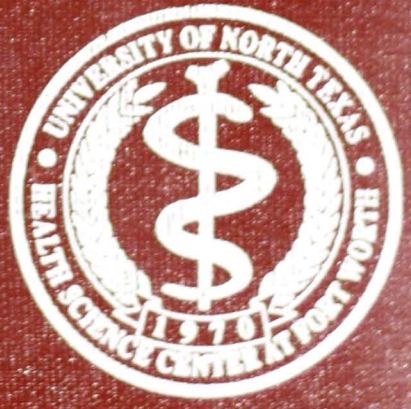
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A SYSTEMATIC SCREEN OF THE *SACCHAROMYCES CEREVISIAE* DELETION MUTANT COLLECTION FOR NOVEL GENES REQUIRED FOR DNA DAMAGE-INDUCED MUTAGENESIS

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Ph.D. Dissertation

In Partial Fulfillment of the Requirements for the

Degree of Doctorate of Philosophy

By

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Dedicated to my dear grandparents who both passed away in 1992 within two months due to lack of professional medical resources before I was going to college. Their demise made me do the decision to go to medical university. Their love and belief make me go all the way and never give up what I have trusted in and dreamed of.

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ABBREVIATIONS AND DEFINITIONS

AP: Apurinic and apyrimidinic

ATM: Ataxia-telangiectasia mutated

ATR: ATM and Rad3 related

BER: Base Excision Repair

CPD: Cyclobutane Pyrimidine Dimers

DSBs: Double Strand Breaks

HNPCC: Hereditary Non-Polyposis Colon Cancer

HR: Homologous Recombination

MBF: MCB binding factor

MCB: *Mlu*I cell cycle box

mtDNA: Mitochondria deoxyribonucleic acid

MMR: Mismatch Repair

NER: Nucleotide Excision Repair

NHEJ: Non-Homologous End Joining

4NQO: 4-Nitroquinoline 1-oxide

8oxoG: 7, 8-dihydro-8-oxoguanine

PCNA: Proliferating Cell Nuclear Antigen

ROS: Reactive Oxygen Species

SBF: SCB binding factor

SC: Synthetic Complete media

SCB: Swi4/6-dependent cell cycle box

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

ssDNA: Single-stranded DNA

TLS: Trans-lesion DNA Synthesis

XP-V: Xeroderma Pigmentosum Variant

YPD: Yeast-extract Peptone Dextrose

A SYSTEMATIC SCREEN OF THE *SACCHAROMYCES CEREVISIAE* DELETION MUTANT COLLECTION FOR NOVEL GENES REQUIRED FOR DNA DAMAGE-INDUCED MUTAGENESIS. Jinjun Gong Department of Cell Biology & Genetics, University of North Texas Health Science Center, Fort Worth, TX 76107

SUMMARY

Deoxyribonucleic acid (DNA) damage is common in a cell's lifetime. DNA can be damaged by endogenous factors such as reactive oxygen species (ROS) or exogenous agents such as ultraviolet (UV) or industrial chemicals. DNA damage will trigger cell responses including cell cycle arrest, transcription activation, DNA repair or apoptosis. In addition to various DNA repair mechanisms including damage reversal, base excision repair, nucleotide excision repair, mismatch repair, homologous recombination and non-homologous end joining, translesion DNA synthesis is an important DNA damage tolerance pathway that can bypass the lesion on template DNA to finish the replication for cell survival but at the risk of potential mutation in the daughter cells. Accumulation of mutation may lead to cancer occurrence.

Translesion DNA synthesis components are highly conserved from yeast to humans. Important players in trans-lesion synthesis pathway such as Rev1, Rev3 and Rev7 were first discovered in budding yeast *Saccharomyces cerevisiae*. Homologues were found later in human cells. I used the

Saccharomyces cerevisiae deletion mutant collection to do a systematic screen to search for novel genes required for DNA damage induced mutagenesis in yeast. After *CAN1* forward mutation assay for the systematic screen and reverse mutation assay for further confirmation, two candidate genes *SWI6* and *DOA4* were detected. Deletion of *SWI6* or *DOA4* decreases mutagenesis of cells. At the molecular level, Swi6, a transcription cofactor, is involved in mutagenesis by regulating expression of *REV7* at the mRNA and protein levels. Rev7 is a regulatory subunit of DNA polymerase zeta, which is essential for DNA damage induced mutagenesis as well as spontaneous mutagenesis. Rev7 is not UV inducible or cell cycle regulated. The regulation of Rev7 at the transcriptional level by Swi6 is essential. Future experimental approaches are planned to address the mechanism by which *DOA4* is involved in mutagenesis.

CONTENTS

CHAPTER 1

INTRODUCTION

The introduction is divided into three parts. The first part provides an overview of DNA damage, the second part provides the general view of one of the important cell responses to DNA damage --- DNA repair and the third part introduces the budding yeast *Saccharomyces cerevisiae* used as a model in our experiment to study genes involved in DNA damage-induced mutagenesis.

1. DNA damage

DNA damage is not a rare but a regular event in a cell's lifetime. About 18,000 purine residues are lost per cell per day by hydrolysis of the bond between base and the phosphate backbone of DNA[1] and more than 74,000 damage incidences occur per cell per day in human DNA. DNA damage affects the primary structure of the DNA double strands and thus interferes with DNA replication or transcription process.

DNA damage can be caused by endogenous cellular processes or exogenous environmental factors. Endogenous cellular process-triggered DNA damage includes: 1) oxidation of bases such as 7,8-dihydro-8-oxoguanine (8oxoG) formation from guanine (Figure 1), 5-hydroxyuracil from cytosine and DNA strand breaks caused by reactive oxygen species (ROS) which are produced from metabolic byproducts mainly in mitochondria; 2) alkylation of

bases, alkylating agents can transfer methyl or ethyl groups to a guanine and form N^7-alkylguanine and O^6-alkylguanine; 3) hydrolysis of bases, hydroxyl radical attack on the deoxyribose moiety can cause the release of free bases from DNA and lead to deamination, depurination or depyrimidination; 4) mismatch of bases. These events occur when DNA is replicated. That is the wrong DNA base is incorporated into place in a newly replicating DNA strand.

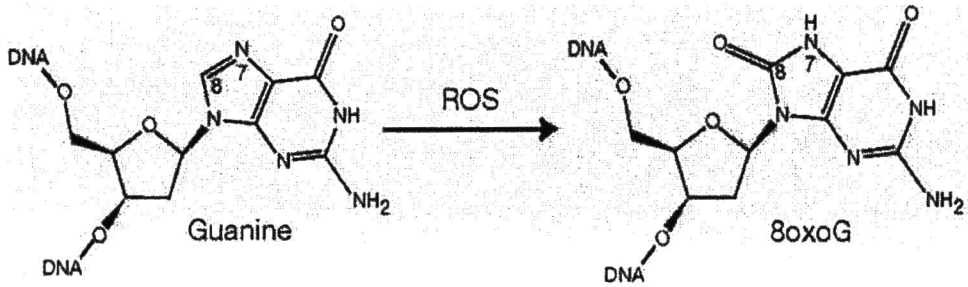

Figure 1. Oxidation of guanine at C8 by reactive oxygen species to form 8oxoG

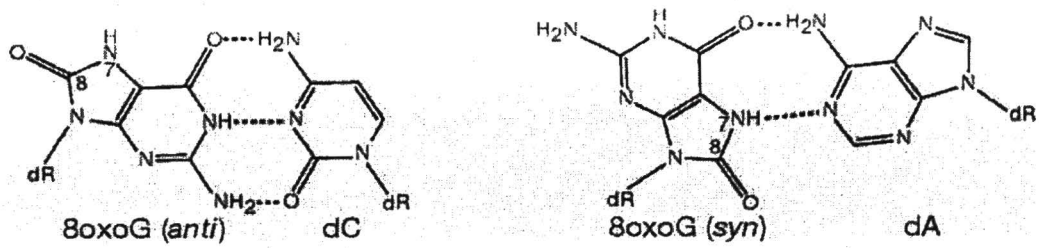

Figure 2. 8oxoG forms a base pair with dC. Dashed lines indicate potential hydrogen bonds. 8oxoG (*syn*) forms a Hoogsteen base pair with dA (*anti*)

Exogenous DNA damage is usually caused by agents including UV-B light (295-320nm), which causes dimeric photoproducts between adjacent pyrimidine bases on the same strand.[2,3] The two main types are cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts. They can cause distortion of the DNA double helix.

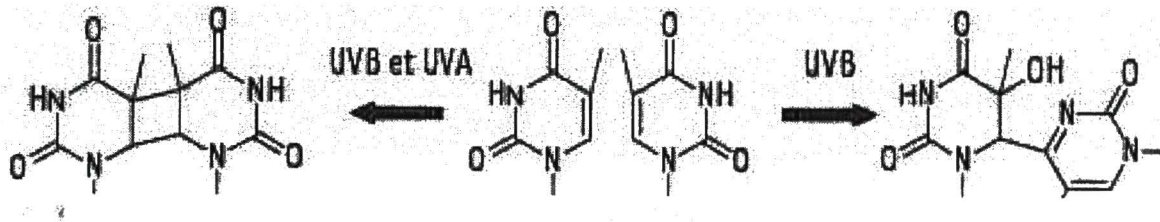

Figure 3. Formation of cyclobutane pyrimidine dimers and (6-4) photoproducts after UV exposure

The cause of the dimer formation after UV-B light is because nucleotides direct absorb energy from UV-B. CPDs are formed by the covalent binding of carbon atoms at C5 and C6 positions of two adjacent pyrimidines (thymine and/or cytosine), (6-4) pyrimidinone photoproducts are formed by the covalent binding between C6 and C4 positions. (Figure 3)

UV-A light (320-400nm), damage of UV-A to DNA depends on reactive oxygen species (ROS) generated through photoactivation of endogenous photosensitizers because UV-A can penetrate the human skin tissue and its energy is absorbed by photosensitizers in the cell and photosensitizers is supposed to produce ROS.[2] Single-strand breaks and alkali-sensitive lesions are commonly detected after UV-A exposure.[4, 5] The damage caused by ROS after UV-A exposure is called indirect DNA damage because ROS is the intermediate

that causes DNA damage. The damage caused by UV-B exposure is called direct DNA damage because DNA directly absorbs energy from UV-B and form T-T dimers. In the Lab UV-C (254nm) is usually used to damage DNA.

Ionizing radiation such as gamma- or X-rays causes breaks in DNA strands. Ionizing radiation damages DNA by direct and indirect (through reactive species produced during ionization) ways.[1,6] Thermal effect: high temperature increases the rate of hydrolysis such as depurination and also single-stranded breaks.[7] Chemicals such as hydrogen peroxide, industry chemicals styrene and styrene 7, 8-oxide and environmental chemicals such as polycyclic hydrocarbons can create different DNA bulky adducts such as oxidized bases by hydrogen peroxide, alkylated phosphotriesters and DNA double helix crosslinking.[8]

The replication using damaged DNA as a template can lead to the incorporation of bases not complementary to opposite damaged ones on the template. The next generation cells containing these incorrect bases may carry mutations that lead to protein dysfunction, thus leading to phenotype change in the organisms.

In addition to damage on chromosomal DNA in the cell nucleus and mitochondria, reactive oxygen species (ROS) are produced that can damage mitochondria DNA (mtDNA). ROS radicals are chemical species possessing an unpaired electron. ROS includes non-radicals hydrogen peroxide (H_2O_2) and singlet oxygen as well as radicals superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), and nitric oxide (NO).[9]

DNA damage can induce mutation and chromosome instability. It not only causes cancer, but also is now used as a way to cure certain cancers through radiotherapy or chemotherapy and, at the same time, is responsible for the side effects of these treatments such as number of normal white blood cells is decreased.

2. DNA repair

DNA damage will trigger multiple cellular responses including induction of multiple genes (activation of transcription), cell cycle arrest, DNA repair, or apoptosis.

After DNA damage, cell cycle checkpoints are activated and cell cycle is arrested. DNA damage checkpoints occur at the G1/S, intra-S phase and G2/M phase transitions.[10,11] Checkpoint activation is controlled by two kinases, ataxia telangiectasia mutated (ATM) kinase and Rad3-related kinase (ATR). ATM responds to DNA double strand breaks and disruptions in chromatin structure, ATR responds to stalled replication forks. p53, a transcription factor, regulates the transcription of many genes in response to stresses. p53 is an important downstream target of ATM and ATR, as it is required for inducing apoptosis following DNA damage. p53 is also activated to play a role to activate p21 which is a cyclin-dependent kinase inhibitor at G1/S transition. Multiple genes are also activated after DNA damage, for example, exposure of yeast *Saccharomyces cerevisiae* to DNA damaging agents such as UV results in distinct transcriptional

profiles. In yeast, *RNR1* and *RNR3* genes encode subunits of ribonucleotide-diphosphate reductase which are highly inducible by DNA-damaging agents.[12]

DNA repair is highly conserved from prokaryotes to eukaryotes, but generally speaking more complex organisms with more complex genomes have more complex repair mechanisms. Depending on the types of damage on the DNA double strand, several repair systems have evolved to correct damaged DNA sequence information. Damage to DNA alters primary structure of DNA and then affects the spatial configuration of the double helix and such alterations can be sensed by the cell. Once damage is detected, specific DNA repair proteins bind at the site of damage, recruiting other proteins to bind and form a complex that makes the DNA repair take place. The types of molecules involved in the repair depend on the type of damage and the cell cycle stages when damage occurs.

The target of DNA repair can be a modified base, an abasic site, damaged single or double strands. According to the type of DNA damage, cells use different repair mechanisms to repair the damaged DNA.

Thymine dimers can be reversed by enzymatic photoreactivation, alkylated bases can be reversed by O^6-methylguanine-DNA methyltransferase (MGMT). Photoreactivation is a light-dependent process using UV-A (320–400 nm) and blue light (400–500 nm) to monomerize pyrimidine dimers.[13-16] Enzyme is present in bacteria (Ada) and human cells (MGMT). MGMT transfers methyl groups from O^6-methylguanine and other methylated moieties of the DNA to a cysteine residue of its active site, and the enzyme is inactivated and degraded. So MGMT

repairs DNA lesions in a single-step reaction. One O^6-methylguanine-DNA methyl-transferase molecule reverses one lesion and is inactivated and degraded. [17]

When only one of the two strands of a double helix is damaged, the other strand can be used as a template to help the repair of the damaged strand.

Base excision repair (BER) corrects the damage to a single base caused by oxidation, alkylation, hydrolysis or deamination. Base damage is the most common insult to DNA. First, the damaged base is recognized and removed by DNA N-glycosylases such as Ogg1 and then apurinic/apyrimidinic endonuclease (APE) incises the Apurinic/apyrimidinic (AP) site. DNA polymerase beta or delta fills the gap by incoporating the correct nucleotides. Finally ATP dependent DNA ligase 3 seals the nick to complete the repair. For example, 8oxoG is removed by base excision repair mechanism.[18,19]

Nucleotide excision repair (NER) recognizes DNA helix-distorting lesions such as bulky DNA adducts and removes a short single-stranded DNA segment (25-30 nucleotides) that includes the lesion. NER is divided into two subgroups, global genome NER and transcription-coupled NER. NER recognizes bulky, helix-distorting changes such as thymine dimers. The two main types of UV-induced damage products cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts are repaired by NER. About 30 proteins in the human cells are involved in the repair. The process includes lesion recognition, unwinding of the

double helix at the lesion site, repair protein binding, single strand incision, DNA repair synthesis and ligation of the nick.

People with NER defect are characterized by extreme sensitivity to sunlight and predisposition to cancer. Xeroderma pigmentosum (XP) is caused by NER genes mutations in *XPA*, *XPB*, *XPC*, *XPD*, *XPE*, *XPF* and *XPG*. Xeroderma pigmentosum variant (XP-V) is an inherited disorder associated with increased incidence of sunlight-induced skin cancers. Mutations in Rad30 (in yeast or human) make the cell lose ability to bypass CPD in an error-free pathway, thus leading to the UV sensitivity.[20,21,22]

Mismatch repair (MMR) corrects errors of mis-paired nucleotides or due to deletion or insertion which happens during DNA replication. This process is highly conserved from E. coli to human. During DNA replication the wrong bases could be incorporated into the new forming strand, and it is corrected by mismatch repair. A multi-protein complex recognizes the mismatched base on the newly formed strand and degrades a small region of this strand. DNA polymerase and ligase fill in the gap with correct nucleotides using the other intact strand as a template. The multi-protein complex is made up of four proteins Msh2, Mlh1, Msh6 and Pms2. Mutations in *MSH2* or *MLH1* cause a major form of cancer termed hereditary non-polyposis colon cancer (HNPCC) or Lynch syndrome. Approximately 70–85% of the gene mutations identified in families with Lynch syndrome occur in the *MLH1* or *MSH2* gene. Mutations in *MSH6* account for 10% of the families.[23,24] In E. coli, DNA is methylated at the N^6

position of adenine in dGATC sequences. In replicating DNA, the new forming strand is transiently un-methylated, and it is the hemimethylated dGATC sequences that make the E. coli distinguish the newly synthesized daughter strand from the parental DNA strand. In E. coli, MutH recognizes hemimethylated dGATC sequences[25,26] and while other complex can distinguish the newly formed strand from the template strand and correct the incorrect nucleotides in the new strand. It is not clear how cells distinguish the newly formed strand in humans.

When both strands in the double helix are broken, this can lead to genome instability and rearrangements. The two mechanisms exist in cell to repair double-stranded breaks (DSBs) in cells are non-homologous end joining (NHEJ) and homologous recombination (HR) repair. More than 90% of the double strand breaks are repaired by NHEJ in human, this pathway is error-prone. In yeast, HR is preferred to repair DSBs. In NHEJ, Ku70/Ku80 recruit DNA-PKc to the sites of DSBs and form DNA-PK complex and PKc is activated. Active PKc phosphorylates itself and other proteins such as XRCC4 (X-ray repair complementing defective repair in Chinese hamster cells 4). DNA Ligase IV forms a complex with XRCC4 to join the two ends of broken DNA. HR repair requires the presence of a similar sequence to be used as a template for repair of the break. This pathway allows a damaged chromosome to be repaired using a sister chromatid or a homologous chromosome as a template. Double strand breaks (DSBs) can occur when cells are exposed to radiation. DSBs are difficult for the cell to repair accurately but can be fixed by recombination with the second

undamaged copy of the chromosome or by non-homologous end joining of the broken strands. Homologous recombination (HR) is the most accurate mechanism for repair of DSBs and it is error-free and relies on repeated sequences in the genome. HR mechanism is also a frequently used method to knock out target genes or insert a DNA sequence to the chromosome.

ATM and ATR kinase activation is the first step in a signal transduction pathway that halts cell cycle progression after DNA damage is induced.[27] When a DSB is induced, ATM separates into two active monomers and then move to the site of the DNA damage to phosphorylate and activate various substrates such as Chk1 and Chk2 which together aid in the phosphorylation and activation of p53. p53 activates p21 which is an inhibitor of G1/S cyclin dependent kinase and cause the cell to arrest. If the DNA damage can not be repaired, p53 is also involved for the apoptosis and cells die.

3. DNA damage tolerance

Translesion DNA synthesis is a DNA damage tolerance process that allows the DNA replication machinery to replicate past DNA lesions such as thymine dimers or AP (apurinic and apyrimidinic) sites. When DNA replication complex reaches the lesion sites, replicative polymerases are stalled. To finish the replication and allow the cell to survive, translesion polymerases take over. Translesion DNA polymerases often have non-restrictive active sites that can facilitate the insertion of bases opposite lesions. The takeover of DNA replicative polymerase by DNA TLS polymerase is thought to be mediated by mono-

ubiquitinated proliferating cell nuclear antigen (PCNA). Translesion synthesis polymerases often insert the wrong bases when compared with the action of replicative DNA polymerases. So TLS rescues cells from severe DNA injuries and allows cell survival but at the expense of potential mutation incurred during DNA lesion bypass; most polymerases in this system are potentially mutagenic and sometimes introduce mutations in the next generation of cells. DNA polymerase zeta [28,29] (complex of Rev3 and Rev7), Rev1 [30,31] (deoxycytidyl transferase) are known to be involved in most damage-induced as well as spontaneous mutations.

Before a moving DNA replication complex encounters damage in the template strand, one of the excision mechanisms such as BER or NER repairs the damage. In some cases, the damage may not be repaired, facing this dilemma, cells resort to translesion DNA synthesis to tolerate and bypass the damage. Experiments in yeast demonstrate that damage bypass is an important component of the overall cellular response to DNA damage.

Eukaryotic DNA damage bypass has been studied most extensively in budding yeast *Saccharomyces cerevisiae.* Characterization of mutants sensitive to ultraviolet or ionizing radiation has identified TLS "epistasis" group important for cell mutagenesis.

Components involved in TLS pathway include Rad5, Rad6, Rad18, Rad30, Rev1, Rev3, Rev7, Mms2, Ubc13, PCNA and Srs2. The TLS pathway is divided into error–free and error-prone pathway. Components included in the error-prone

pathway are Rad6, Rad18, Rev1, Rev3 and Rev7. Mono-ubiquitination of PCNA is thought to be able to promote TLS event while poly-ubiquitination of PCNA is thought to be in error-free pathway.

Rev3 and Rev7 form a complex and this complex functions as DNA polymerase zeta which is essential for DNA damage induced mutagenesis in eukaryotes.[32,33,34] Rev7 is the accessory subunit and Rev3 is the catalytic subunit. In response to DNA damage, PCNA is mono-ubiquitinated on Lys164 by Rad6.Rad18 complex, and mono-ubiquitination of PCNA is the first step for post-replicative DNA repair.[35] Polymerase zeta frequently makes mistakes in replicating past thymine dimmers in vitro. Rev1 usually inserts dCTP opposite an apurinic and apyrimidinic (AP) lesion at the template strand. One current hypothesis is that, *in vivo,* Rev1 inserts the first nucleotide opposite the lesion and extension is then performed by polymerase zeta to sufficiently bypass the lesion. Because Polymerase zeta does not bind tightly to its DNA substrate, so after the lesion replicative DNA polymerases take over again and the normal DNA synthesis can continue.

The proteins Rad18 and Rad6 are essential for error-prone and error-free damage bypass. Mutations in these genes make the cells more sensitive to killing by UV and other DNA damaging chemicals. Rad18 forms tight complex with Rad6. Rad18 has function of ATPase and ssDNA binding. Rad6 is a ubiquitin-conjugating enzyme that can transfer ubiquitin from a ubiquitin-

activating enzyme to PCNA after DNA is damaged. Rad18 is a ubiquitin ligase (E3).

The Srs2 functions as 3'-to-5' DNA helicase and DNA-dependent ATPase involved in DNA repair and affects genome stability by suppressing unscheduled homologous recombination.[36] Srs2 blocks homologous recombination at the lesions where it binds by disrupting Rad51-ssDNA filaments. In the absence of the Srs2 helicase, the cell may attempt to directly repair these lesions by homologous recombination, rather than allowing replication forks to pass through the lesion.

Rad5 (E3) protein is a DNA-dependent ATPase containing zinc-binding domain capable of binding ssDNA, Rad5 also functions as helicase. Rad5 can bind to a ubiquitin-conjugating protein, Ubc13. Ubc13 forms a heterodimer with Mms2, which is homologous to Ubc13 but lacks the active cysteine residue and does not have ubiquitin-conjugating activity.[37,38] Ubc13 prefers to catalyze the formation of Lys63 linked ubiquitin chains.

Finally, DNA damage and mutations are related because DNA damage is a major cause of mutations, but it is important to distinguish DNA damage from mutation. DNA Damage causes structural abnormalities in the DNA. Mutations are changes in the base sequence of the DNA different from the original sequence information. Mutations are stable and can not be recognized once the base changes are present in both DNA strands, so mutations cannot be repaired.

4. Yeast

Yeasts are single-cell fungi belonging to eukaryotes. Yeast cells divide by budding or binary fission. Yeast size can vary depending on the species, usually about 3 to 4 μm in diameter, but some yeast can reach 40 μm in diameter.

The budding yeasts are classified in the order *Saccharomycetales*. The most well-known and commercially significant yeasts are the related species and strains of *Saccharomyces cerevisiae*. Yeast was first isolated in pure culture in 1883. *Saccharomyces cerevisiae* is commonly used as baker's yeast and for some types of fermentation. Yeast is also taken as a vitamin supplement because it is a rich source of B vitamins, niacin, and folic acid.

Yeasts use organic compounds as a source of energy including hexose sugars such as glucose and fructose or disaccharides such as sucrose and maltose. Yeasts grow best in a neutral or slightly acidic PH environment with an optimal temperature range of 30°-37°C depending on the type of species. *S. cerevisiae* grows best at about 30°C. The cells can survive freezing under certain conditions, with viability decreasing over time. Yeast strains are always stored in glycerol at -80°C. Yeast is generally grown in the laboratory on solid growth media or in liquid medium such as yeast-extract peptone dextrose (YPD) or synthetic complete media (SC). Yeasts can grow and bud as haploids.

For the past three decades, *S. cerevisiae* has been the model organism for much of molecular genetic research because the basic cellular mechanics of

replication, recombination, DNA repair, cell division and metabolism are generally similar in yeast and mammalian cells. The cell cycle in a yeast cell is very similar to the cell cycle in humans, and also the basic cellular mechanics of DNA replication, recombination, cell division and metabolism are relatively similar. Many proteins important in human biology were first discovered by studying their homologues in yeast such as hRev1, hRev3 and hRev7.

S. cerevisiae was the first eukaryote to have its genome sequenced in 1996.[39] 12 million base pairs are located on 16 chromosomes. In 2002, genome sequence of yeast *Schizosaccharomyces pombe* was completed.[40]

In 2000, yeast knock-out (YKO) deletion collection was released, about 18% of the genes are essential for growth on rich glucose media. About one-third of yeast genes have homologues in the human genome including genes involved in DNA copying, DNA repair, protein synthesis, transport, and metabolic processes. In cancer research, *S. cerevisiae* has been an important model for studying control of the eukaryotic cell cycles.

Hypothesis We hypothesize that novel mutagenesis-related genes will be discovered after systematic screen in *S. cerevisiae* after canavanine forward mutation assay.

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CHAPTER 2

Systematic Screen for Novel Genes Required for UV-Induced Mutagenesis in *Saccharomyces cevevisiae*

SUMMARY

Translesion DNA synthesis (TLS) proteins are conserved from yeast to human . When DNA is damaged it will trigger cellular responses to repair the damage, sometimes the damage can not be repaired and the cells use TLS system to bypass the lesion to complete the DNA replication and survive. However, the bypass can be potentially mutagenic. A systematic screen was performed to look for novel genes required for mutagenesis in *S. cerevisiae*. After a series of experiments two candidate genes *SWI6* and *DOA4* were identified. The results of this screen show that *SWI6* or *DOA4* deletion makes the cells show a decreased mutagenesis phenotype. It suggests that Swi6 functions in mutagenesis by regulating expression of related genes such as *REV1*, *REV3* or *REV7* and Doa4 may indirectly be involved in mutagenesis by posttranslational protein modification.

RATIONALE FOR SYSTEMATIC SCREEN

As a eukaryote model organism, yeast *S. cerevisiae* genome DNA was completely sequenced in 1996 and yeast knock-out (YKO) mutant collection was available commercially in 2000. Among the 6,200 open reading frames (ORFs) in yeast genome, about one third of yeast genes have homologues in human genome. Genes encoding proteins involved in DNA copying and repair of damaged DNA, protein synthesis and transport across membranes, and control of metabolic processes are highly conserved from yeast to human.

TLS system is also conserved in yeast and human cells. Rev1, Rev3, Rev7, Rad6, Rad18 and PCNA in yeast have homologues in human cells. Most of the TLS components were first discovered in yeast and then homologues were identified in human cells.

In the light of the above knowledge of TLS conservation and availability of yeast gene knockout mutant collection, the first aim was to do a systematic screen to identify novel genes involved in DNA damage-induced mutagenesis. Similar screens have been done to identify genes involved in suppressing mutagenesis.

Three logics are kept in mind during the systematic screen

A: Analysis of the phenotype of mutants lacking the gene is a powerful method for determining gene function. Over 4,700 strains on the same genetic background were available and a specific non-essential gene was deleted in each strain. Each strain was given the same treatment and the phenotype differences were observed and then relate the change of phenotype to function of gene deleted in each strain, then do further research to find out how the genes affect the phenotype at the molecular level.

B: DNA damage-induced mutation is the best index to evaluate TLS in vivo. It takes a longer time to observe spontaneous DNA damage-induced mutagenesis and, much longer to observe the spontaneous mutation level because it is very low. To facilitate a study of TLS, the cells were treated with DNA damaging agents to produce multiple DNA damage and observe the response of cells.

C: Genes will be identified that make the cells show lower mutant phenotypes after deletion. Deletion of genes may not change the incidence of mutant phenotype. If deletion of a gene makes the strain show a higher mutation phenotype, then the gene functions to suppress mutation occurrence in the cell. On the contrary, if deletion of a gene makes the cell show lower mutant phenotype, the gene may function to be involved in facilitating the TLS events.

The following section outlines the rationale of methods that were employed to address the aims to do a systematic screen to identify novel genes involved in DNA damage-induced mutagenesis, details of which have been included in following sections.

CAN1 forward mutation assay for systematic screen

Genetic methods for deciphering mutagenic mechanisms in yeast use forward or reverse mutation assays to identify and characterize strains with elevated or reduced mutagenesis. The power of forward mutation assays resides in their ability to detect any mutation that inactivates its corresponding gene product. The yeast gene used in forward mutation assay in this experiment is *CAN1*. *CAN1* in yeast encodes an arginine permease that is located in the cell membrane and can transport arginine into the cell from the environment for the cell growth.[1] If there is no arginine available in the environment, yeast itself can also synthesize arginine to survive.

L-canavanine is a non-protein amino acid present in various beans, seeds and some higher plants.[2] It is a natural homologue of L-arginine and can compete with L-arginine to be transported into the cell by arginine permease located at the cell membrane (Figure 1). Inside the cell, L-canavanine can compete with L-arginine as target of arginyl tRNA synthetase, inducible nitric oxide synthase (iNOS)[3], and arginase. L-canavanine can replace L-arginine and

be incorporated into protein chain during protein synthesis, creating canavanyl proteins. Up to 30% of arginine residues can be replaced by L-canavanine based on analysis of proteins synthesized *in vitro*. L-canavanine substitution disrupts the tertiary and/or quaternary structure of the protein, thus disrupts the normal function of the protein and further interferes with DNA and RNA synthesis. The cell can not survive in the environment of high canavanine concentration.

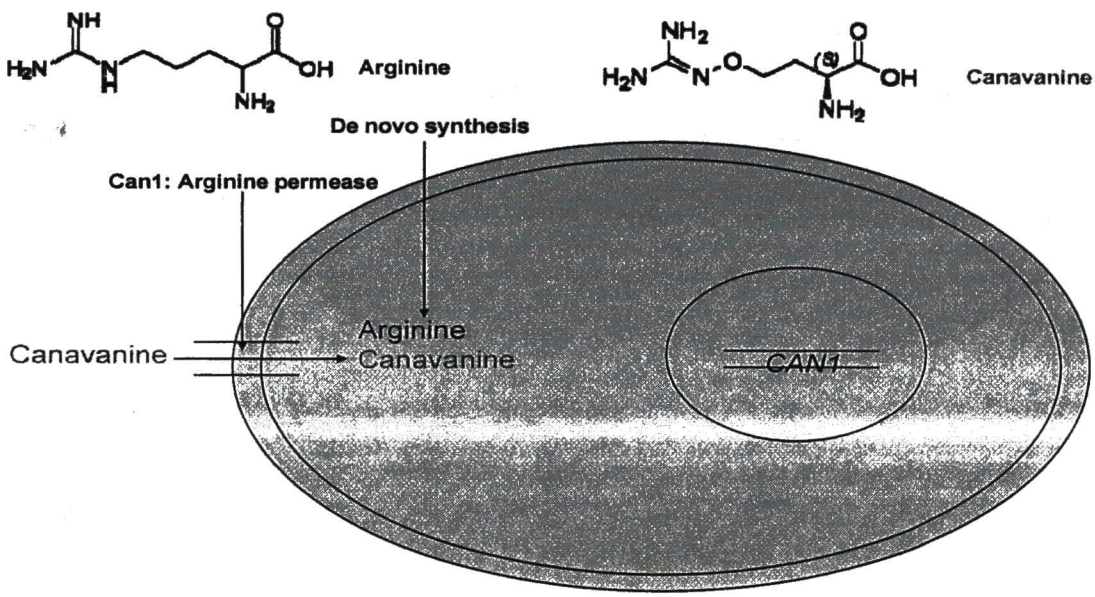

Figure 1. *CAN1* forward mutation assay system

The utility of the arginine permease system for studies of mutation in *Saccharomyces cerevisiae* was described in 1979.[4] The advantage of this system results from the fact that all mutations selected as canavanine resistant occur only in gene *CAN1* encoding arginine permease (Figure 2). In utilizing this system to study the mutagenic action after DNA damage, it became necessary to

evaluate the mutation level change after specific gene deletion. But a limitation with *CAN1* forward mutation assay is that some missense mutations do not perturb gene function and so escape detection.

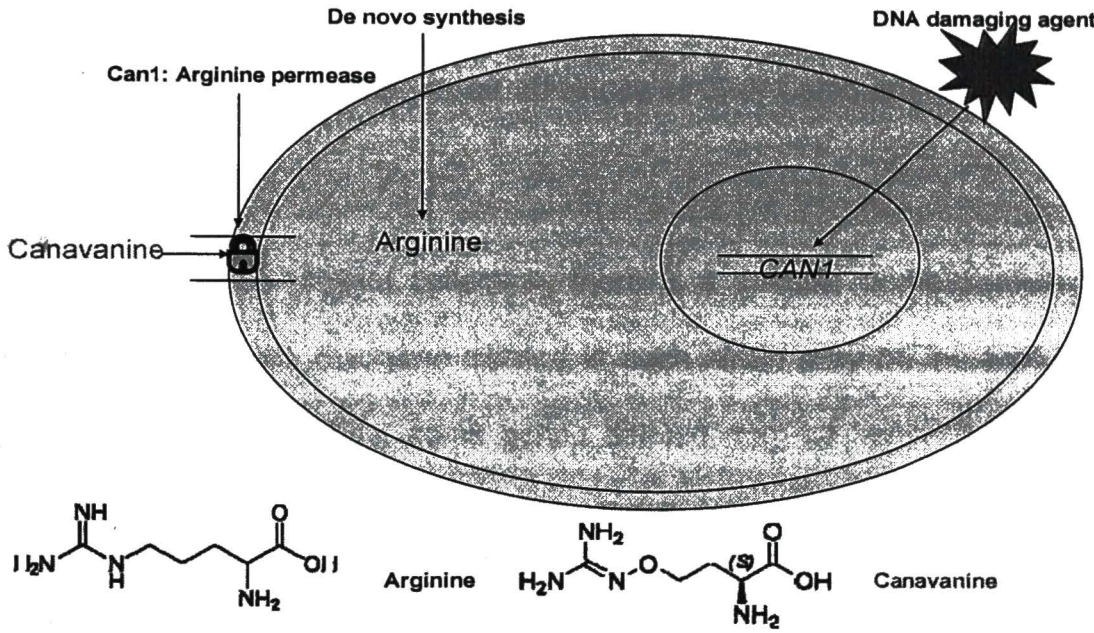

Figure 2. *CAN1* forward mutation assay system

Canavanine sensitivity test

After *CAN1* forward mutation assay, it is necessary to detect canavanine sensitivity of each strain in which candidate genes are knocked out. In the yeast *Saccharomyces cerevisiae,* the extent of resistance to L-canavanine, after mutagenesis, depends on the metabolic state of the cell. Data show that the number of mutants is determined by three factors: (1) the potential mutants may

possess enough permease activity to take up enough canavanine, and so are killed before they can express the mutant Can1 protein and form canavanine resistant colony. The sensitivity is influenced by the endogenous arginine synthesis level; (2) the decay efficiency of the arginine permease and the inability of the potential mutants to resynthesize this protein results in a rapidly increasing chance of expression when selection is delayed; (3) during the time when the permease activity is decaying, repair efficiency of *CAN1* also affect the mutation of the cells.

In the deletion mutant collection, there is a specific non-essential gene deletion in each strain, the gene deleted in each strain may be involved in the arginine synthesis, and also, some genes deleted may be involved in arginine permease expression or decay. Some genes are involved in transport of permease to the cell membrane.[5] Some genes may be involved in the export of canavanine in cytosol. Deletion of these genes may affect canavanine sensitivity of the cell. The change in canavanine sensitivity after gene deletion will affect the screening results and may make the strains show a mutation-less or more false-positive phenotype.

Quantitative *CAN1* forward mutation assay and UV sensitivity assay

To compare the mutagenesis level change among strains with specific gene deletion, it is necessary to quantitatively detect UV sensitivity and ratio of number of mutants to number of surviving cells after UV. For each strain, certain number of cells will be placed on the surface of medium and exposed to different doses

of UV and after incubation at 30°C for 3 or 4 days, the number of surviving colonies and mutant colonies will be counted.

Quantitative reverse mutation assay

With reverse mutation assays the target size for mutations is typically small and mutations are either base substitutions or small insertions/deletions. Examples of such assays include the reversion of nonsense or missense alleles to identify base substitutions, or reversion of frame-shift alleles to identify insertions or deletions. In nonsense mutations and in this study, reversion event can correspond to locus events that mutate the relevant stop code within coding sequence to a wild type sense code. The power of this system is that only a single mutation is capable of reversing a given allele, so that we automatically know the identity of the underlying mutation without resorting to DNA sequence analysis. In this experiment, a point mutation in *TRP1* gene in CRY1 strain makes the cell lose the ability to synthesize amino acid tryptophan. Only after the point mutation is reversed to its wild type nucleotide after DNA damage induction can the cell restore its ability to synthesize tryptophan and survive on minus tryptophan medium (-Trp).

TRP1 gene encodes phosphor-ribosylanthranilate isomerase that catalyzes the third step in tryptophan biosynthesis. *TRP1* coding sequence is listed below:

1 ATGTCTGTTA TTAATTTCAC AGGTAGTTCT GGTCCATTGG TGAAAGTTTG

51 CGGCTTGCAG AGCACAGAGG CCGCAGAATG TGCTCTAGAT TCCGATGCTG

101 ACTTGCTGGG TATTATATGT GTGCCCAATA GAAAGAGAAC AATTGACCCG

151 GTTATTGCAA GGAAAATTTC AAGTCTTGTA AAAGCATATA AAAATAGTTC

201 AGGCACTCCG AAATACTTGG TTGGCGTGTT TCGTAATCAA CCTAAG**T**AGG

251 ATGTTTTGGC TCTGGTCAAT GATTACGGCA TTGATATCGT CCAACTGCAT

301 GGAGATGAGT CGTGGCAAGA ATACCAAGAG TTCCTCGGTT TGCCAGTTAT

351 TAAAAGACTC GTATTTCCAA AAGACTGCAA CATACTACTC AGTGCAGCTT

401 CACAGAAACC TCATTCGTTT ATTCCCTTGT TTGATTCAGA AGCAGGTGGG

451 ACAGGTGAAC TTTTGGATTG GAACTCGATT TCTGACTGGG TTGGAAGGCA

501 AGAGAGCCCC GAAAGCTTAC ATTTTATGTT AGCTGGTGGA CTGACGCCAG

551 AAAATGTTGG TGATGCGCTT AGATTAAATG GCGTTATTGG TGTTGATGTA

601 AGCGGAGGTG TGGAGACAAA TGGTGTAAAA GACTCTAACA AAATAGCAAA

651 TTTCGTCAAA AATGCTAAGA AATAG

The mutation of G to T (underlined 247th nucleotide) in the *TRP1* gene makes CRY1 strain unable to survive on SC minus tryptophan medium because the cell itself loses its ability to synthesize tryptophan. Only after the reversion from T to G can the cell survive and form colony on the SC minus tryptophan medium. So this assay system will be used to detect the mutagenesis level of candidate strains obtained after *CAN1* forward mutation assay.

CAN1 expression level in different titers of cells and its effect on mutation assay

In the *CAN1* forward mutation assay system, arginine permease expression level plays an important role and affects the results of this screen. Cell growth conditions such as presence or absence of nutrients may affect the arginine permease level. To determine if *CAN1* expression level is stable, mRNA level will be detected for log phase and stationary phase cells, respectively. Cells at

different titers will be used to detect the stability of the quantitative assay for *CAN1* forward mutation assay and also reverse mutation assay.

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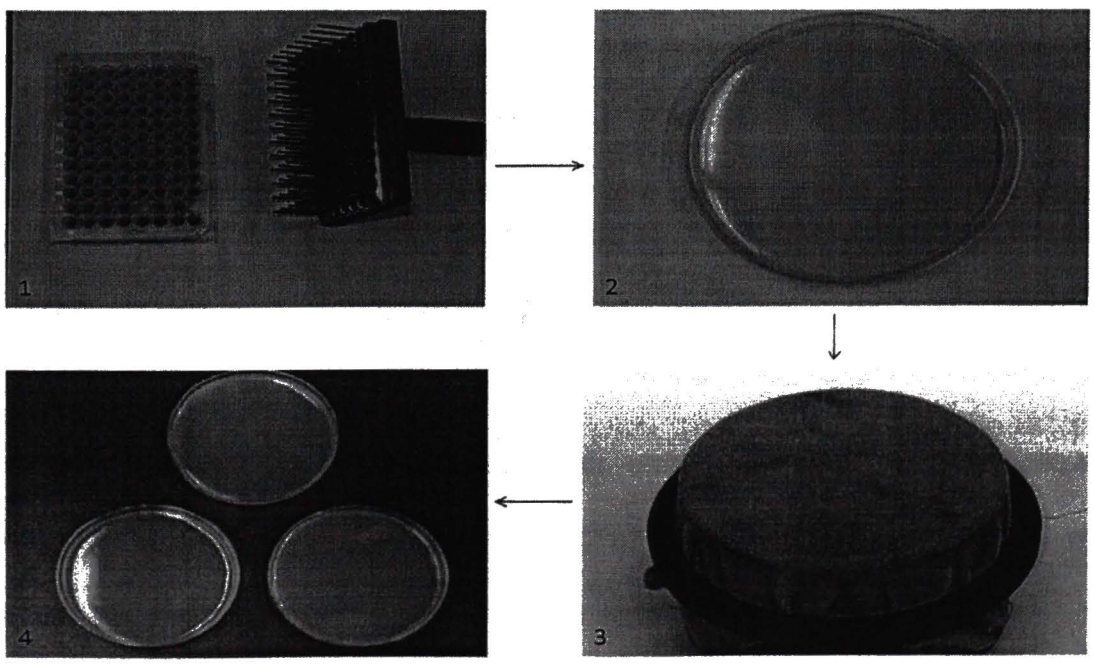
Yeast cells and cell culture

Yeast gene knockout mutant collection was purchased from Open Biosystems for systematic screen using *CAN1* forward mutation assay method. They are based on the W303 genetic background (BY4741, MATa *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0). Strains used for reverse mutation assay are based on CRY1 genetic background (MATa *ade2*-1, *trp1*-1, *ura3*-1, *his3*-11.15, *leu2*-3,112, *can1*-100). Cells are cultured in YPD (Yeast extract, Peptone and Dextrose) medium at 30°C shaking (200rpm) incubator or YPD agar media. Synthetic Complete (SC) media without arginine but containing L-canavanine are mainly used for the screen. L-Canavanine was purchased from United States Biological (Swampscott, Massachusetts, USA)

***CAN1* forward mutation assay**

Plates containing YPD agar medium or SC minus arginine but containing 60mg/l canavanine medium were prepared. Lids and the aluminum seal were removed from the source plates to allow the source plates to thaw completely. A sterile multiprong replica-plating tool was placed into the source plate to mix the yeast cells in each well. The multiprong replica-plating tool was gently removed from the source plate and the tips of device touch the YPD medium surface. The replicating tool was sterilized to transfer all other source strains to YPD media plates. Above steps were repeated until all strains have been replicated to YPD

media plates. The plates with the replicated strains on YPD agar medium were incubated at 30°C incubator for 24 hours. Each growing strain was spread using the sterilized loop on the YPD medium to make sure each strain occupy a circular area. Then the strains were Incubated in the incubator for another 24 hours. All of the strains were transferred onto 3 plates containing SC minus arginine medium rich with 60mg/l L-canavanine using the replica-plating approach. One plate was the control, the second plate was exposed with 60 Joules/m^2 UV to induce the DNA damage in each strain after replica-plating. The third plate contained 0.2 µg/ml 4-nitroquinoline 1-oxide (4NQO) to induce DNA damage in the yeast strains. The cells will be incubated at 30°C incubator for 4 to 5 days. The canavanine resistant mutant colonies formation was observed for each strain compared to that of the wild type cells. Gene deletion mutant strains which showed a mutation-less phenotype were identified and genes deleted in these strains are recorded.



Canavanine sensitivity test

Canavanine sensitivity test was done for the candidate strains which show a phenotype with low incidence of mutations after *CAN1* forward mutation assay. Cells from each strain were spread on the surface of SC minus arginine media and at the center of the plate, a small circular sterile filter paper was placed tightly onto the media. 30µl water solution containing 3mg canavanine was added to the paper filter so that canavanine can diffuse and form a gradient of concentration around the filter paper. Cells will be incubated for 5 days to observe the canavanine sensitivity for each strain.

Quantitative forward mutation assay and UV sensitivity assay

$2x10^7$ yeast cells were placed on each of 4 SC minus arginine containing 60mg/l canavanine medium plate for each candidate strain. One plate was the control without UV exposure, and the other 3 are treated with 20, 40 and 60 Joules/m^2 of 254nm UV-C light. UV sensitivity is also detected for the strains. A total of 400, 400, 800, 2000 cells were placed on SC minus arginine without canavanine media plate and subjected to 0, 20, 40, 60 Joules/m^2 of UV treatment. After UV treatment, plates are put in 30°C incubator for 5 days. Surviving colony number on each SC minus arginine plate and number of canavanine resistant mutant colonies on each SC minus arginine with canavanine plate were counted for each strain. Survival percent following different doses of UV exposure and ratio of mutants to surviving cells were calculated. Data was shown as dose response to UV exposure.

PCR based gene disruption in CRY1 strain

Primer pairs flanking coding sequence downstream and upstream used to synthesize the PCR products for gene disruption use or to verify gene deletion are listed below for each selected candidate gene. The first pair for each gene is used to produce PCR product for gene disruption transformation and the second pair is used to verify if gene is deleted after strain selection.

LDB7:
5'-CTGATGCAGTTGTCGCCTAAGACCT-3'
5'-TATGCGCTAAATATTCGTTCTG-3'

5'-CTGATGCAGTTGTCGCCTAAGACCT-3'
5'-CATAGACGGCCCTATAGATG-3'

DOA4:
5'-GCACTTGGTACAAACTCGTATATGC-3'
5'-GCGTGATCTCATCTTTACACC-3'

5'-GCACTTGGTACAAACTCGTATATGC-3'
5'-CCAAGAGATGCACAGGAGGAAGAC-3'

HNT3:
5'-GAAGAGCTGAGAGCGATGATTGAAG-3'
5'-ATGACCGTGATCACATCAACCCTGA-3'

5'-GAAGAGCTGAGAGCGATGATTGAAG-3'
5'-AGATCCTGCTTTGTTGAGGCCCGGT-3'

LDB19:
5'-TGCGATTTGAGCTGACTCGAACACT-3'
5'-ATCATAACAAAGCTATGTACCTACG-3'

5'-TGCGATTTGAGCTGACTCGAACACT-3'
5'-GGCATGGCTACCTACGTGGCAGATT-3'

ARP5:
5'-ACGTAGTGGCATCATGTTAGCATAG-3'
5'-GAAGGAGCTAAGCAATCATGATCCG-3'

5'-ATAGAAGGGTCTTGACCGATGTAAT-3'

5'-GTATCATTCACTCTGACTTCGCAAC-3'

INO2:
5'-TCATCGGCAGGGCGTTGACACCTGT-3'
5'-AGATGCTGGTAATTCTTGTCCGCA-3'

5'-TCATCGGCAGGGCGTTGACACCTGT-3'
5'-GCAGACACTCAGCAGTTAAT-3'

SDS3:
5'-CATAGCTCTTGTCTGTAATC-3'
5'-CGTTTTCCACTTGTATCCGAGA-3'

5'-AGAAGTCATCGCACGCCATG-3'
5'-CGTTTTCCACTTGTATCCGAGA-3'

SWI6:
5'-GTGGACAATTAGTTTCTAAA-3'
5'-CGTCATGGTAAGGACGCCGC-3'

5'-CTGTCTTCTGCTTCACTCTCGCCG-3'
5'-CTGAGACAAGATTCACTATCATCTG-3'

RSC1:
5'-CCTACTATTGCGCGAAATCAAAA-3'
5'-TTGTTGGAACAACCTCCTAGTTC-3'

5'-CCAAGGATGTGCTTGGTGAAG-3'
5'-CTGGAACTTTATCATCGGTGGAT-3'

Genomic DNA extract from respective BY4741 deletion mutant was used as template for PCR amplification.

A: Yeast DNA extraction

A loop of yeast cells grown on YPD plate was suspended in 0.2 ml 50mM Tris / 50mM EDTA / 1% SDS in an Eppendorf tube. Zirkonium beads were added up to the meniscus and vortexed vigorously for 3 x 30 seconds at 4°C. Then 0.3 ml sterile water was added, extracted with 0.5ml phenol/chloroform (1:1), and spun for 5 minutes at 13,000 rpm. Aqueous phase was transferred to a new tube

and extracted again with 0.5 ml chloroform. The aqueous phase was transferred to new microtube. 50µl 3 M sodium acetate and 1ml 100% ethanol were added, mixed and spun for 10 minutes at 13,000 rpm. The dried pellet was dissolved in 0.5ml TE buffer (pH8.0). 2µl RNase was added and then incubated at 37°C for 3 hours. 0.5 ml chloroform was used to extract once. The aqueous phase was transferred to new microtube for later use. 1µl was used in a PCR reaction (total reaction volume 50µl).

The resulting PCR product contains the kanMX4 selection marker flanked by the upstream and downstream sequence of each ORF of interest and was transformed into CRY1 strain, and successful gene deletion mutants were selected on G418 media and verified by second pair of primers.

B: PCR product transformation for deletion of specific genes

CRY1 strain was inoculated in YPD media and incubated at 30°C shaker (200 rpm) until it contains equivalent to $2x10^7$cells/ml. The culture was harvested in a sterile 50ml centrifuge tube centrifuged at 3000g for 5 min. The liquid medium was poured off and the cells were re-suspended in 25 ml of sterile water and centrifuged again. The cells were re-suspended in 1 ml 100 mM lithium acetate (LiAc) and transferred to a 1.5ml microfuge tube. The cells were pelted at maximum speed for 15 seconds and the LiAc was removed with micropipette. The cells were re-suspended in LiAc to a final volume of 500µl (about $2x10^9$cells/ml). Cell suspension was vortexed and 50µl samples were pipetted

into labeled microfuge tubes. LiAc solution was removed with a micropipette. Then 240µl PEG (50%), 36µl 1.0 M LiAc, 10µl ss-DNA (2.0mg/ml), 40µl PCR product and 34µl sterile ddH_2O were added carefully in the order listed. Each tube was vortexed vigorously until the cell pellet has been completely mixed. And then was incubated at 30°C incubator for 30 minutes. The samples were subjected to heat shock in a water bath at 42°C for 30 minutes. Samples then were centrifuged at 3000 rpm for 15 seconds and solution was removed with a micropipette. 1.0ml of sterile water was added into each tube and the pellet was re-suspended by pipetting it up and down gently. The cells were transferred to YPD and incubated in the 30°C shaker for 2 or 3 hours. Then cells were centrifuged, washed and re-suspended with 1ml sterile water. 200µl of the transformation mix was spread onto G418-containing YPD plates (200µg/ml) which were incubated at 30°C incubator for 3 to 4 days to select gene deleted strains.

After selection of G418 resistant colonies for each strain, the DNA samples were extracted for PCR using the second pair of primers to verify the deletion of candidate genes in CRY1 strain. The strains were stored for reverse mutation assay.

Quantitative reverse mutation assay and UV sensitivity test

Synthetic complete (SC) medium minus tryptophan was used for the assay. SC medium was used for cells subject to UV sensitivity test. Cell number placed

($2x10^7$) on each plate and UV doses were the same as it is in forward mutation assay.

CAN1 expression level in different titers of cells and its effect on mutation assay

CAN1 mRNA level detection was performed using RT-PCR method for log phase cells and stationary phase cells. *PDA1* mRNA was the loading control.

Comparison between _CAN1_ forward mutation assay and reverse mutation assay for different titer of cells

Wild type BY4741 cells were collected separately when they reached different titers and quantitative forward mutation assay was performed to observe if there were obvious UV sensitivity change and mutagenesis level change. CRY1 wild type cells were collected when they reached different titers and quantitative reverse mutation assay was performed to observe the same index.

RESULTS

In the present study, a systematic screen was performed among yeast non-essential genes to look for novel genes required in DNA damage-induced mutagenesis. 4786 strains were detected. Based on the results after *CAN1* forward mutation assay, the canavanine sensitivity for each candidate strain was determined. Then a quantitative forward mutation assay was performed. Nine genes were deleted in CRY1 strain using PCR-based gene disruption method. Quantitative reverse mutation assay was done for 9 candidate strains selected after forward mutation assay.

Candidate genes after CAN1 forward mutation assay

After the systematic screen using the *CAN1* forward mutation assay (see Figure 1), 108 genes were identified and separated into groups according to their known functions (listed below). Essential TLS components such as *REV1*, *REV3*, *REV7* and *RAD6* were also included in the results. UV sensitive cells such as *RAD2* deleted strain was shown in the screen result because of the UV treatment killed the cells and thus showed a low incidence of false positive phenotype. 4NQO-has the similar effect on DNA damage. Proteins encoded by the candidate genes may regulate mutagenesis process by affecting chromatin structure, regulating TLS components expression or protein modification.

Candidate genes which make the cells show a low incidence of mutation

phenotypes if deleted were listed below.

DNA transactions: *REV1, REV3, REV7, RAD6, RAD2, MRE11, UAF30, HNT3, HOP2*

Chromatin structure: *TAF14, RSC1, ARP5, ARP8, HTL1, IES6*

Gene expression regulation: *RTG1, INO2, GCN4, MOT2, SWI6, WHI3, SDS3, IMP2, SRB5*

Unknown genes: *YOR305W, LDB19, LDB16, YGR064W, IRC14, YDL118W, YDL156W, YLR358C, YEL045C*

Cell structure/cytoskeleton: *HOP1, SHE4, CDC10, MDM30, MDM32, SHS1, HSL7, VRP1, CLC1*

Transcription/translation: *FUN12, CDC40, CBC2, NSR1, WTM1, PFD1, REF2, PRP18, SSN3, STO1, ISY1, MSL1, MRPL28, MRM2, BDF1, DHH1, ROX3, TAF14, RPL1B, RPL12B, RPL21B, RPL34B*

A.A. metabolism: *GCV3, AAT2, PRO2, SER2, GGC1, GLY1*

Membranes/transport/sorting: *SNF7, SNF8, SRN2, STP22, DID4, PEP12, BFR1, BRO1, LDB7, TIR3, FYV5, FUR4, SRV2, CHC1, VPS3, VPS20, VPS25, VPS27, VPS28, VPS36, VPS65, VPS66, VPS69.*

Other metabolism: *POS5, IDH2, LTV1, ATP15, KRE6, GPH1, FAA3, SFA3, FMP35, VAN1, AAC3, GPM2, HEM14, RIB1, DOA4*

Canavanine sensitivity difference among candidate strains

As a toxic antagonist, canavanine was used in our screening system. Some proteins may be involved in the export of canavanine. Deletion of genes

encoding these proteins will cause increased accumulation of canavanine in the cytosol, thus making the cell more sensitive to canavanine (Figure 2). The level of arginine permease located on the cell membrane also affects the assay system. Specific proteins may regulate expression or function of arginine permease. And deletion of genes encoding such proteins will make the cells have shorter or longer time to get mutant at *CAN1* before they die of toxic canavanine in the media. Deletion of genes involved in arginine synthesis in the cell also makes the cell more susceptible to toxic canavanine. Two thirds of the candidate genes were removed from the list due to the super sensitivity of the strain to canavanine after gene deletion. The remaining 36 gene candidates are listed below:

DNA transactions: *REV1, REV3, REV7, RAD6, RAD2,* UAF30, *HNT3, HOP2*

Chromatin structure: *RSC1, ARP5, IES6*

Gene expression regulation: *INO2, SWI6, WHI3, SDS3, IMP2*

Unknown genes: *LDB19, YDL118W, YDL073W, LDB7*

Cell structure/cytoskeleton: *HSL7, CLC1*

Transcription/translation: *STO1, BDF1, ROX3, RPL34B*

Membranes/transport/sorting: *BRO1, SRV2, CHC1, VPS66*

Other metabolism: *KRE6, VAN1, AAC3, GPM2, RIB1, DOA4*

Quantitative forward mutation assay and UV sensitivity assay

It is necessary to do quantitative mutation assay for each candidate strain for

the convenience of comparison to wild type cells (Figure 3, only *SWI6*- or *DOA4*-deleted strain was shown here). Compared to the wild type strain all of the 36 strains with candidate gene deletion show lower incidence of mutagenesis. And the lower incidence of mutagenesis phenotypes may suggest that genes deleted in these strains are directly or indirectly involved in DNA damage-induced mutagenesis.

Quantitative reverse mutation assay and UV sensitivity test

Nine genes were selected and deleted according to known protein functions encoded by these genes in the CRY1 strain background including *INO2, SDS3, LDB7, RSC1, SWI6, DOA4, LDB19, HNT3* and *ARP5.* Among the 9 genes, only deletion of *SWI6* or *DOA4* makes the cell show a lower mutagenesis phenotype. Deletion of the other 7 genes does not affect its mutagenesis phenotype in the reverse assay. UV sensitivity curve and mutagenesis level for *SWI6* or *DOA4* deleted strains as well as wild type strain are shown following different doses of UV treatment (Figure 4).

CAN1 mRNA level change for log phase and stationary phase cells and its effect on stability of CAN1 mutation forward assay.

There is a lower level of expression of *CAN1* gene at the mRNA level in stationary phase cells than in log phase cells (Figure 5). For *CAN1* forward mutation assay, the higher titer cells reach, the more canavanine resistant mutants form after DNA damage in BY4741 strain (Table 1). This may be

because in log phase, cells need and have enough amino acid in their environment. This could induce the expression of *CAN1* gene, so there is more arginine permease activity at the cell membrane. But at the same time log phase cell will also become more susceptible to the presence of canavanine than stationary phase cells because more canavanine will be transported into cytosol for log phase cells. Then, after DNA damage, these cells have a shorter time to mutate at *CAN*1 before they die of the cytotoxicity. This explains why stationary phase cells form more canavanine resistant colonies than same number of log phase cells before they die after DNA damage.

BY4741	UV dose: 40J/m^2
Titer	Mutants/10^6surviving cells
$2x10^7$/ml	5.27
$1.5x10^8$/ml	45.85
$2x10^8$/ml	106
$2.5x10^8$/ml	196.75

CRY1	UV dose: 40J/m^2
Titer	Mutants/10^6surviving cells
$2x10^7$/ml	9.49
$5x10^7$/ml	10.35
$1x10^8$/ml	13.44
$1.2x10^8$/ml	20.83

Table 1 BY 4741 strain is used for *CAN1* forward mutation assay, CRY1 strain is used for reverse mutation assay.

DISCUSSION

CAN1 gene in *Saccharomyces cerevisiae* genome encodes a plasma membrane protein arginine permease which requires phosphatidyl ethanolamine (PE) for localization[1,2] and is exclusively associated with lipid rafts. This gene uniquely determines sensitivity or resistance to the toxic arginine analog, L-canavanine. *S. cerevisiae* takes up arginine via the activity of arginine permease located at the cell membrane. Canavanine as arginine analog can also be transported into cells by arginine permease, so canavanine competitively inhibits arginine uptake. Resistance to canavanine is associated with loss of arginine permease function so that toxic canavanine can not get into the cell and the cell itself synthesizes arginine for survival. Mutations to canavanine resistance occurred at *CAN1* gene and this makes *CAN1* gene very useful for study of mutagenesis. This system was used to detect the levels of change of DNA damage-induced mutagenesis after UV exposure or 4NQO treatment in each strain, and then relate the change of mutagenesis to genes deleted in the strain.

After the blind, systematic screen using *CAN1* mutation assay system, it was not surprising to find that *REV1*, *REV3*, *REV7* and *RAD6* are included in the result list. These genes are essential to perform major roles in the production of both spontaneous and DNA damage-induced mutations. *REV1*, *REV3* and *REV7* were identified in budding yeast by screening specifically for mutants that showed a greatly reduced capability for induced mutagenesis.[3-6] The

homologues of these genes were discovered in humans[7,8] and they are also conserved in other eukaryotic organisms. Rev1 was originally found to exclusively utilize only dCTP and insert dCTP opposite G; later Rev1 was found to have preference for adding dCMP to the primer opposite a template abasic site[8]. This may lead to mutation in the daughter cell. dCMP transferase activity has been maintained throughout eukaryotic evolution, suggesting that this function contributes to survival but at the risk of occurrence of mutation. Rev3 and Rev7 form a complex and functions as DNA polymerase zeta which plays the most important roles in translesion bypass system. Yeast polymerase zeta is capable of replicating past a T–T cyclobutane dimer *in vitro*[3] and also has a remarkable facility for extending terminally mismatched primers. Rad6 is a ubiquitin-conjugating enzyme (E2) which together with Rad18 can ubiquitinate PCNA[9] after DNA damage. The monoubiquitinated PCNA facilitates the recruitment of TLS polymerases such as Rev1, polymerase zeta, or polymerase eta. Rad6 mutants make the cells show deficiency in mutant induction because PCNA can not be mono-ubiquitinated and TLS polymerase can not be recruited to lesions. The essential TLS components in these results show that the forward mutation system works for our systematic screen even we missed some, such as *RAD18*.

In this study, genes encoding proteins involved in vacuolar protein sorting were identified, including *VPS3, VPS20, VPS25, VPS27, VPS28, VPS36, VPS65, VPS66* and *VPS69*. Data also show that deletion of some *VPS* genes

can make the cells become more sensitive to chemicals. In this system, high concentration of toxic canavanine was used to treat the cells, so after canavanine sensitivity test, two-thirds of the candidate genes were removed from the list. For example, *GCN4* encodes a basic leucine zipper transcriptional activator which activates amino acid biosynthetic genes in response to amino acid starvation.[10,11] Expression of *GCN4* is tightly regulated at both the transcriptional and translational levels. Deletion of *GCN4* makes the cell deficient in synthesizing some amino acids and presence of canavanine in the media makes the cell survival more difficult. Even it is not related to mutagenesis, *GCN4* deletion mutant shows a low-mutation-like phenotype due to high canavanine sensitivity. After the canavanine sensitivity test, only 31 candidate genes were remaining in addition to the known TLS components because deletion of the other genes makes the cells more sensitive to canavanine. The quantitative forward mutation assay is used to compare the difference of mutagenesis level among strains, especially comparing to that of the wild type cells. All the 31 strains showed a decreased level of mutation phenotype after the gene deletion, some strains showed more UV sensitivity after gene deletion such as *SWI6* deletion.

Because of the involvement of arginine permease and toxic canavanine in this assay system, even after canavanine sensitivity test, other factors affecting the result can not be ruled out. So reverse mutation assay system was used to verify if strains show decreased mutagenesis after gene deletion. Reverse mutation assay system used in this experiment is based on a point mutation (T to

G) in CRY1 strain which can reverse the stop code TAG within *TRP1* coding sequence to a glutamic acid code GAG This reverse point mutation can make the cell restore its activity to synthesize amino acid tryptophan. Thus, the cell can survive on SC media without tryptophan, but only after mutation is reversed from T to G. Nine genes were selected from 31 candidate genes obtained from the canavanine assay and deleted in the CRY1 strain, including *INO2, SDS3, LDB7, RSC1, SWI6, DOA4, LDB19, HNT3* and *ARP5.* Quantitative reverse mutation assay showed that among the 9 strains with specific gene deletion, only two strains show decreased mutagenesis in this system, which are the *SWI6*- or *DOA4*-deleted mutants. Therefore, *SWI6* or *DOA4* deletion induces the strain to decrease mutagenesis in two assay systems.

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Figure legends

Figure 1. Systematic screen for novel genes involved in mutagenesis using *CAN1* forward mutation assay method 4786 gene knockout mutant strains based on W303 genetic background were detected on SC media without arginine, but containing 60mg/l canavanine after 60 Joules/m^2 of UV treatment or exposure to 0.2μg/ml 4NQO. Strains from source plate 7 are shown. Each patch of cells on the media represents one specific gene deletion mutant strain. Strains at the same positions but on different plates are the same mutant strain. **(A) Control plate.** Cells can not survive on the medium because of the presence of canavanine, for some strains, several canavanine resistant mutant colonies form. For example, more canavanine resistant mutant colonies formed for strain at position 7D3, no canavanine resistant mutant colony formed for strain at position 7B8. **(B) UV treatment plate.** After UV treatment (60 Joules/m^2), most of the strains form many canavanine resistant mutant colonies compared to the control, but for some strains, such as strains at 7B8 and 7B9, no canavanine resistant colony form which is called mutation-less phenotype. **(C) 4NQO treatment plate.** Similar to UV treatment, most of the strains show same level of mutation phenotype, but some show mutation-less phenotype. Genes deleted in strains with mutation-less phenotype are candidates of TLS components.

Figure 2. Canavanine sensitivity test to candidate strains 108 strains were tested for canavanine sensitivity compared to wild type cell. Sterile paper filter in the center of medium contains 3mg canavanine was distributed and formed a

gradient concentration of canavanine around the center. Compared to the wild type cells (on the left plate), *GCN4* deleted cells show high sensitivity to canavanine and were unable to form a colony. About two thirds of 108 candidate strains were removed from the list after the sensitivity assay.

Figure 3. Quantitative *CAN1* forward mutation assay for strains after canavanine sensitivity test 2×10^7 cells for each strain were put on SC minus arginine with 60mg/l canavanine media and then treated with different doses of UV (0, 20, 40, 60 Joules/m^2). The number of canavanine resistant mutant colonies was counted after incubation at 30°C. A certain number of cells put on SC complete media was treated with different doses of UV, number of surviving colonies was counted and survival percent is calculated. Ratio of mutants to surviving cells was calculated and shown as curves. *SWI6* or *DOA4* deleted strains were shown compared to the wild type strain. Deletion of *SWI6* makes the cell show a little more UV sensitivity (3A), deletion of *SWI6* or *DOA4* makes the cell show decreased mutagenesis (3B).

Figure 4. *Quantitative reverse mutation assay in CRY1 strain* The same number of cells are placed on each SC minus tryptophan media. Cell survival (4A) and the cell mutagenesis level (4B) are shown as a curve following different doses of UV. Deletion of *SWI6* or *DOA4* makes the cell show decreased mutagenesis. UV sensitivity does not produce a significant change after gene deletion.

Figure 5. *CAN1 mRNA level change in log and stationary phase cells* RT-PCR result shows that *CAN1* mRNA level is decreased when cell reaches stationary phase. *PDA1* is mRNA loading control.

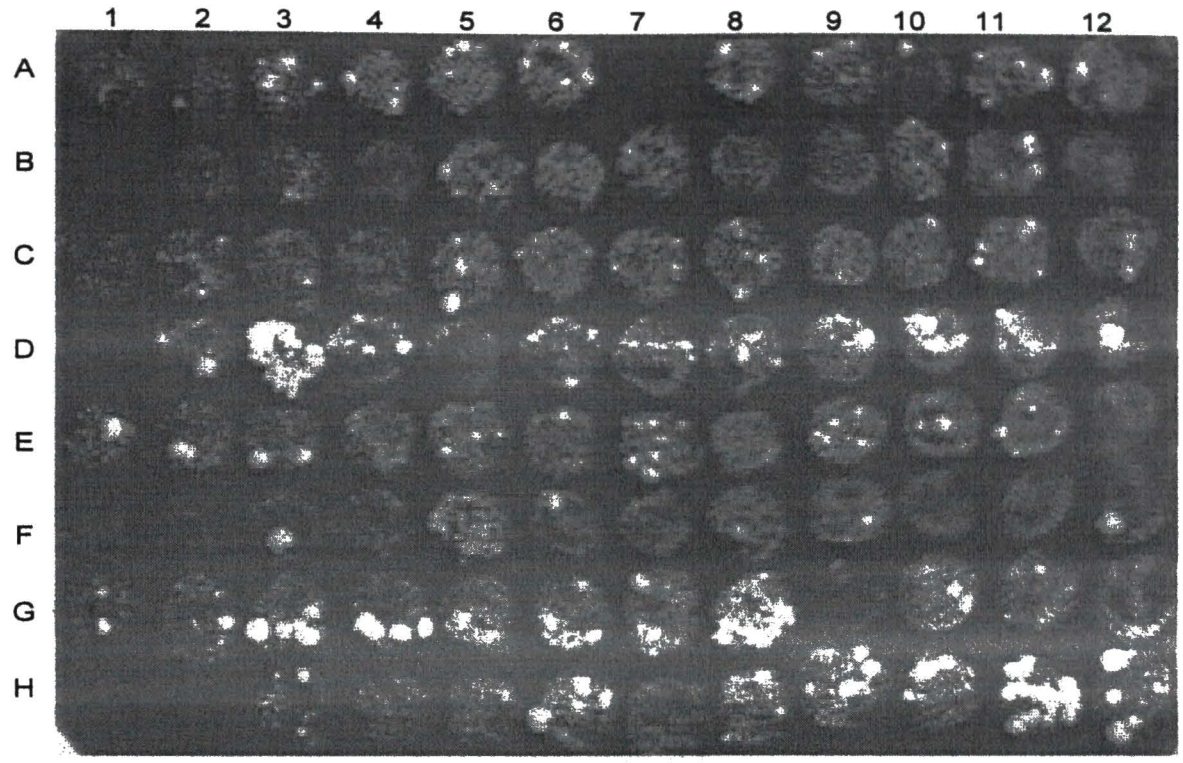

Figure 1A. Control strains

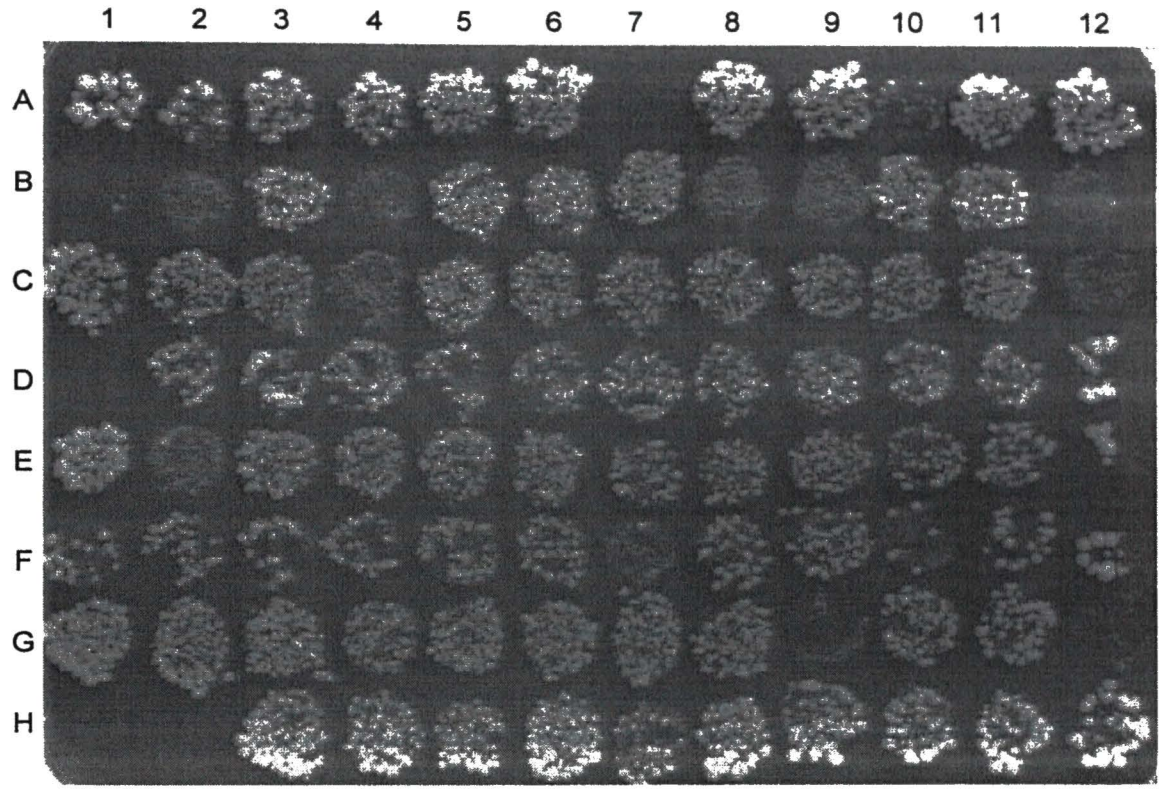

Figure 1B. UV-treated strains

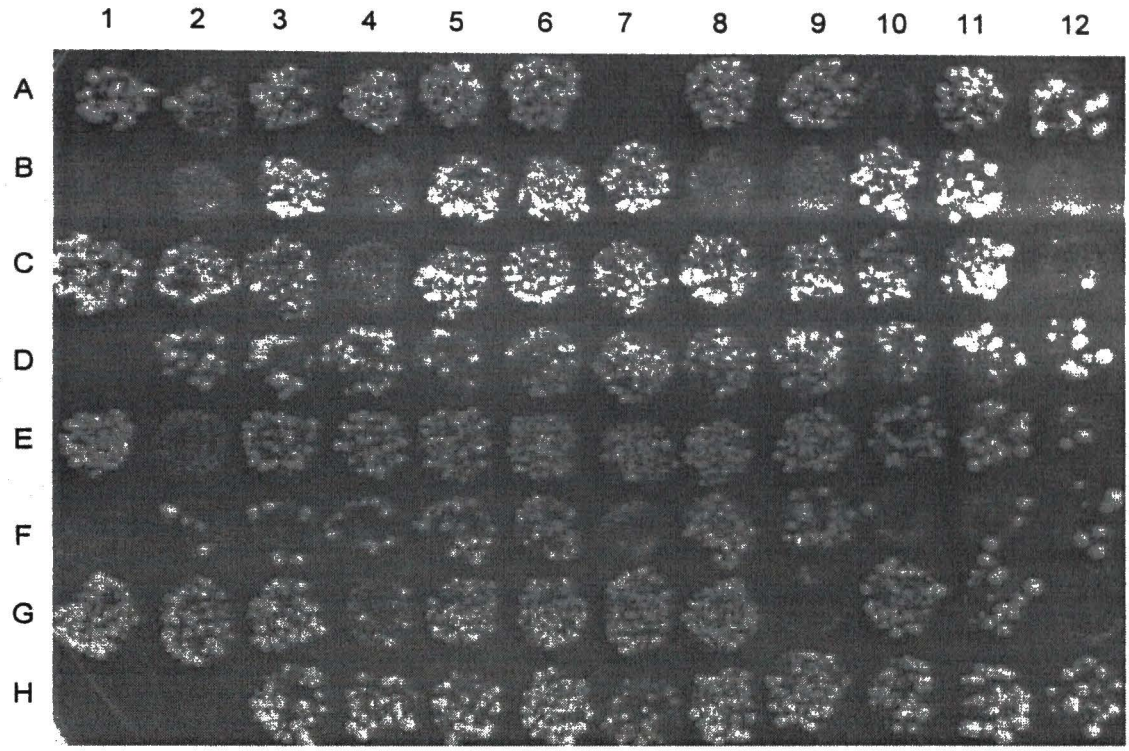

Figure 1C. 4NQO-treated strains

Wild type cells *GCN4Δ* cells

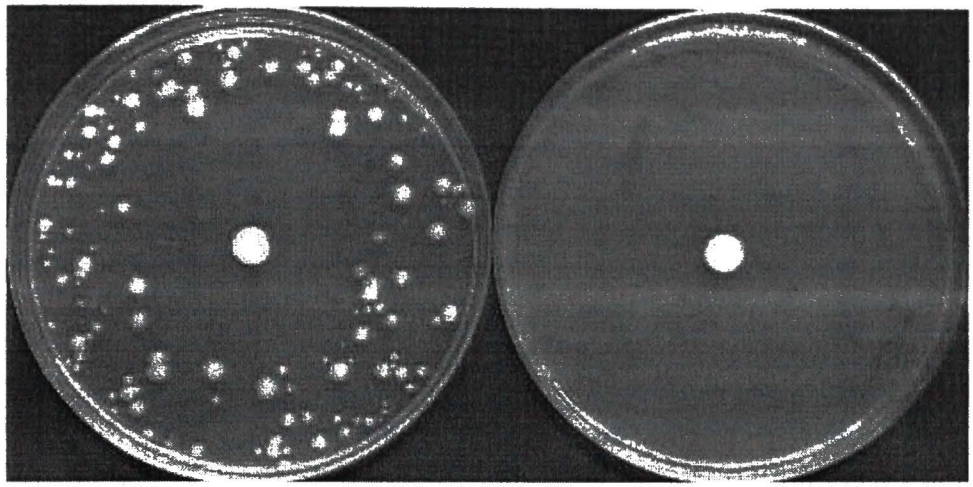

Figure 2. Canavanine sensitivity test for each candidate strain

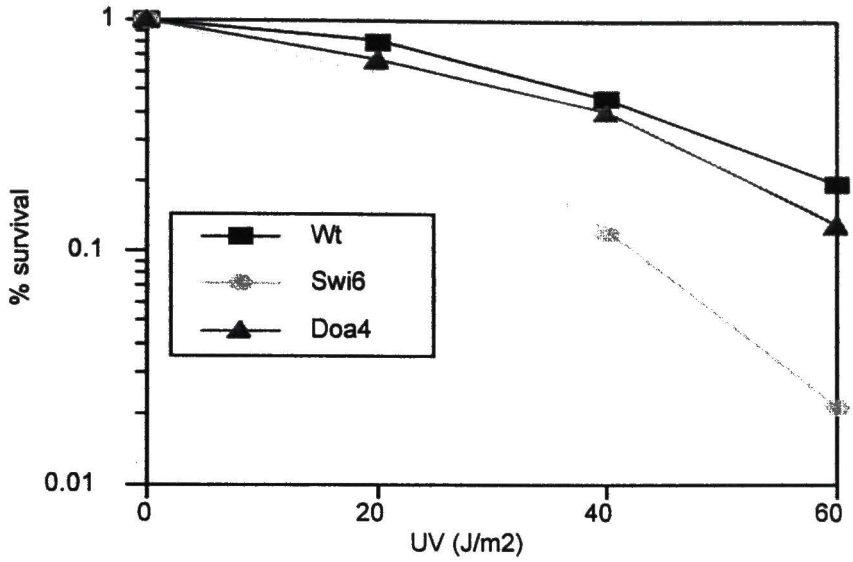

Figure 3A. UV sensitivity after gene deletion

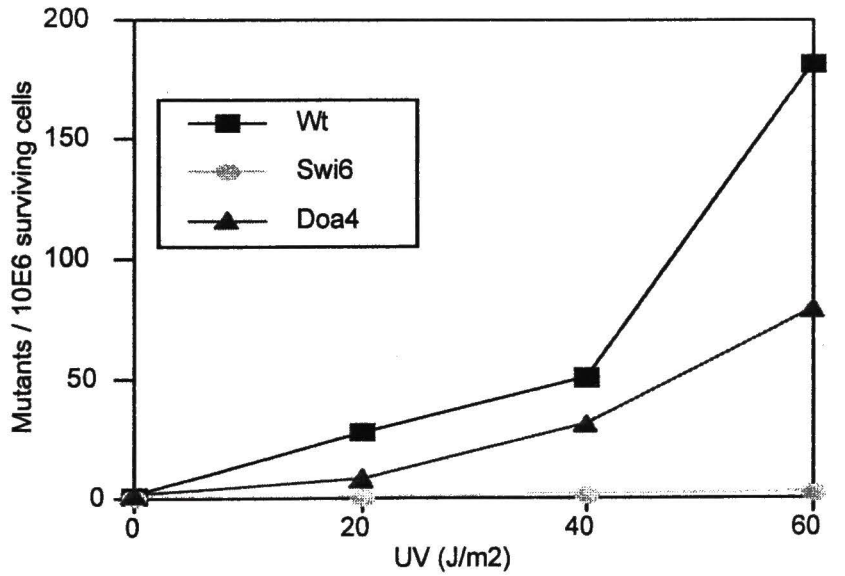

Figure 3B. Mutagenesis level change after gene deletion

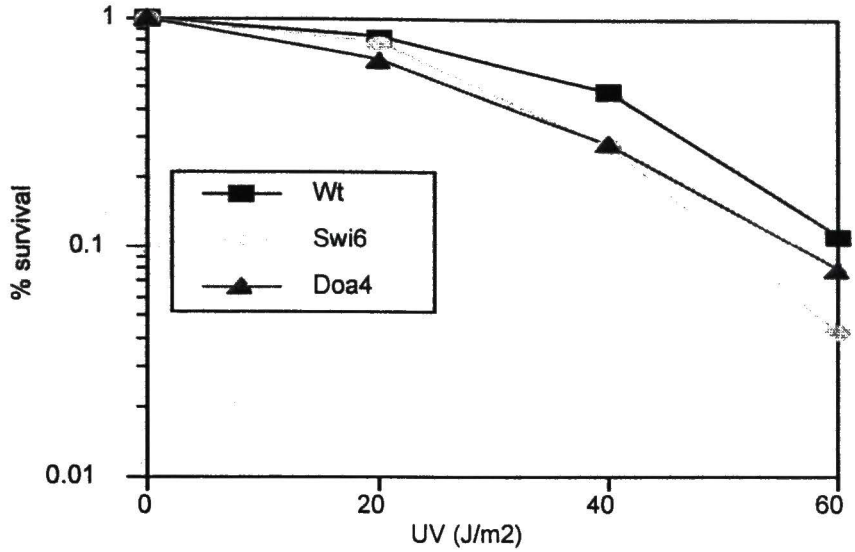

Figure 4A. UV sensitivity after gene deletion

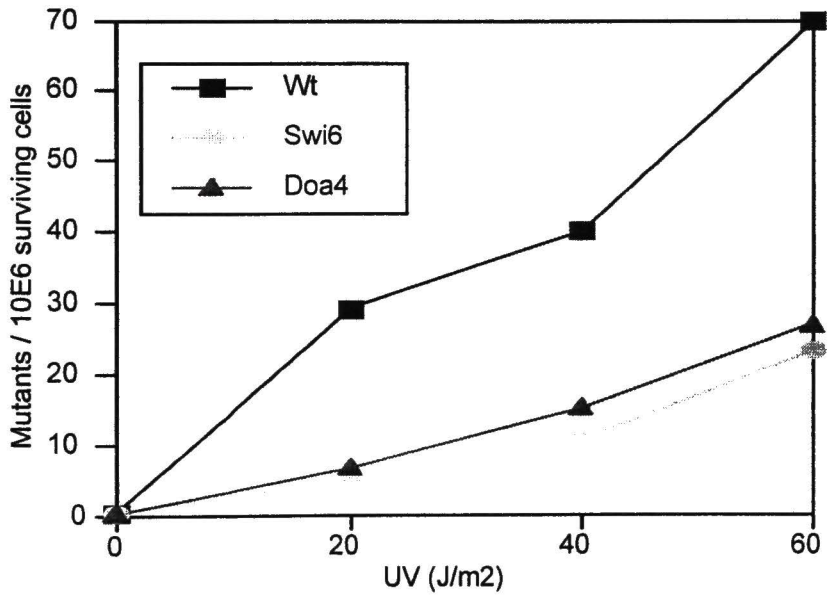

Figure 4B. Mutagenesis level after gene deletion

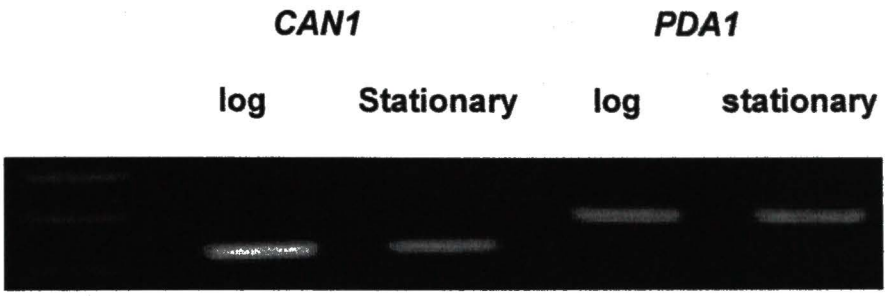

Figure 5. RT-PCR analysis

Swi6 Is Involved in UV-induced Mutagenesis by Regulating Rev7 Expression at the mRNA and Protein Level in *Saccharomyces cerevisiae*

SUMMARY

SWI6 encodes a transcriptional cofactor that can form a complex with DNA binding protein Swi4 or Mbp1 to regulate gene expression at G_1/S phase transition in *S. cerevisiae*. As a candidate gene that may be involved in UV-induced mutagenesis after a systematic screen among non-essential genes in *S. cerevisiae*, this study was undertaken to determine if Swi6 regulates expression of key components Rev1, Rev3 and Rev7 in TLS pathway. The mRNA level of these genes after deletion of *SWI6* was detected using Northern blotting analysis and further verification with RT-PCR. Rev7 protein level was also analyzed after *SWI6* deletion. The results suggest that Swi6 is involved in mutagenesis by regulating expression of *REV7*, but not *REV3* or *REV1*. Additionally, the results suggest that *REV7* is not inducible by UV and its level is not regulated by cell phases. This makes *REV7* expression regulation more important because deletion of *REV3* is lethal in the mouse embryo. The function of DNA polymerase zeta can be regulated through expression of *REV7*.

INTRODUCTION

SWI6 encodes a transcription cofactor in S.cerevisiae.[1] Swi6 forms complex with DNA binding protein Swi4 or Mbp1 to regulate transcription at the G1/S transition of the cell cycle in yeast. Complex formed by Swi6 and Swi4 is known as SCB binding factor (SBF), complex of Swi6 and Mbp1 is known as MCB binding factor (MBF).[2,3] SBF and MBF are sequence-specific transcription factors. SBF usually binds to DNA consensus sequence CGCGAAA known as Swi4/6-dependent cell cycle box (SCB), while MBF binds to ACGCGN (N can be any base) known as Mlu1 cell cycle box (MCB).[4] About ten percent of all intergenic elements contain SBF consensus sequence and about twenty percent of the elements contain MBF consensus. Even though there are consensus sequences for SBF or MBF within some gene coding sequences, SBF and MBF selectively bind to promoters and not coding sequences. Swi6 itself cannot bind to DNA directly. Also, not every promoter bound by SBF or MBF contains the consensus sequences listed above. Twenty-five percent of the MBF target genes are involved in DNA replication, recombination and repair. SBF is the main controller of membrane and cell wall formation.

As a transcription cofactor, Swi6 needs to bind to Swi4 or Mbp1 to regulate their functions. Swi4 and Mbp1 can bind to DNA *in vitro* without the help of Swi6, but Swi6 is critical. One theory is that Swi6 may control the accessibility of DNA binding domain in Swi4 to DNA consensus sequence. C-terminus of Swi4 is sufficient for association with Swi6.[5] *In vitro*, Swi6 shows no SCB binding ability

by itself. Swi6 is highly polyphosphorylated *in vivo*.[6] Serine 160 is the only site of phosphorylation in Swi6 that varies during the cell cycle. It is phosphorylated before S phase and remains phosphorylated until late M phase. This suggests that phosphorylation of serine 160 disrupts the function of nuclear localization signal peptide of Swi6, thus phosphorylation of Swi6 controls its nuclear localization to affect the function of SBF.

When DNA is damaged, especially in the S phase, replicative DNA polymerases with high fidelity are stalled, translesion system may be used to bypass the lesion if the damage is not repaired for restoration of its original sequence information. DNA polymerase eta (Rad30), DNA polymerase zeta (Rev3, Rev7) and deoxycytidyl transferase (Rev1) may be recruited at the lesion site to take over replicative polymerases by mono-ubiquitinated PCNA which is modified by Rad6 and Rad18. Rad30 is thought to be involved in error-free bypass of *cis-syn* pyrimidine dimers at the template strand.[7,8] DNA polymerase zeta plays important roles in error-prone pathway. It is essential for DNA damage-induced as well as spontaneous mutagenesis in eukaryotes.[9] Rev1 is required for bypass of abasic lesions as well as other lesions.[8,10] Rev1 also functions as scaffolding protein which can associate with polymerase zeta.[11,12]

The primary goal of this study was to determine if Swi6, a transcription cofactor, regulates expression of TLS components *REV1*, *REV3* and *REV7*. Due to the low expression level of these genes, after Northern blotting analysis for

mRNA, RT-PCR experiments were also performed to enhance the sensitivity of the analysis. After the mRNA analysis for *SWI6* deleted strains, *REV7* gene was tagged with a 3HA epitope sequence and protein level was detected for Rev7. Finally the incidence of cell mutagenesis level was analyzed quantitatively for *SWI6* or *SWI4* deleted strains after overexpression of Rev7.

MATERIALS AND METHODS

Yeast strains

Protein studies were performed in strains derived from BY4741 (MATa *his3*Δ, *leu2*Δ, *met15*Δ, *ura3*Δ). Rev7 protein was epitope-tagged with 3HA at its C-terminus after transformation and homologous recombination based gene insertion at the chromosome genome with a PCR product, using a plasmid-borne, his3 gene marked module as a template. In the constructed strain, *SWI6* deletion was performed by replacement of KanMX4 which makes the strain resistant on G418 containing media, G418 is an aminoglycoside antibiotic similar in structure to gentamicin B1, which blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells. KanMX4 encodes an aminoglycoside 3'-phosphotransferase which confers resistance to G418 and is the selection marker.

Strains derived from CRY1 (MATa *ade2*-1, *trp1*-1, *ura3*-1, *his3*-11,15, *leu2*-3,112, *can1*-100) were used to be transformed with plasmids which can be induced to overexpress Rev7 when galactose is in the medium.

Antibodies

The primary monoclonal mouse antibody against HA epitope was purchased from Covance company, Emeryville, California. The anti-mouse secondary antibody IgG conjugated to HRP was purchased from Amersham Biosciences, Piscataway, NJ. The primary antibody dilution in the western blotting analysis is

1:1000, and the secondary is 1:2000. Immun-Star HRP peroxide buffer and Luminol were purchased from BioRad.

Northern blotting analysis

Northern blotting analysis was used to detect mRNA level of *REV1*, *REV3* and *REV7* after *SWI6*, *SWI4*, or *MBP1* deletion compared to that of wild type cells.

RNA extraction

Cells were centrifuged and re-suspended in 400µl of AE buffer (50mM Na acetate pH 5.3, 10mM EDTA). 40µl of 10% SDS was added to the 2 ml tube. The suspension was vortexed and an equal amount of phenol was added. The vortexed mixture was incubated at 65°C for 4 minutes and then rapidly chilled in a dry ice ethanol bath, until ethanol crystals appeared. Samples were centrifuged for 2 min at max speed at 4°C to separate the phenol and aqueous phase. The aqueous phase was transferred into new tubes and 500µl of phenol/chloroform was added to the mixture and vortexed. The samples were incubated at room temperature for 5 minutes and centrifuged for max speed at 4°C. The top phase was transferred to new tubes and 40µl 3M Na acetate (pH5.3) and 2.5 volume of ethanol were added. The mixure was centrifuged to isolate the precipitated RNA at maximum speed at 4°C. The pellet was washed with 80% ethanol and dried. The dried pellet was re-suspended in 40µl of distilled H_2O and treated with

DNase 1, then extracted with chloroform. The aqueous phase was collected and stored at -80°C freezer.

Total RNA (2µg) was separated on a 1% agarose gel in formaldehyde/MOPS buffer. Samples were heated at 80°C for 10 minutes in a denaturing agarose gel loaded on buffer with 100µg/µl ethidium bromide before electrophoresis (100 volts).

The RNA was transferred to the Nylon membrane overnight, pre-hybridized and hybridized with specific probes labeled with $dCTP^{32}$. The non-specific bound probes were washed away and the film was exposed to the hybridized membrane at -80°C and developed.

The membrane was stripped and hybridized with probes specific to *PDA1* mRNA as a loading control. *PDA1* primer pairs used to synthesize PCR the products that were used as templates to produce ^{32}P-labeled probes are as following:

5'-GAAACCACATTTGTGCCAATGCT-3'

5'-TCTCGTCTCTAGTTCTGTAGGTAGTACC-3'

REV7 primer pair used to make PCR product which was used as templates to produce ^{32}P-labeled probes are as following:

5'- TGTATACCCACCTCAGTCATTCGACT- 3'

5'- CACGTCAGAACCGACTAAAGA- 3'

RT-PCR

Due to the low copy number of mRNA for *REV7*, RT-PCR was used to increase the detection sensitivity. Primer pair used for *REV7* (547bps) is as following:

5'- TGTATACCCACCTCAGTCATTCGACT- 3'

5'- CACGTCAGAACCGACTAAAGA- 3'

Primer pair used for *PDA1* (715bps) is as following:

5'- AGATATGGTCATCATCAGAAGA- 3'

5'- TCTAGTTCTGTAGGTAGTACCG- 3'

Superscript one-step RT-PCR with Platinum Taq kit was used to perform the RT-PCR reactions. The system consists of two major components: RT/Platinum Taq mix (reverse transcriptase and Taq DNA polymerase), Taq is *taq* DNA polymerase complexed with a proprietary antibody that inhibits polymerase activity at ambient temperatures. The antibody is denatured at high temperature and polymerase activity is restored during the denaturation step in PCR cycling at 94°C and 2 x reaction mix (buffer system optimized for reverse transcription and PCR amplification, Mg^{2+}, deoxyribonucleotide triphosphates.) RT-PCR reaction mix formula is listed as follows: RT-PCR: 2 x reaction mix (25µl),

template RNA (50pg), sense primer (10μM) (1μl), anti-sense primer (1μl), RT/Taq mix (1μl), autoclaved distilled water to a final volume of 50μl.

RT-PCR Protocol: 1 cycle at 52°C for 30 minutes followed by 94 °C for 2 minutes for cDNA synthesis and pre-denaturation was performed, then 28 cycles of denature, 94°C for 15 seconds, anneal, 55-60°C for 30 seconds, extend, 72°C for PCR amplification were performed, then 1 cycle of 72°C for 5 minutes for final extension was performed.

To detect if *REV7* is UV inducible or not, the wild type cell was treated with 60 Joules/m^2 of UV and then cultured in liquid YPD medium and cells were collected at 30 and 60 minutes to produce an mRNA sample for RT-PCR analysis.

Western blotting analysis for Rev7 level

A: 3HA tagged *REV7* gene construction

Primer 1, 2 are used for PCR production using plasmid pFA6a-3HA-His as template to synthesize products which include 3 HA coding sequence followed by a gene *HIS3* including its promoter required for synthesis of histidine (selection marker) by the yeast cell. Primer pair using the plasmid as template is as follows:

(1).5'-

(GAGTGTATTCTCAATATGAAGAGGGCGAGAGCATTTTTGGATCTTTGTTT)C

GGATCCCCGGGTTAATTAA-3'

(2)5'-

(ACTTAGAGACATTTAATTTTAATTCCATTCTTCAAATTTCATTTTTGCAC)GAA

TTCGAGCTCGTTTAAAC-3'

To verify if 3HA coding sequence is inserted into the 3' end of *REV7* coding sequence. Primer pair flanking *REV7* terminal code was used and the sequence was (3) 5'-GTGACGACAAAATTTTGAATG-3'

(4) 5'-ATTAGTGGTTTACAGATACC-3'

B: Strain selection after transformation

After transformation of the PCR product using primer 1 and 2, the cells were placed on SC minus histidine medium and incubated at 30°C incubator for 3 days. Single colony was selected and DNA was extracted from the cells and then primers 3 and 4 were used for PCR and PCR product was sequenced.

C: Confirmation of 3HA DNA sequence insertion at 3' end of REV7 coding sequence

After PCR using the confirmation primer pair, the sample was sequenced and the DNA sequence analysis result listed below showed that the tag was inserted successfully to Rev7. REV7 coding sequence:

5'....AGAGCATTTTTGGATCTTTGTTT (TAA) GTG CAAAAATGA...

The following DNA sequence was inserted before the stop code of *REV7* (the 3' DNA sequences underlined will encode 3 HA epitopes, 3 HA sequences

are separated by linker sequence and TGA underlined is the stop code.) The sequence after the stop code is a sequence of *HIS3* promoter and its coding sequence which makes the cell able to synthesize amino acid histidine, so histidine is the selection amino acid for selection.

CGGATCCCCGGGTTAATTAACATCTTTTACCCATACGATGTTCCTGACTATG
CGGGCTATCCCTATGACGTCCCGGACTATGCAGGATCCTATCCATATGAC
GTTCCAGATTACGCTGCTCAGTGCTGAGGCGCGCCACTTCTAAATAAGCGA
ATTTCTTATGATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAATAAGTGT
ATACAAATTTTAAAGTGACTCTTAGGTTTTAAAACGAAAATTCTTATTCTTGAG
TAACTCTTTCCTGTAGGTCAGGTTGCTTTCTCAGGTATAGTATGAGGTCGCT
CTTATTGACCACACCTCTACCGGCAGATCCGCTAGGGATAACAGGGTAATA
TAGATCTGTTTAGCTTGCCTCGTCCCCGCCGGGTCACCCGGCCAGCGACAT
GGAGGCCCAGAATACCCTCCTTGACAGTCTTGACGTGCGCAGCTCAGGGG
CATGATGTGACTGTCGCCCGTACATTTAGCCCATACATCCCCATGTATAATC
ATTTGCATCCATACATTTTGATGGCCGCACGGCGCGAAGCAAAAATTACGG
CTCCTCGCTGCAGACCTGCGAGCAGGGAAACGCTCCCCTCACAGACGCGT
TGAATTGTCCCCACGCCGCGCCCCTGTAGAGAAATATAAAAGGTTAGGATTT
GCCACTGAGGTTCTTCTTTCATATACTTCCTTTTAAAATCTTGCTAGGATACA
GTTCTCACATCACATCCNAACATAAACAACCATGGCAGANCNGCCCAAAAAA
GCAAAAACAAACTGTTCNNGGNGCANGGNGTTTATCTCCCGTATCACTAAT
GAAACTAAAATTCAAATCGCTATTTCGCTGAANGGNNGNNATATTC

Amino acid sequence in the fusion Rev7 is Rev7-RIPGLINIF-YPYDVPDYA-G-YPYDVPDYA-GS-YPYDVPDYA-AQC

D: Knockout of *SWI6* in the strain *REV7* is tagged with 3HA

Protein samples were prepared for Western blotting analysis to detect Rev7 protein using antibody against HA. Three strains used were: BY4741 wild type as a negative control, BY4741 wild type strain with Rev7-3HA, *SWI6* deleted BY4741 strain with Rev7-3HA.

E: Protein sample preparation

$4x10^8$ cells were centrifuged and washed once with water. The pellet was washed with 0.5 ml 20% TCA, re-suspended with 200µl 20% TCA. Zirconium beads were added up to two thirds of volume, and vortexed at 4°C for 20 minutes. Liquid was transferred into new tube, the beads were washed twice with 200µl 5% TCA and the liquid was collected into the same tube. The samples were centrifuged for 10 minutes at 3,000 rpm at room temperature. The pellet was re-suspended in 200µl of SDS-loading buffer. 100µl of 1M Tris base was added. The sample was boiled and clarified by centrifugation for loading. For protein samples from different cell phases, cells were first arrested in G1 phase using alpha factor. In liquid YPD, for first 75 minutes, the alpha factor concentration was 5µg/ml, and alpha factor was added to make its final concentration reach 10µg/ml (about 30 minutes). After cells were arrested in G1 phase, alpha factor was washed away and the cells were cultured in new YPD

media and at different time points, cells were collected for protein extraction to detect Rev7 protein levels.

F: Western blotting analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel was made of ten percent of resolving gel (11.9ml water, 10ml 30% acrylamide mix, 7.5ml 1.5M Tris, pH 8.8, 0.3ml 10% SDS, 0.3ml ammonium persulfate and 12µl TEMED were added and mixed) with a five percent stacking gel (5.5ml water, 1.3ml 30% acrylamide mix, 1.0ml 1M Tris, pH6.8, 80µl 10%SDS, 80µl 10% ammonium persulfate and 8µl TEMED were added and mixed). SDS-PAGE gel was used to separate the protein samples (100 volts for the first hour when the samples were in the stacking gel followed by 120 volts when the samples were run in resolving gel). The separated proteins were transferred to the nitrocellulose membrane using transfer buffer soaked filter and membrane. The membrane was pre-incubated with 5% nonfat milk in TTBS buffer for 1 hour at room temperature. The solution was disposed and new 5% nonfat milk with 1:1000 diluted primary antibody against HA epitope was added and membrane was incubated overnight at 4°C. The unbound primary antibody was washed away and the membrane was incubated with secondary antibody-HRP (1:2000) for one hour at room temperature. After the rinse, the membrane was put in the buffer and substrate mix solution for reaction and then exposed to the film for development.

Over-expression of REV7 in *SWI6* or *SWI4* deleted strain. The plasmid used was pEGLH6yhREV7 (Leu2 is the selection marker). The *REV7* is constructed with galactose inducible promoter, so high concentration of galactose in the medium can induce *REV7* overexpression after plasmid transformation.

The plasmid was transformed into *SWI6* or *SWI4* deleted cells and the cells were put on SC minus leucine, minus tryptophan medium (1.5% galactose, 0.5% dextrose) and their mutagenesis level change was recorded.

Overexpression of Rev7 in *SWI6* or *SWI4* deleted CRY1 strain--plasmid transformation

The cells were inoculated in liquid YPD at 30°C on a shaker at 200 rpm until it's equivalent to $2x10^7$ cells/ml. Cells were harvested in a sterile 50ml centrifuge tube at 3000x g for 5 minutes. The medium was poured off and the cells were re-suspended in 25 ml of sterile water and centrifuged. The cells was re-suspended in 1.0 ml 100mM LiAc and transferred to a 1.5 ml microfuge tube. The cells were centrifuged at top speed for 15 seconds and LiAc solution was removed with micropipette. The cells were re-suspended to a final volume of 500µl ($2x10^9$ cells/ml) and vortexed. 50µl sample was pipetted into labeled microfuge tubes. The cells were pelleted and LiAc solution was removed with a micropipette. 240µl polyethylene glycol (PEG) (50%), 36µl 1.0 M LiAc, 10µl SS-DNA (2.0mg/ml), 40µl plasmid DNA and 34µl sterile ddH2O were added carefully. Each tube was vortexed vigorously until the cell pellet has been completely mixed and incubated

at 30°C for 30 minutes. The sample mixure was subjected to heat shock in a water bath at 42°C for 30 minutes. The transformation solution was removed with a micropipette after centrifuge. 1.0ml of sterile water was pipetted into each tube and the pellet was re-suspended by pipetting it up and down gently. 200µl of the transformation mix was spread on SC-minus histidine media plates. Transformants were incubated at 30°C for 2 to 4 days for selection of successful transformed cells.

Results

In the present study, *SWI6* was shown to be involved in DNA damage-induced mutagenesis in *Saccharomyces cerevisiae*. TLS components may be regulated at the transcription level by transcription factors such as Swi6. BY4741 wild type strain and *SWI6*, *SWI4* or *MBP1* deletion mutant derivatives were used for comparison of TLS components mRNA level or protein expression level change.

The *mRNA level analysis of REV1, REV3 and REV7 after SWI6, SWI4 or MBP1 deletion was determined using Northern blotting.* As shown in Figure 1, *REV1* and *REV3* mRNA level are not decreased compared to that of wild type cells after deletion of *SWI6*, *SWI4* or *MBP1*. *REV7* mRNA is decreased in *SWI6* or *SWI4* deleted strains, but *REV7* mRNA level is not decreased in *MBP1* deleted strain. These suggest that the complex formed by Swi4 and Swi6 regulates expression of *REV7*, complex formed by Swi6 and Mbp1 does not.

mRNA level of REV7 is decreased after deletion SWI6 or SWI4 using RT-PCR. mRNA level of *REV7* is extremely low even in wild type cells. To further confirm that Swi6 and Swi4 regulate mRNA level of *REV7*, RT-PCR was used. The result was similar to that in the Northern blot study. Deletion of *SWI6* or *SWI4* decreased the *REV7* mRNA expression level, deletion of *MBP1* did not have effect on *REV7* mRNA level (Figure 2A). RNA sample was also added with only *Taq* DNA polymerase mix to determine if there was *REV7* DNA

contamination. A band was not detected, thus RNA sample was clean for RT-PCR anaysis.

REV7 is not inducible by UV-C. To check if *REV7* was UV inducible, cells were exposed to 60 J/m^2 of UV and then were cultured in YPD media and clollected at 30 and 60 minutes. The cell samples were collected and total mRNA was extracted and RT-PCR was performed to analyze if *REV7* level was stable or not compared to the sample without UV treatment. No significant change of *REV7* mRNA was detected in the sample without UV treatment and samples after 30 and 60 minutes of UV treatment. (Figure 2B)

Rev7 protein level is affected by deletion of SWI6. HA tag DNA sequence was inserted into the 3' end of *REV7* coding sequence at the chromosome to make the cell express HA-tagged Rev7 protein. An antibody against HA was used to detect the Rev7 protein level change after *SWI6* deletion. The results show that Rev7 protein is significantly decreased after *SWI6* deletion (Figure 3). This further demonstrated that Swi6 regulated expression of *REV7* and affected mutagenesis indirectly. Rev7 protein level was stable in different cell phases.

Overexpression of REV7 rescued the cells partially from low mutagenesis phenotype caused by deletion of SWI6 or SWI4. Previous experiments showed that *SWI6* was involved in mutagenesis after the screen and at the mRNA level and protein level, Swi6 affected Rev7 level. Overexpression of Rev7 in *SWI6* or *SWI4* deleted strain should increase the level of mutagenesis. Plasmid with galactose inducible *REV7* was transformed into the *SWI6* or *SWI4* deleted cells.

After transformation of the plasmid, mutagenesis increased in *SWI6* or *SWI4* deleted cells, but wild type cells with transformed plasmid did not change the mutagenesis level (Figure 4).

Discussion

After a systematic screen among 4786 non-essential genes to search for novel genes involved in DNA damage-induced mutagenesis using *CAN1* forward mutation assay system, a reverse mutation assay system was used for further verification, *SWI6* is one of the candidate genes. Deletion of *SWI6* makes the cell show a decreased mutagenesis phenotype in the *CAN1* forward mutation and reverse mutation assays. As a transcription cofactor, Swi6 forms a complex with DNA binding protein Swi4 or Mbp1 to function to regulate transcription at G1/S phase transition.[2,4]

SBF (Swi6.Swi4 complex) binds to SCB (Swi4/6-dependent cell cycle box) to regulate gene expression. MBF (Mbp1.Swi6 complex) binds to MCB (*Mlu*I cell cycle box) to regulate gene expression. About 25% of MBF target genes are involved in replication, recombination, and repair of DNA.

Protein activation can be regulated at the transcription and translation levels, or by post-translational modifications including phosphorylation, acetylation, methylation and ubiquitination. For TLS components, the mono-ubiquitination of PCNA initiates TLS pathway[13]. Some genes can also be induced to respond to environmental insults. For example, *RNR1, RNR3* and *RNR4* can be induced by UV[14,15]. Some proteins function as a complex such as Rev3 and Rev7. These proteins form a complex and function as DNA polymerase zeta essential for translesion DNA synthesis. Rev3 is the catalytic subunit and Rev7 the regulatory subunit. Deletion of either protein will make the cell show a moderate UV

sensitivity and significant decreased mutagenesis. Rev1 is a deoxycytidyl transferase which can bypass abasic lesions and lead to mutation. Deletion of *SWI6*, *SWI4* or *MBP1* does not affect expression of *REV1* or *REV3* at mRNA level. Deletion of *SWI6* or *SWI4* did affect mRNA level of *REV7*, but the deletion of *MBP1* did not. At the protein level, deletion of *SWI6* makes the cell show an extremely low Rev7 protein level which may directly influence TLS efficiency and make the cells show a low mutagenesis phenotype. Overexpression of Rev7 in *SWI6* deleted cells makes the cell show partially recovered mutagenesis relative to wild type cells.

DNA damage can trigger several cellular responses including cell cycle arrest, gene transcription activation, DNA repair, and apoptosis. In cancer treatment, chemotherapy and radiotherapy are used to damage the cancer cell DNA and ultimately kill the cell. DNA repair and TLS will be involved in the attempt to repair the damaged DNA and bypass the lesion to make the cell survive. It is possible that the TLS components can be the anticancer targets to decrease the bypass efficiency to kill more cells. DNA polymerase zeta is the most important error-prone polymerase essential for DNA damage-induced mutagenesis and spontaneous mutagenesis in yeast and human. In yeast, Rev3 and Rev7 are not essential. In mice, knockout of *REV3* makes mice die at midgestation of embryogenesis[17]. This makes the Rev7 more important in mammalian cells as an anticancer target. Rev7 can regulate activity of DNA polymerase zeta. Knockdown of Rev7 can make the TLS less efficient and as a small protein Rev7 can be better targeted at the transcription level.

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Figure legends

Figure 1. *Northern blot analysis for mRNA level of REV1, REV3, REV7.*

After deletion of *SWI6* or *SWI4* in BY4741, mRNA level of *REV7* is decreased compared to the wild type cells, deletion of *MBP1* does not have obvious effect on the level of *REV7* mRNA. Deletion of *SWI6*, *SWI4* or *MBP1* does not affect mRNA levels of *REV1*, *REV3*.

Figure 2A. *RT-PCR analysis to further detect REV7 mRNA level after gene deletion.*

RT-PCR results also further confirm that deletion of *SWI6* or *SWI4* decreased the level of *REV7* mRNA, but deletion of *MBP1* does not have detectable effect on *REV7* mRNA level. *PDA1* is a loading control (pyruvate dehydrogenase subunit).

Figure 2B. *REV7 is not UV inducible in wild type cell.*

After exposure to 60 Joules/m^2 of UV, samples were collected after 30 minutes and 60 minutes to determine if UV induces *REV7* at the mRNA level. *REV7* mRNA level is similar in cells collected before UV treatment.

Figure 3A. *Western blot analysis for Rev7 protein level after SWI6 deletion.*

Rev7 protein level was significantly decreased after deletion of *SWI6* compared

to sample from wild type cells. 3-phosphoglycerate kinase (Pgk1) is the loading control.

Figure 3B. *Cell phases have no effect on level of Rev7 protein.*

Alpha factor-arrested G1 phase and released cell samples at different time point were collected and extracted for analysis by Western blot analysis against Rev7 protein. Rev7 protein maintains the same level at different cell phases.

Figure 4. *Overexpression of Rev7 in SWI6 or SWI4 deleted cells partially rescue the low mutagenesis after UV compared to the wild type in CRY1 strain.*

Galactose inducible promoter fused with *REV7* coding sequence was included in plasmid. Transformation of the plasmid into *SWI6* or *SWI4* deleted cells makes the cells show increased mutagenesis. Overexpression of Rev7 in wild type cells does not affect its mutagenesis level.

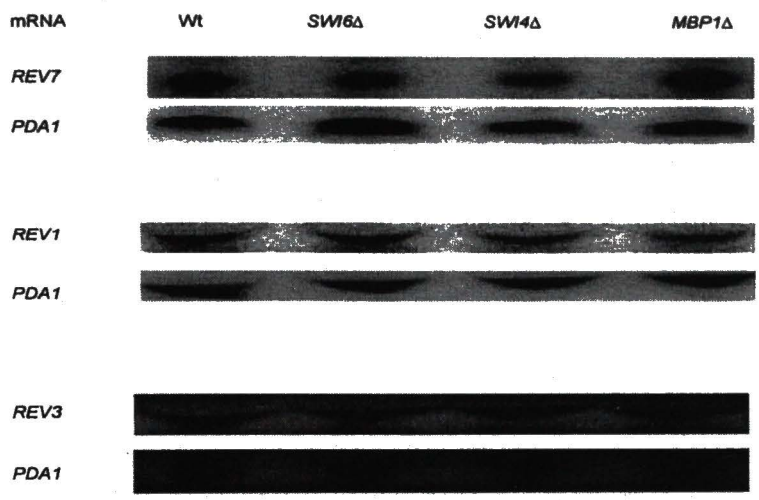

Figure 1. Northern blot analysis

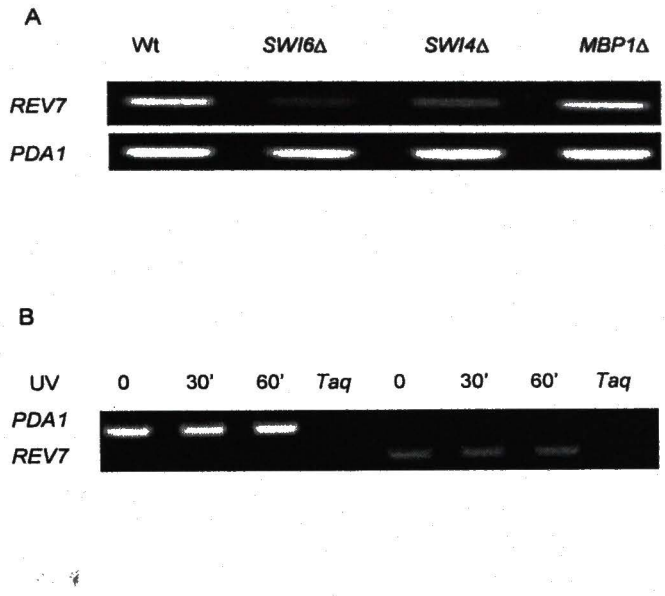

Figure 2. RT-PCR analysis A: *REV7* mRNA level after gene deletion B: *REV7* mRNA level after UV treatment

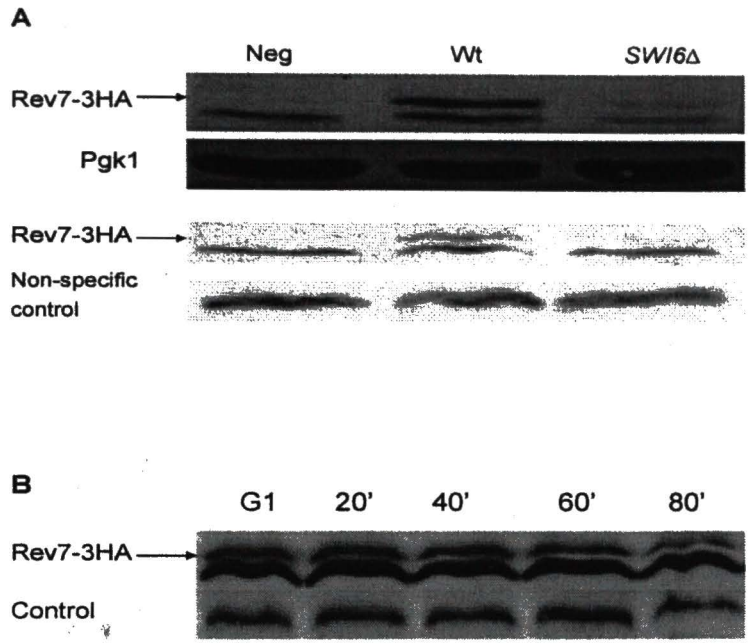

Figure 3. Western blot analysis A: Rev7 protein level after SWI6 deletion
B: Rev7 protein level at different cell phase stages

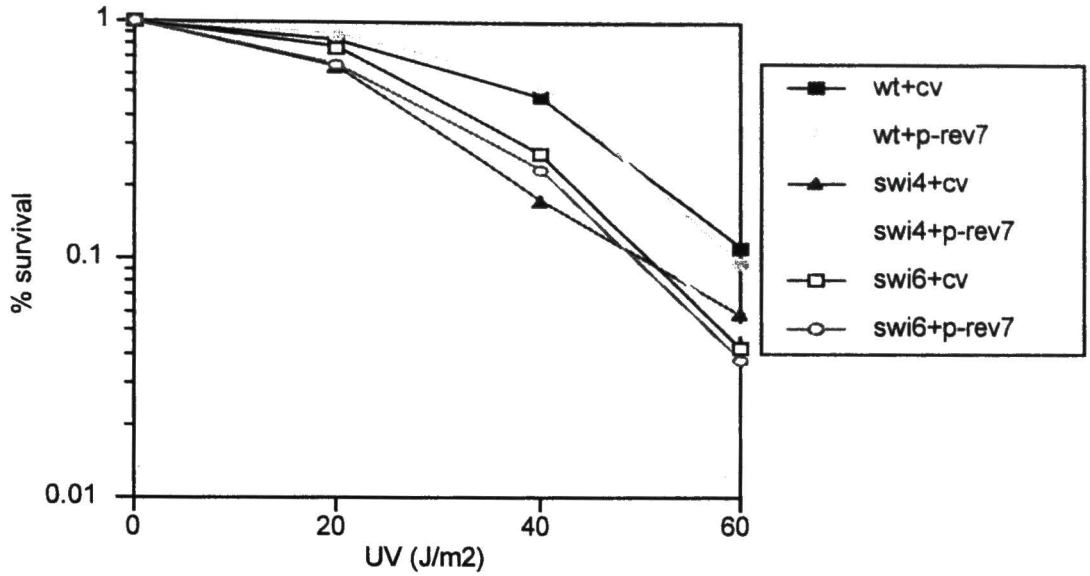

Figure 4A. UV sensitivity after overexpression of *REV7*

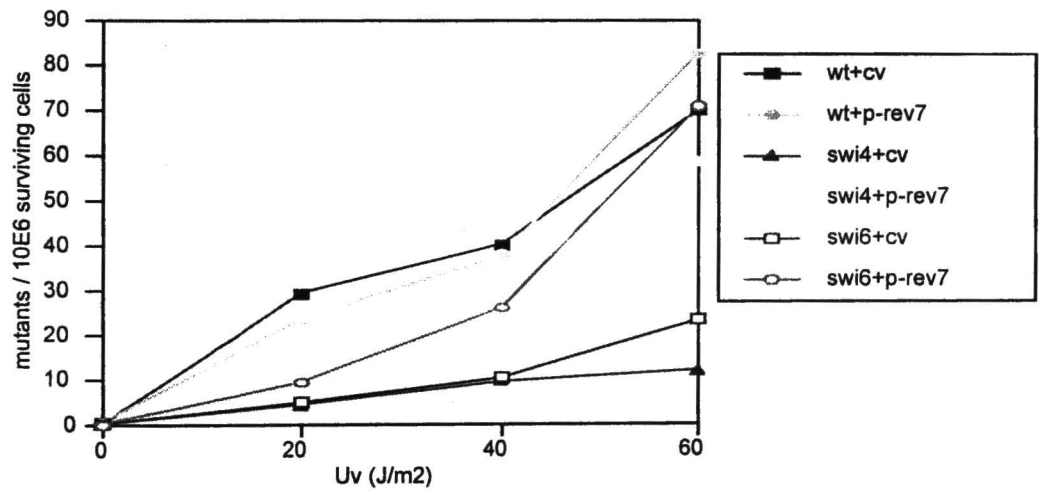

Figure 4B. Mutagenesis level change after overexpression of *REV7*

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

This study examined novel genes *SWI6* and *DOA4* involved in DNA damage-induced mutagenesis in *Saccharomyces cerevisiae*. *SWI6* encodes a transcription cofactor that can form a complex with DNA binding protein Swi4 or Mbp1 to regulate gene expression at the G1/S transition in *Saccharomyces cerevisiae*. *DOA4* encodes a ubiquitin isopeptidase that is required for recycling ubiquitin from proteasome-bound ubiquitinated intermediates. Deletion of *SWI6* or *DOA4* makes the cell show decreased mutagenesis phenotype after different doses of UV treatment. At the molecular level, Swi6 affects *REV7* mRNA level, but not levels of *REV1* or *REV3*. Swi6 affects Rev7 mRNA level by forming a complex with Swi4, not Mbp1. At the protein level, deletion of *SWI6* significantly decreased the Rev7 protein level. *REV7* is not UV inducible or cell cycle regulated. Therefore, expression level of Rev7 plays an important role in affecting DNA damage-induced mutagenesis.

DNA damage triggers cell responses to the damage which include cell cycle arrest, gene transcription activation, DNA repair, DNA damage tolerance and apoptosis. DNA repair includes direct reversal, base excision repair, nucleotide repair, mismatch repair, non-homologous end joining and homologous recombination. If the DNA damage cannot be repaired, the cell will tolerate the damage or cell apoptosis can be induced. Translesion DNA synthesis is a tolerance process and can potentially lead to mutation occurrence. DNA damage

can initiate cancer and now radiological and chemical agents are used to damage cancer cell DNA to treat cancer patients. Potentially inhibition if components involved in DNA repair and DNA damage tolerance can be used together with radiological or chemical agents to kill cancer cells and make cancer cells lose the ability to repair DNA. DNA polymerase zeta is an important error-prone polymerase involved in translesion DNA bypass. DNA polymerase zeta consists of two subunits Rev7 and Rev3 in yeast as well as in mammalian cells. Rev7 is an accessory subunit and Rev3 is the catalytic subunit. Deletion of *REV7* or *REV3* makes the cell show extremely low mutagenesis level. In yeast, deletion of *REV7* makes cells become more UV sensitive and mutagenesis is decreased significantly because the cell loses most of the ability of translesion bypass, the cell cannot complete the DNA replication for reproduction, so the cell dies. In mouse cells, *REV3* deletion is lethal for its embryo, so *REV7* could be a target coupled with radiotherapy or chemotherapy to treat cancer patients and enhance the therapy.

Cancer can happen in people of all ages but more often in middle-aged or older people than in young people. It is because cancer cell occurrence is a process of cell selection after mutation. Only accumulation of mutations in DNA reaches a certain level may cancer occur, accumulation of DNA mutations in cells is a long process. It is possible to inhibit or delay the process of cancer cell formation in one's lifespan by inhibiting translesion DNA bypass system. Rev7 and Rev3 can both be the targets, Rev3 defect is lethal to the mouse embryo, so there may be some side effects if Rev3 is inhibited, Rev7 could be partially

inhibited at the protein level or its expression could be regulated at the transcriptional level and thus reduce the activity of DNA polymerase zeta. Copy number of Rev7 is far more than that of Rev3 in yeast cells, so inhibition of Rev7 at the transcriptional level is better to control the activity of DNA polymerase zeta. Complex of Swi6 and Swi4 regulates expression of *REV7* in yeast and affects cell mutagenesis indirectly, Further experiments will be performed to explain how *REV7* expression is regulated by Swi6 and Swi4.

Future experiments with the following design have been planned.

1. An electrophoretic Mobility Shift Assay (EMSA) analysis can be used to determine if Swi4 binds to promoter of *REV7*. The EMSA technique is based on the observation that protein:DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide gel electrophoresis. PCR will be used to amplify the 400 nucleotides pairs upstream of the coding sequence for *REV7* and the product will be labeled with P^{32}. The P^{32}-labeled DNA fragment with nuclear extract from *SWI4*-deleted cells and wild-type cells. The mixture will be subjected to non-denaturing polyacrylamide gel eletrophoresis. The dried gel will be subjected to auto-radiography (-80°C). Furthermore, the purified Swi4 will be used detect the binding to the promoter sequence, GST coding sequence will be inserted to the 3' end of *SWI4* so that the cell can encode a fusion protein of Swi4 tagged with GST at its C terminus. This tag will be used to purify Swi4 protein for an EMSA

experiment. A DNA band shift will be observed after mixing with P^{32}-labeled PCR product compared to the PCR product alone.

2. To detect how *DOA4* is involved in mutagenesis, a study will be undertaken to determine the level of monoubiquitination of PCNA is decreased after *DOA4* deletion. After DNA damage, the Rad6.Rad18 complex is involved in monoubiquitination of PCNA. The monoubiquitination of PCNA initiates the translesion DNA systhesis. Doa4 regulates free ubiquitin pool in the cell. Decreased concentration of free ubiquitin in the cell may lead to decreased level of monoubiquitinated PCNA, thus affecting translesion synthesis efficiency.