging chemicals.

Listed below are the chemical agents used in specific aim 2:

- 4-Nitroquinoline 1-oxide
- Hydroxyurea
- Hydrogen Peroxide
- Nocodazole
- Methyl-Methane Sulfonate

CHAPTER 2:

MATERIALS & METHODS

2.1 Experimental Approach:

Cells were grown and harvested at 30ºC and plated onto YEPD media (D-media). Following this, the yeast cells were grown overnight (o/n) in a 5mL culture at 30ºC on shaker at 130rpm. The next day, the cells were diluted into a 50mL D-Broth with a starting O.D of 0.3. Cells were grown to 0.7 O.D reading at 600nm. 2ul of a 10-fold serial dilution were spotted on the appropriate plates. Cells were then incubated for up to 3 days at 30ºC. Pictures were taken after each day. All cells were grown at 30ºC at 130rpm unless otherwise stated. This protocol was used to address both specific aims.

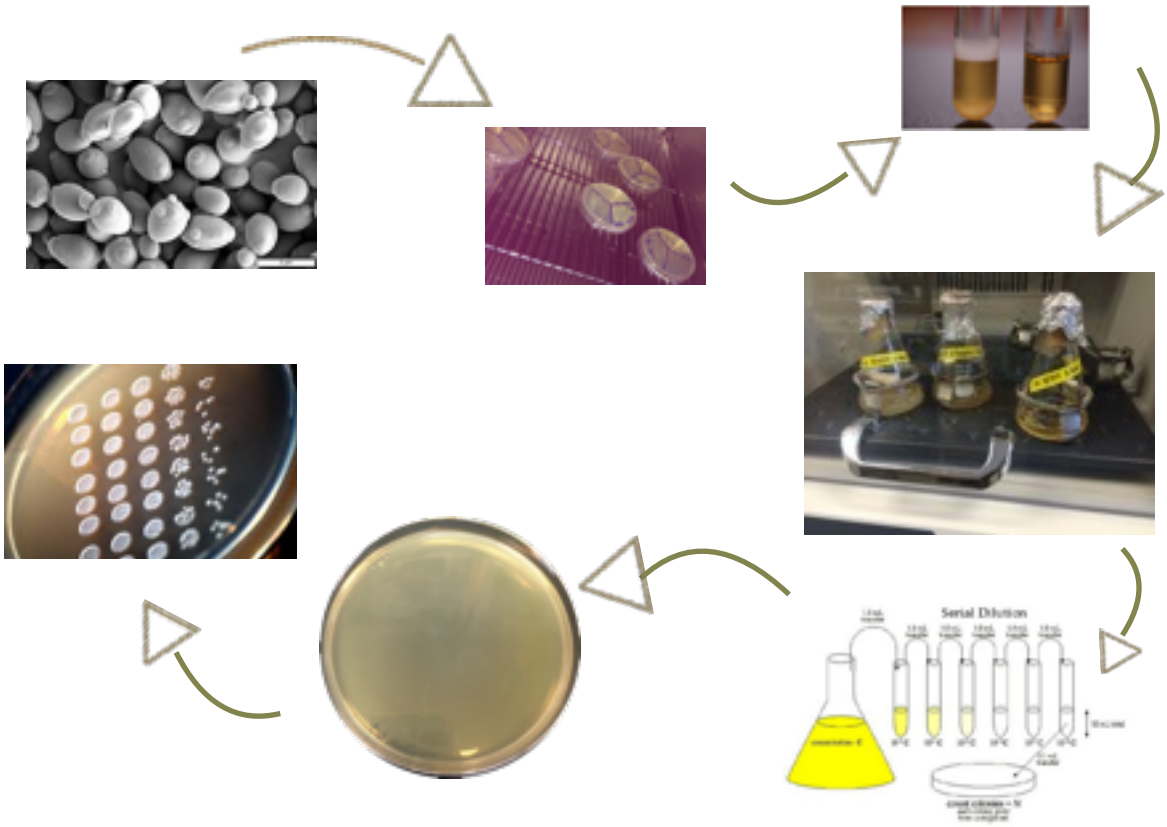


Figure 6. Experimental Approach
Cells were grown on D-plates at 30ºC for 24 hours. Next day, a colony was inoculated into a 5mL D-Broth and grown o/n at 30ºC with shaking at 130rpm. After 24 hrs., the appropriate volume was diluted into a 50mL broth to obtain a starting O.D reading of 0.3. Cells were then grown until an O.D. of 0.7 (log phase). 2ul of a 10-fold serial dilution of the culture were spotted onto appropriate media for the studies.

Materials & Methods:

2.2 ***Yeast Strains***

Yeast Strains	***Genotype***	**Source**
(1) JC746-9D	***MATα leu2-3, 112 ura3-52 can1-100 trp1 his3-11,15***	Aikins/Cannons Lab
(2) JC1447-2C	***MATα PCL6-TAP: HIS3 his3 leu2 ura3***	Aikins/Cannons Lab
(3) JC1448-3A	***MATα PCL7-TAP: HIS3 his3 leu2 ura3***	Aikins/Cannons Lab
(4) JC1570	***MATα PCL6-TAP: HIS3 elc1:: kanMX4 his3 leu2 ura3***	Aikins/Cannons Lab
(5) JC1574-2A	***MATα PCL6-TAP: HIS3 ela1:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0***	Aikins/Cannons Lab
(6) JC1576-2A	***MATα PCL6-TAP: HIS3 rad7:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0***	Aikins/Cannons Lab
(7) JC1575-1A	***MATα PCL6-TAP: HIS3 rad16:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0***	Aikins/Cannons Lab
(8) JC1579-1A	***MATα PCL6-TAP: HIS3 rad23:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0***	Aikins/Cannons Lab

Table 3. Yeast strains used for this work

Yeast media

YEPD media

The yeast strains are grown in YEPD media (1% Yeast Extract, 2% Peptone, 2% Dextrose) or synthetic complete (SC) medium which contains yeast nitrogen base lacking the appropriate amino acids that will be used as the basis for our strain selection. YEPD is non-selective media that contains all of the nutrients necessary for cell growth. All D-media was prepared in total volumes of 250mL and autoclaved. Plates were poured and allowed to solidify at room temperature.

-HIS media

Many of the JC746-9D background strains, excluding JC746-9D, JC1447-2C and JC1448-3A, contain the HIS3 marker. HIS3 is a marker for the amino acid histidine. All strains containing HIS3 are expected to grow on -HIS media because it will compensate for the absence of histidine in the minimal media. This drop-out medium was prepared with 5g agar and 5g of dextrose, which were then autoclaved in up to 250mL dH_2O. Upon autoclaving, the minimal media cooled down before adding the following filtered additional components; 6.25mL of Yeast Nitrogen Base (YNB), 2.5mL -HIS amino acid mixture, 1.25mL tyrosine, 1mL uracil and .25mL adenine were added to this medium. Plates were poured and allowed to solidify at room temperature.

G418 media

Geneticin, formally known as G418 is a chemical agent that inhibits protein synthesis. This media was used to select NER mutant strains containing the *kanMX4* (Kanamycin) cassette. Kanamycin is an antibiotic that is resistant to G418, therefor strains containing the *kanMX4* (strains 4-8 listed in **Table 3**) were expected to grow on this selective plate. The 250mL media was prepared by the addition of 2.5g Yeast Extract, 5g Peptone, 5g Dextrose and 5g Agar. After autoclaving 1.12mL of the active G418 stock was added. The active concentration of G418 ranges from [100µg/µl] to [800µg/µl]. Our stock concentration was [50mg/ml]. The final concentration is 0.3mg/mL.

4-NQO media

4NQO is a drug that mimics the effects of UV light, inducing DNA damage via exogenous damage. From [15mg/ml] of 4NQO stock, the appropriate volume was added to D-media after autoclaving to obtain a final concentration of [2µg/ml], [4µg/ml], and [8µg/ml].The media was mixed well and plates were poured and allowed to solidify.

Hydroxyurea Media

Hydroxyurea is an anticancer drug that is responsible for inhibiting DNA synthesis. Once the YEPD media was prepared and cooled, the approximate volume of a 2M stock was added to the media to obtain a final concentration of [10mM], [25mM] and [50mM]. The media was mixed well and plates were poured to solidify.

H_2O_2 Media

Hydrogen Peroxide disrupts the cells viability by inducing both single and double strand DNA breaks. This occurs because this chemical agent mimics the effects of environmental stress, UV light or irradiation. From an 8.8M (30% H_2O_2) stock of Hydrogen Peroxide, we prepared D-media and after autoclaving, the appropriate volume of H_2O_2 was added to obtain a final concentration of [.25mM], [.5mM], [1mM], and [2mM] for further analysis.

Nocodazole Media

An anti-mitotic drug that play a role in cell arrest. When this chemical agent is added to the cells it is expected to interrupt polymerization of microtubules, necessary for the structure and function of the cytoskeleton. To further analyze this, Nocodazole was added to the YEPD to reach a final concentration of [15µg/ml].

Methyl-Methane Sulfonate Media

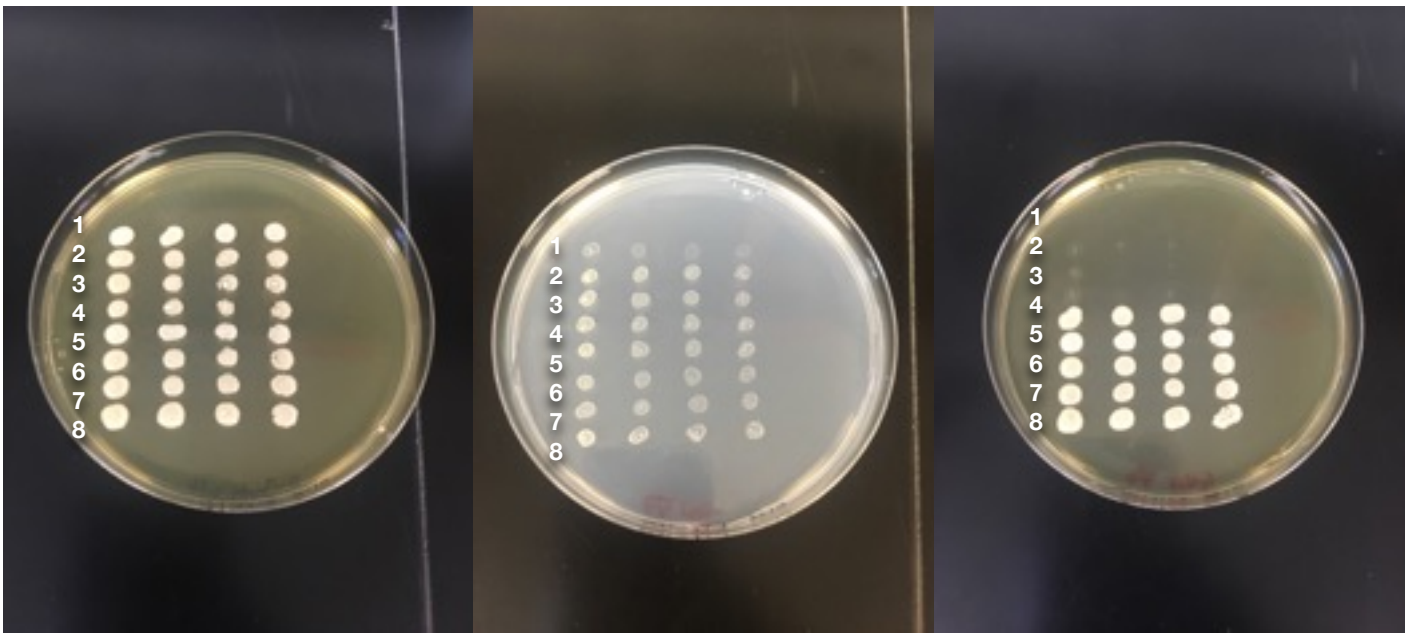
Methyl-Methane Sulfonate (MMS) is a carcinogen responsible for inducing DNA damage via double strand breaks and lesions. Upon this damage, DNA replication is blocked and base pairs get altered. 99% stock was prepared prior to the preparation of MMS media. From this stock we added the appropriate volume to the YEPD media to reach a final concentrations of [0.01%] , [0.02%] and [0.5%].

CHAPTER 3:
RESULTS

Results:

Genotype confirmation of yeast strains

Cells were grown in YEPD media (as described in Materials and Methods). 2µl of a 10-fold dilution was spotted on -HIS, G418 and D-Plate (control plate). All strains were expected to grow on the D-Plates. Only strains with the HIS3 marker were expected to grow on the -HIS plate and likewise, strains containing *kanMX4* were expected to grow on the G418 plate. The presence of the kanMX4 in the strain genome causes resistance to G418.



1	JC746-9D	MATα leu2-3, 112 ura3-52 can1-100 trp1 his3-11,15
2	JC1447-2C	MATα PCL6-TAP: HIS3 his3 leu2 ura3
3	JC1448-3A	MATα PCL7-TAP: HIS3 his3 leu2 ura3
4	JC1570	MATα PCL6-TAP: HIS3 *elc1.:: kanMX4 his3 leu2 ura3*
5	JC1574-2A	MATα PCL6-TAP: HIS3 *ela1:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
6	JC1576-2A	MATα PCL6-TAP: HIS3 rad7:: *kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
7	JC1575-1A	MATα PCL6-TAP: HIS3 rad16:: *kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
8	JC1579-1A	MATα PCL6-TAP: HIS3 rad23:: *kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*

Figure 7. Yeast strains genotype confirmed

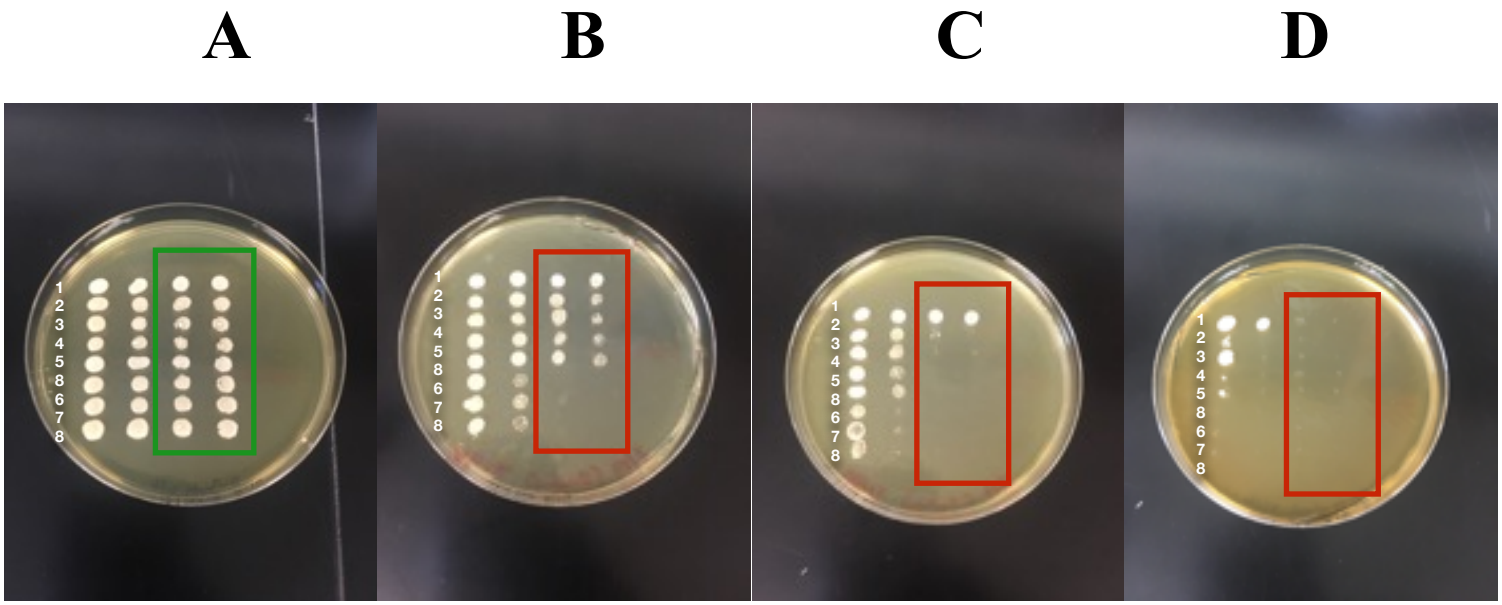
All plates were prepared accordingly (see materials and methods) and allowed to solidify at room temperature. In the Genetic Confirmation of our NER mutant strains, we expected all NER mutant strains to be viable on YEPD media **(Fig. 7A)**. We were expecting cell viability of all strains on -HIS excluding *WT* (row 1). Furthermore we expected no growth of the *WT* (row 1) or Pcl6-TAP and Pcl7-TAP controls (row 2-3) on G418 plates.

3.1 Genetic Analysis Results

Our results were consistent with our expectations. Strains 1-3 were unable to grow on the G418 plates due to the absence of the *kanMX4* cassette, which is necessary for G418 resistance. Strain #1 did not grow on both -HIS and G418, which was also expected, because its missing both HIS3 and *kanMX4* markers, necessary for growth on both plates. All strains grew on YEPD rich media as expected (control plate). Our results successfully confirmed the genotype of the strains for the experiment.

3.2 Assessing the Sensitivity of Yeast Cells to Chemical Agents

After confirming the genotype of the NER mutant strains we wanted to assess the sensitivity of the mutant strains to DNA damaging chemicals. Plates were prepared with the selected chemical agent. Cells were grown overnight in a 5mL culture. The next day the cells were diluted into a 50mL culture with starting O.D at 0.3 and grown to exponential phase (O.D of 0.7). 2µl of a 10-fold serial dilution were spotted on the plates containing the appropriate concentrations of the drug.



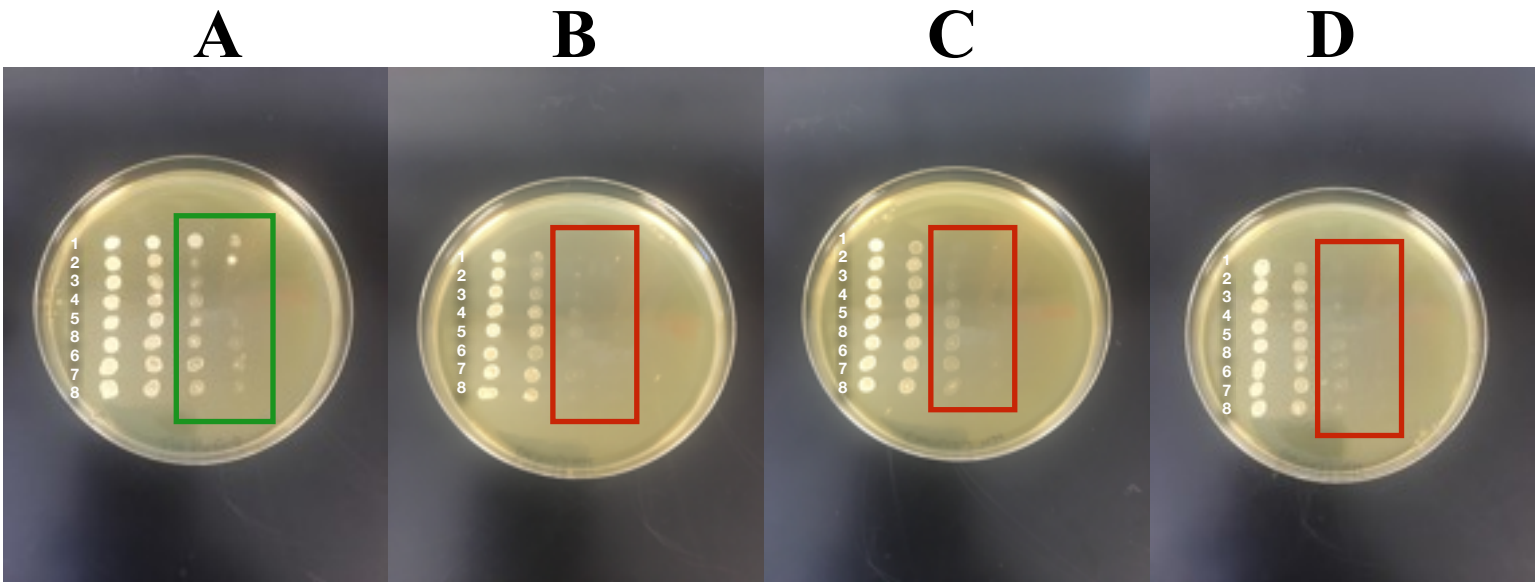
YEPD **[2µg/µl]** **[4µg/µl]** **[8µg/µl]**

1	JC746-9D	MATα leu2-3, 112 ura3-52 can1-100 trp1 his3-11,15
2	JC1447-2C	MATα PCL6-TAP: HIS3 his3 leu2 ura3
3	JC1448-3A	MATα PCL7-TAP: HIS3 his3 leu2 ura3
4	JC1570	MATα PCL6-TAP: HIS3 *elc1.:: kanMX4 his3 leu2 ura3*
5	JC1574-2A	MATα PCL6-TAP: HIS3 *ela1:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
6	JC1576-2A	MATα PCL6-TAP: HIS3 *rad7:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
7	JC1575-1A	MATα PCL6-TAP: HIS3 *rad16:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
8	JC1579-1A	MATα PCL6-TAP: HIS3 *rad23:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*

Figure 8. NER mutant strains are sensitive to 4NQO at [4µg/µl] and [8µg/µl]
All plates were prepared accordingly (see materials and methods) and allowed to solidify at room temperature. In the 4NQO sensitivity assay we introduced three different concentrations; [2µg/µl] ,[4µg/µl], and [8µg/µl]. We expected all NER mutant strains to be viable on YEPD media. We also expected an increase in cell sensitivity as the drug concentration increased. Furthermore we expected not only the *WT* (row 1) to remain viable, but also the Pcl6-TAP and Pcl7-TAP controls (row 2-3) to show cell viability since the NER repair machinery is not compromised in these strains.

Cell Sensitivity to 4NQO:

4NQO is a chemical agent that induces DNA damage by creating lesions, repaired through the NER pathway. This drug induces exogenous DNA damage through binding to macromolecules resulting in bulky adducts. Our results show that as the 4NQO concentration increased, the cell sensitivity increased as well. Greater level of sensitivity was evident at the higher concentrations of [4µg/µl] and [8µg/µl] **(Fig. 8B-C)**. Interestingly only strains 6-8 were sensitive to 4NQO at the lowest tested concentration of 2µg/µl. Perhaps the mutated NER components (Rad7, Rad16, Rad23) are more essential in NER response to DNA damage. Furthermore our control strain (strain #1) grew on all tested plates. This could be due to the fact that strain #1 had an intact NER complex, that was able to fix the DNA damage incurred by the 4NQO, thus leading to cell growth. Overall our data confirms that the DNA damage induced by 4NQO is repaired by the NER pathway. We were expecting all strains to grow on the rich media (YEPD). Furthermore we expected that our three controls (*WT,* Pcl6-TAP, Pcl7-TAP) would grow because their NER repair machinery is not compromised. Our results were consistent with these expectations for WT until our highest concentration of [8µg/ml]. Additionally our strain #2-3 were only viable at [2µg/ml].



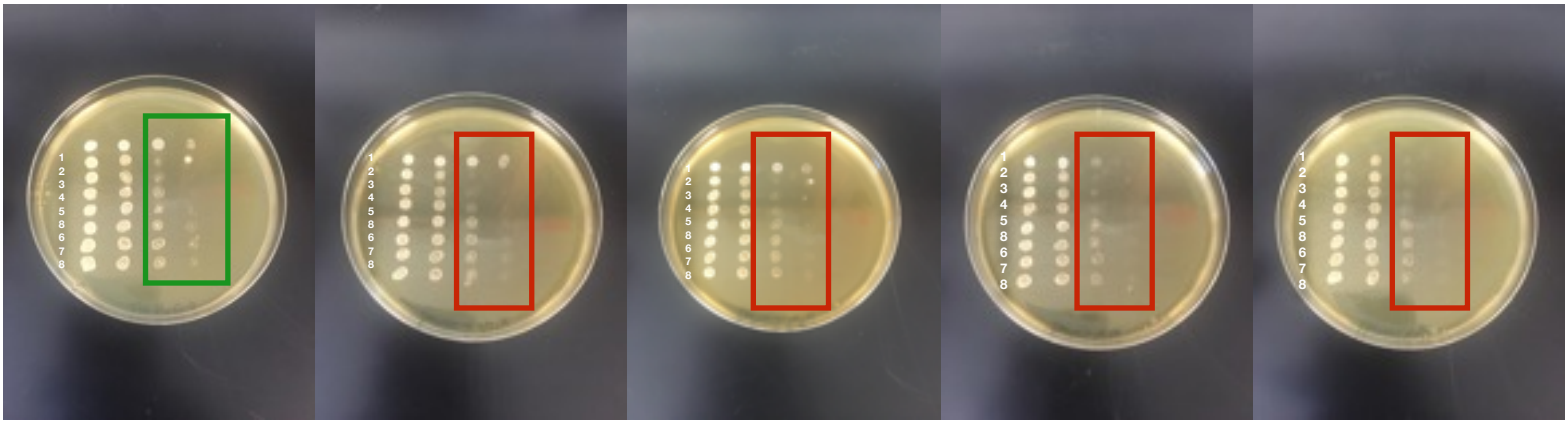
1	JC746-9D	MATα leu2-3, 112 ura3-52 can1-100 trp1 his3-11,15
2	JC1447-2C	MATα PCL6-TAP: HIS3 his3 leu2 ura3
3	JC1448-3A	MATα PCL7-TAP: HIS3 his3 leu2 ura3
4	JC1570	MATα PCL6-TAP: HIS3 *elc1.:: kanMX4 his3 leu2 ura3*
5	JC1574-2A	MATα PCL6-TAP: HIS3 *ela1:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
6	JC1576-2A	MATα PCL6-TAP: HIS3 *rad7:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
7	JC1575-1A	MATα PCL6-TAP: HIS3 *rad16:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
8	JC1579-1A	MATα PCL6-TAP: HIS3 *rad23:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*

Figure 9. Cell sensitivity to Hydroxyurea (HU)

All plates were prepared accordingly (see materials and methods) and allowed to solidify at room temperature. In the HU sensitivity assay we introduced three different concentrations; [10mM] , [25mM], and [50mM]. We expected all NER mutant strains to be viable on YEPD media. We also expected an increase in cell sensitivity as the drug concentration increased. Furthermore we expected not only the *WT* (row 1) to remain viable, but also the Pcl6-TAP and Pcl7-TAP controls (row 2-3) to show cell viability since the NER repair machinery is not compromised in these strains.

Cell Sensitivity to HU:

Hydroxyurea inhibits the replication of DNA necessary for synthesis, which further inhibits the production of dNTP's. The dNTP's are crucial in the sense that they act as building blocks for DNA. Our studies show that at all tested concentrations of HU resulted in a noticeable level of sensitivity in comparison to our control plate (YEPD). The tested concentrations were drug was introduced at [10mM], [25mM] and [50mM], yet the level of sensitivity appears to have remained constant throughout **(Fig. 9A-C)**. Perhaps the *WT* strains responded to this drug similarly with all the strains because at these particular concentrations, this drug may be lethal to the cells and their repair machinery. For this assay we were expecting all strains to grow on the rich media (YEPD). Furthermore we expected that our three controls (*WT,* Pcl6-TAP, Pcl7-TAP) would grow because their NER repair machinery is not compromised. Our results were not consistent with these expectations at all concentrations.



1	JC746-9D	MATα leu2-3, 112 ura3-52 can1-100 trp1 his3-11,15
2	JC1447-2C	MATα PCL6-TAP: HIS3 his3 leu2 ura3
3	JC1448-3A	MATα PCL7-TAP: HIS3 his3 leu2 ura3
4	JC1570	MATα PCL6-TAP: HIS3 *elc1.:: kanMX4 his3 leu2 ura3*
5	JC1574-2A	MATα PCL6-TAP: HIS3 *ela1:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
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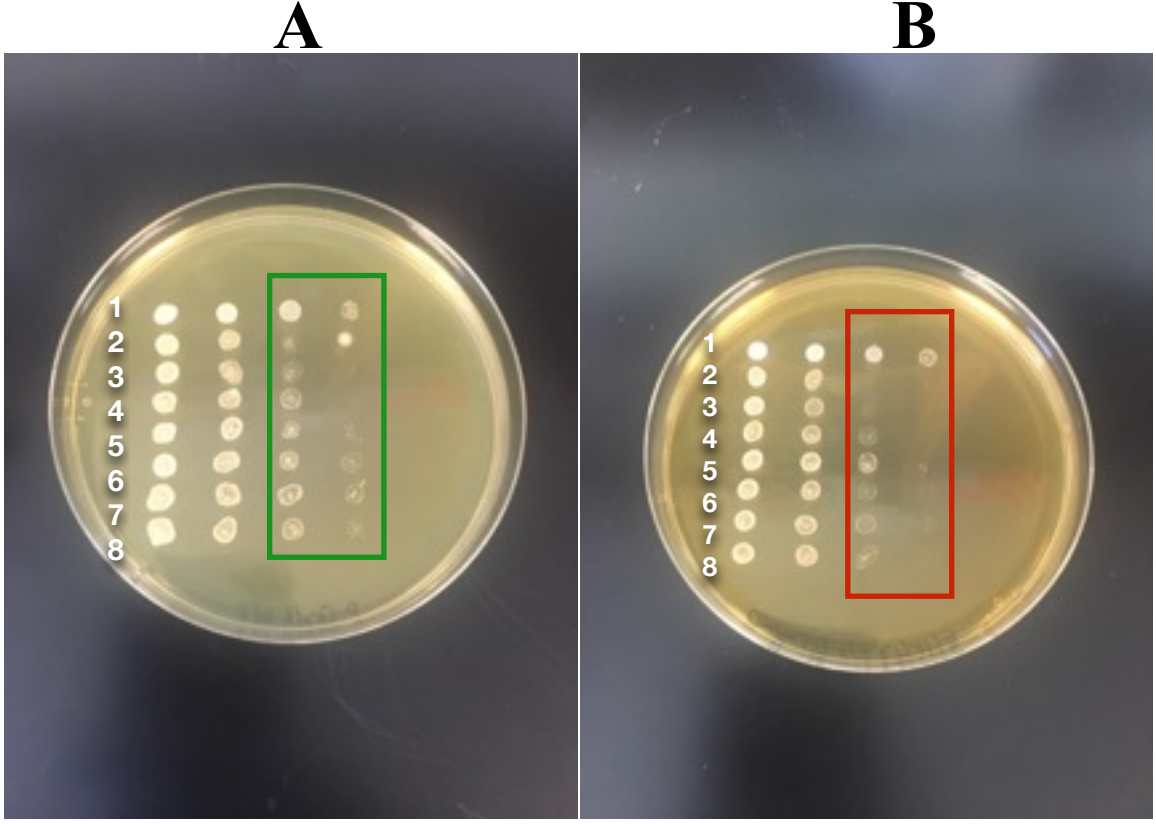
7	JC1575-1A	MATα PCL6-TAP: HIS3 *rad16:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
8	JC1579-1A	MATα PCL6-TAP: HIS3 *rad23:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*

Figure 10. Cell Sensitivity to Hydrogen Peroxide (H_2O_2)
All plates were prepared accordingly (see materials and methods) and allowed to solidify at room temperature. In the H_2O_2 sensitivity assay we introduced four different concentrations; [.25mM] , [.5mM], [1mM], and [2mM]. We expected all NER mutant strains to be viable on YEPD media. We also expected an increase in cell sensitivity as the drug concentration increased. Furthermore we expected not only the *WT* (row 1) to remain viable, but also the Pcl6-TAP and Pcl7-TAP controls (row 2-3) to show cell viability since the NER repair machinery is not compromised in these strains.

Cell Sensitivity to H_2O_2:

Hydrogen Peroxide is an oxidizing agent that may induce both single or double strand breaks as a response to DNA damage. The cells were spotted on plates containing different concentrations of H_2O_2 at [.25mM], [.5mM], [1mM] and [2mM]. After thorough analysis, our data shows cell sensitivity on the treated plates at high concentrations tested; [1mM] and [2mM] when compared to our YEPD control plate. Furthermore it appears that at lower concentrations of [.25mM] and [.5mM], the cell viability seems relatively similar to that of our YEPD control. Interestingly our control strains had very little to no growth, indicating a greater level of sensitivity **(Fig. 10B-E)**. As stated earlier, the NER pathway is pivotal for DNA damage repair. If these particular strains are not missing necessary components of its repair machinery, then we should expect growth. For this assay we were expecting all strains to grow on the rich media (YEPD). Furthermore we expected that our three controls (*WT,* Pcl6-TAP, Pcl7-TAP) would grow because their NER repair machinery is not compromised. Our results were consistent with these expectations for WT until our highest concentration of [.25mM] and [.5mM]. Additionally our strain #2-3 were not viable at any test concentrations.



YEPD **[15µg/ml]**

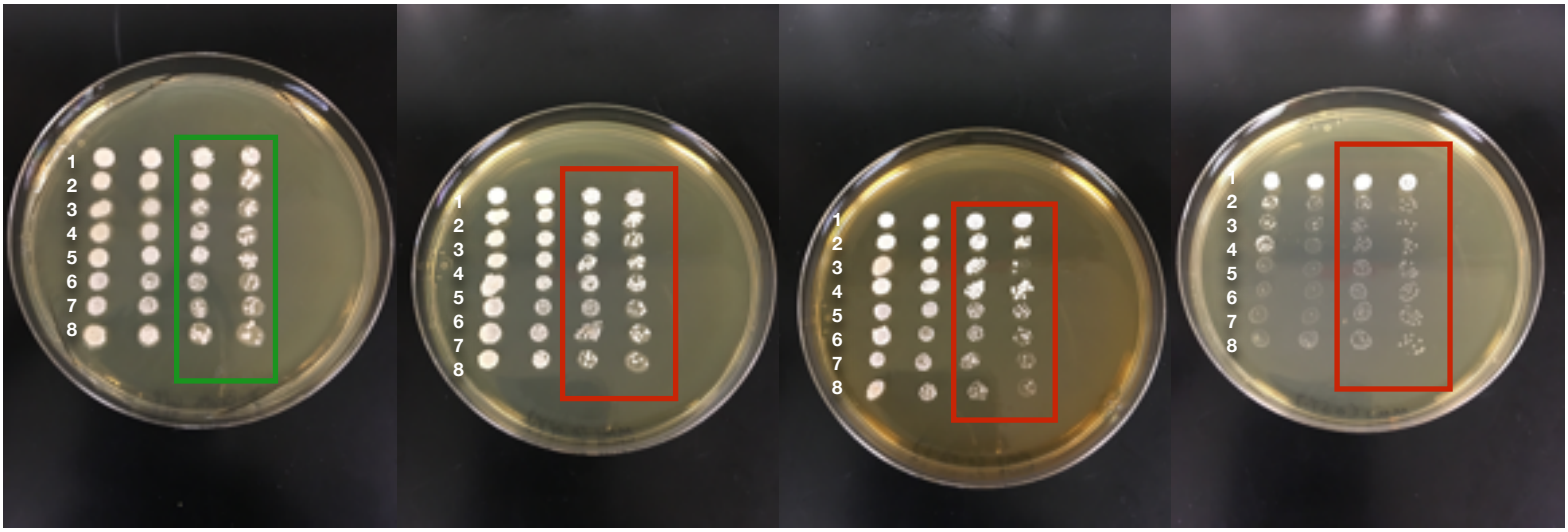
1	JC746-9D	MATα leu2-3, 112 ura3-52 can1-100 trp1 his3-11,15
2	JC1447-2C	MATα PCL6-TAP: HIS3 his3 leu2 ura3
3	JC1448-3A	MATα PCL7-TAP: HIS3 his3 leu2 ura3
4	JC1570	MATα PCL6-TAP: HIS3 *elc1.:: kanMX4 his3 leu2 ura3*
5	JC1574-2A	MATα PCL6-TAP: HIS3 *ela1:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
6	JC1576-2A	MATα PCL6-TAP: HIS3 *rad7:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
7	JC1575-1A	MATα PCL6-TAP: HIS3 *rad16:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
8	JC1579-1A	MATα PCL6-TAP: HIS3 *rad23:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*

Figure 11. Cell sensitivity to Nocodazole (No)

All plates were prepared accordingly (see materials and methods) and allowed to solidify at room temperature. In the No sensitivity assay we introduced one final concentration; [15µg/ml]. We expected all NER mutant strains to be viable on YEPD media. We also expected an increase in cell sensitivity as a response to the treated plate. Furthermore we expected not only the WT (row 1) to remain viable, but also the Pcl6-TAP and Pcl7-TAP controls (row 2-3) to show cell viability since the NER repair machinery is not compromised in these strains.

Cell Sensitivity to No:

Nocodazole induces apoptosis in cells. This chemical agent is an anti-mitotic drug that interrupts polymerization of microtubules, which is essential for structure and function of cell cytoskeleton. Cells were grown and spotted on Nocodazole plates at a final concentration of [15µg/ml].We used this concentration due to studies reported in the literature (Clémenson, Marsolier-Kergoat,2006). Our data shows a noticeable cell sensitivity of all tested strains compared to the control plate. Interestingly our strains #2 and 3 had the most level of sensitivity. This was not expected since the NER complex is intact in both strains. Perhaps, this is due to its genotype or their repair and recovery machinery are not suitable for this kind of DNA damage. Additionally strain #5 (as listed in **Table 3.**) having a deletion of a component in its repair complex, appears to be a bit more resistant to the tested Nocodazole concentration indicated by its cell growth. Both JC746-9D and JC1570 have a lower level of sensitivity in comparison to the remainder NER mutants. For this assay we were expecting all strains to grow on the rich media (YEPD). Furthermore we expected that our three controls (*WT,* Pcl6-TAP, Pcl7-TAP) would grow because their NER repair machinery is not compromised. Our results were consistent with these expectations for WT at the final concentration of [15µg/ml]. Additionally our strain #2-3 were not viable as expected.



1	JC746-9D	MATα leu2-3, 112 ura3-52 can1-100 trp1 his3-11,15
2	JC1447-2C	MATα PCL6-TAP: HIS3 his3 leu2 ura3
3	JC1448-3A	MATα PCL7-TAP: HIS3 his3 leu2 ura3
4	JC1570	MATα PCL6-TAP: HIS3 *elc1.:: kanMX4 his3 leu2 ura3*
5	JC1574-2A	MATα PCL6-TAP: HIS3 *ela1:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
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7	JC1575-1A	MATα PCL6-TAP: HIS3 *rad16:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*
8	JC1579-1A	MATα PCL6-TAP: HIS3 *rad23:: kanMX4 his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*

Figure 12. Cell sensitivity to 0.5% Methyl-Methane Sulfonate (MMS)
All plates were prepared accordingly (see materials and methods) and allowed to solidify at room temperature. In the MMS sensitivity assay we tested three different concentrations; [0.01%] , [0.02%], and [0.5%]. We expected all NER mutant strains to be viable on YEPD media. We also expected an increase in cell sensitivity as the drug concentration increased. Furthermore we expected not only the *WT* (row 1) to remain viable, but also the Pcl6-TAP and Pcl7-TAP controls (row 2-3) to show cell viability since the NER repair machinery is not compromised in these strains.

Cell Sensitivity to MMS:

Our final chemical agent tested in the cell sensitivity assay was Methyl-Methane Sulfonate (MMS). Methyl-Methane Sulfonate creates DNA lesions and double strand breaks. Cells were treated with MMS at concentrations of [0.01%], [0.02%], and [0.5%]. At [0.01%] the cells viability appear to be relatively similar to the YEPD control plate. Strains 5-8 showed the most noticeable cell sensitivity at [0.02%] MMS **(Fig. 12C).** This affirms the need of these NER complexes in fixing DNA damage caused by MMS. Additionally strain #4 appears to be less sensitive compared to the other NER mutant strains. Additionally, at our highest concentration of [0.5%], the NER mutants exhibited a greater level of cell sensitivity. At a final concentration of [0.5%] strain #1 was noticeably resistant to the tested MMS concentration. This affirms the function of an intact NER complex responding and fixing DNA damage induced by MMS. Strain #1 has an intact NER complex. For this assay we were expecting that all strains will grow on the rich media (YEPD). Furthermore we expected that our three controls (*WT,* Pcl6-TAP, Pcl7-TAP) would grow because their NER repair machinery is not compromised. Our results were consistent with these expectations for WT at all concentrations. Additionally our strain #2-3 were only viable at [0.01%] and [0.02%].

CHAPTER 4:
SUMMARY

Discussion

The goal of this project was to gather information about Pcl6 to enhance our understanding of its role in yeast cell biology. Preliminary data supported the idea of Pcl6 functioning in the NER pathway. To proceed with this project we confirmed the genotype of the yeast strains generated. This was done by selecting the cells on a -HIS and G418 plate., with YEPD media as the control. There were a series of trials to confirm this data. Next we tested the sensitivity of the cells to DNA damaging chemical agents. These chemical agents included 4-Nitroquinoline, Hydroxyurea, Hydrogen Peroxide, Nocodazole and Methyl-Methane Sulfonate. Each of these drugs have their own unique role in inducing DNA damage. All the strains were mostly sensitive to the drugs at high concentrations. Our 4NQO studies showed [8µg/ml] to be the concentration with the most sensitivity. Our data shows that the NER mutant strains JC1576-2A, JC1575-1A, and JC1579-1A **(Fig. 8)** appear to have an extreme or much higher level of sensitivity to 4NQO. Hydroxyurea studies showed a moderate level of sensitivity because cell viability appeared to remain constant in spite of differential concentrations of the drug **(Fig. 9)**. Further investigations of the cell sensitivity assay led to the treatment of NER mutant strains with Hydrogen Peroxide. We found that our lower concentrations of [.25mM] and [.5mM] had very little effect on the cells viability in comparison to the YEPD control. This data also shows that the cells experienced induced DNA damage at higher concentrations of [1mM] and [2mM] by assessing the cell sensitivity **(Fig. 10)**. In continuing to analyze cell sensitivity, we treated plates with Nocodazole at a fi-

nal concentration of [15µg/ml], which showed selective sensitivity to all strains excluding *WT* and strain 5 **(Fig. 11).** In our final cell sensitivity assay cells were exposed to MMS. Our results show that there was relatively similar cell viability at [0.01%] compared to the YEPD control plate. In addition, this data also shows selective sensitivity amongst half (strains 1-4 and 5-8) of the NER mutant strains at [0.02%] MMS. Ultimately, the cells experienced their greatest sensitivity at [0.5%] **(Fig. 12)**. In its totality, the cell sensitivity assay has confirmed our hypothesis for this work. We hypothesized that with the introduction of DNA damaging agents our NER mutant strains will result in some level of cell sensitivity due to the compromised NER complex. Furthermore we found it unexpected that in multiple cell sensitivity assays, strain JC1570 appeared to show resistance and recovery, despite Elc1 being deleted. In other assays we found that both our Pcl6-TAP and Pcl7-TAP strain (positive and negative controls) experienced extreme sensitivity. We expected these strains to respond similarly to that of the *WT*. These three control strains were expected to grow despite the presence of chemical agents, because their NER pathway was intact. In knowing that some results were not consistent with our expectations we can further speculate that these controls are highly sensitive to several drugs at different concentrations. In considering their genetic background, perhaps the repair machinery is not suitable to fix DNA damage incurred by the tested drugs.

4.1 Future Directions

These studies are a works in progress so it would be interesting to investigate why the three controls behaved differently from our expectations with each tested drug.This was unexpected because their NER mechanism has not been altered. We would also like to explore the cell sensitivity assay with Pcl7 NER mutant strains, since they share great similarities in structure and function. We are also interested in investigating the levels of both Pcl6 and Pcl7 within different phases of the cell cycle. Cells would be synchronized and halted at the different phases of the cell cycle. We will use Wetern Blot analysis to study the protein levels. Furthermore we would like to determine the levels of Pcl6 and Pcl7 in the NER mutant strains via Western Blot analysis. Lastly we will set up to analyze the NER mutant strains in a cell survival assay with the tested chemical agents. This will validate our spot plate results from the cell sensitivity assay.

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