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Deletion of the RNR4 gene causes hyperresistance to the carcinogen 4-NQO in the yeast model

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Cette thèse intitulée :

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Résumé

La stabilité génomique, qui est essentielle à la vie, est possible grâce à la réplication et la réparation de l'ADN. Une des enzymes responsables de la réplication et de la réparation de l'ADN est la ribonucleotide reductase (RNR), qui est retrouvée chez la levure et chez l'humain. Cette enzyme catalyse la formation de déoxyribonucléotides et maintien le pool de dNTP requis pour la réparation et la réplication de l'ADN. L'enzyme RNR est un tétramère $\alpha_2\beta_2$ constitué d'une grande (R1, α_2) et d'une petite (R2, β_2) sous-unité. Chez *S. cerevisiae*, les gènes *RNR1* et *RNR3* encodent la sous-unité α2 (R1). L'activité catalytique de RNR dépend d'une interaction avec le fer et de la formation d'un complexe entre R1 et R2. L'expression de toutes les sous-unités est inductible par les dommages causés à l'ADN.

Dans cette étude, nous démontrons que des cellules qui n'expriment pas une des sous-unités, Rnr4, du complexe RNR sont sensibles à divers agents endommageant l'ADN, tels que le méthyl méthane sulfonate, la bléomycine, le péroxyde d'hydrogène et les rayons ultraviolets (UVC 254 nm). Au contraire, le mutant est résistant au 4-nitroquinoline-1-oxide (4-NQO), un composé qui engendre des lésions encombrantes. Par conséquent, le mutant *rnr4*Δ démontre une réduction marquée en mutations induites par le 4-NQO comparativement à la souche parentale. Nous voulions identifier la voie de réparation de l'ADN qui conférait cette résistance au 4-NQO ainsi que les protéines impliquées. Les voies BER, NER et MMR n'ont pas aboli la résistance au 4-NQO de la souche *rnr4*Δ. La protéine recombinante Rad51 ne joue pas un rôle critique dans la réparation de l'ADN et dans la résistance au 4-NQO. La délétion du gène *REV3*, qui encode une polymérase de contournement, impliquée dans la réparation post-réplication, a partiellement aboli la résistance au 4-NQO dans *rnr4*Δ. Ces résultats suggèrent que la polymérase Rev3 et possiblement d'autres polymérases translésion (Rev1, Rev7, Rad30) pourraient être

impliquées dans la réparation de lésions encombrantes dans l'ADN dans des conditions de carence en dNTP.

La réparation de l'ADN, un mécanisme complexe chez la levure, implique une vaste gamme de protéines, dont certaines encore inconnues. Nos résultats indiquent qu'il y aurait plus qu'une protéine impliquée dans la résistance au 4-NQO. Des investigations plus approfondies seront nécessaires afin de comprendre la recombinaison et la réparation post-réplication.

Mots-clés : Réparation de l'ADN, RNR, 4-NQO, recombinaison, polymérases de translésion

Abstract

Genomic stability, critical for life, is controlled by DNA replication and repair. DNA replication and repair is mediated through many enzymes, one being ribonucleotide reductase (RNR), an enzyme found in both yeast and humans. RNR catalyzes the reaction involved in the formation of deoxyribonucleotides and is responsible for maintaining dNTP pools required for DNA repair and replication. RNR is an $\alpha_2\beta_2$ tetramer consisting of a large (R1, α_2) and small subunit (R2, β_2). In *S. cerevisiae RNR1* and *RNR3* encode α2 (R1). RNR catalytic activity depends on its interaction with iron and on the formation of the complex between R1 and R2. All subunits are inducible by DNA damage.

Here we show that cells lacking one subunit, Rnr4, of the RNR complex are sensitive to various DNA damaging agents such as methyl methane sulfonate, bleomycin, hydrogen peroxide, and ultraviolet radiation (UVC 254 nm). In contrast, the mutant is resistant to 4-nitroquinoline-1-oxide (4-NQO), an agent which produces bulky lesions. Consistent with this resistance, the *rnr4*Δ showed a sharp reduction in 4-NQO-induced mutations as compared to the parent.

We wanted to determine which pathway was able to confer resistance to 4-NQO and thus targeted DNA repair proteins. The repair pathways BER, NER and MMR did not abolish 4-NQO resistance in *rnr4*Δ. Recombination protein Rad51 (NHEJ) was lethal in an *rnr4*Δ thus indicating no role in DNA repair and 4-NQO resistance. Deletion of the REV3 gene, encoding a DNA bypass polymerase involved in post replication repair, partially abolished 4-NQO resistance in *rnr4*Δ. These results suggest that Rev3 and possibly other translesion polymerases (Rev1, Rev7, Rad30) could play a role in the repair of bulky DNA lesions under low levels of dNTPs.

DNA repair, a complex mechanism in yeast, involves a vast array of proteins, some yet to be discovered. Our results indicate that there is more than one protein involved in 4-NQO resistance and further investigation is required concerning recombination and post replication repair.

Keywords: DNA repair, RNR, 4-NQO resistance, recombination, bypass polymerase

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List of Abbreviations

4-NQO	4-nitroquinoline-1-oxide
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AP	apurinic/apyridimic
BLM	bleomycin
bp	base pair
BSA	bovine serum albumin
C	cytosine
°C	degree Celsius
CDP	cytidine diphosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dNTP	deoxynucleotide triphosphate
DSB	double strand breaks
dTTP	2'-deoxythymidine 5'-triphosphate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra acetic acid
EMS	ethane methanesulfonate
Fig.	figure
G	guanosine
g	gram
GDP	guanosine diphosphate
H_2O_2	hydrogen peroxide
HR	homologous recombination
HU	hydroxyurea
J	Joule
kDa	kiloDalton

L	litre
M	molar (moles/litre)
mA	milliAmp (10^{-3} A)
ml	millilitre (10^{-3} L)
mM	millimolar (10^{-3} M)
MMR	mismatch repair
MMS	methyl methanesulfonate
NER	nucleotide excision repair
ng	nanogram (10^{-9} gram)
nM	nanomolar (10^{-9} M)
NHEJ	non-homologous end joining
o/v	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol (TE/Li + 40% PEG)
PRR	post-replication repair
RNA	ribonucleic acid
RNAse A	ribonuclease A
RNR	ribonucleotide reductase
Rnr1/R1/Y1	ribonucleotide reductase α-subunit
Rnr3/R1/Y3	ribonucleotide reductase α-subunit
Rnr2/R2/Y2	ribonucleotide reductase β-subunit
Rnr4/R2/Y4	ribonucleotide reductase β-subunit
RNR	ribonucleotide reductase gene
RNR1	R1 gene
RNR2	R2 gene
RNR3	R3 gene
RNR4	R4 gene
RPA	replication protein A

RR inhibitor	ribonucleotide reductase inhibitor
SSA	single-strand annealing
SSB	single strand breaks
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
T	thymine
TBS	25 mM Tris pH 7.0, 19.2 mM Glycine and 20% methanol
TBS-T	(TBS + 0.1% Tween)
TE	10 mM tris, 1 mM EDTA pH 8.0
TE/Li	TE + 0.1 mM LiCL
TLS	translesion repair
U	uracil
UDP	uracil diphosphate
UVC	ultraviolet C (wavelength range 220-280 nm)
μg	microgram (10^{-6} gram)
μl	microlitre (10^{-6} litre)
μM	micromolar (10^{-6} M)
V	volt
YGD	Yeast Genome Database
YPD	1% yeast extract, 2% peptone, 2% dextrose
YPDa	YPD + 2% agar

In dedication to my family for their love and support.

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This work would not have been possible without my loving family, especially my mother and father. I would like to dedicate my work to them for always believing in me. I would also like to dedicate this work to my strong support of friends, also scientists, who have brought much to my thoughts and have been there throughout the term of my project.

1. Introduction

1.1. General Review

Ribonucleotide reductase (RNR) is an enzyme that controls cellular levels of deoxyribonucleotides and catalyzes the reaction involved in the formation of deoxyribonucleotides from the four ribonucleoside diphosphates ADP, GDP, CDP and UDP [1-3]. RNR is also responsible for maintaining dNTP pools required for DNA repair and high fidelity replication. RNR differs from other enzymes in that it requires a free radical to complete its reaction [1-3].

Cells contain a small pool of dNTPs used for DNA replication. They are able to produce dNTPs de novo but the high demand for dNTPs is highly regulated by RNR [4]. RNR thus plays a central role for DNA synthesis.

1.2. The Enzyme Classes and Function of RNR

There exist 3 classes of RNR that all have similar mechanisms of NDP reduction and all utilize free-radical chemistry [1, 2]. The first reaction step of all RNR classes involves radical generation and storage. These radicals are transferred to cysteine side chains. The NDP reduction is initiated by the free radical and involves a reduction at the 2'-carbon of ribose 5-phosphate to form dNTPs [1, 2].

With respect to the NDP reduction mechanism, class I RNR utilizes a tyrosyl radical located within one of its smaller subunits that is generated via a dinuclear iron center [1, 2]. This class of enzymes is the most studied and best known of the ribonucleotide reductases. They are found mainly in eukaryotic organisms and in some viruses and prokaryotes. There is a 19% sequence identity between mouse and *E. coli* R2 but there is a 50% or higher identity within eukaryotic R2 proteins [5, 6]. Class 1 enzymes are subdivided into Ia

and Ib categories whereby class Ib are only located within prokaryotic organisms. Class I RNR is oxygen dependent and forms a $\alpha_2\beta_2$ tetramer [5, 6]. The larger alpha subunit R1 contains the active site for substrate binding while the smaller beta subunit R2 contains the tyrosyl free radical. *S. cerevisiae* is the exception to the smaller subunit in that only one of the two subunits making up R2 can house the dinuclear iron radical [1, 2, 6]. The structural model of RNR is shown in Fig. 1.

Class II RNRs generate a transient 5'- deoxyadenosyl radical radical via adenosylcobalamin [1, 2, 6]. Class II RNRs contain only one alpha or beta subunit and while it may be found in some microorganisms, the main model studied is that of Lactobacillus leichmanni [6, 7]. Radical formation is due to the cleavage of the adenosyl-Co bond of adenosylcobalamin [6, 7]. Recently the sequences of several class II RNRs have been characterized and their catalytic domains share some similarity with those of class I and III RNRs [6-8].

Class III enzymes use a glycyl radical that is generated via an iron sulfur protein and S-adenosyl methionine [7, 9]. Class III RNRs are $\alpha_2\beta_2$ tetramers found in prokaryotic organisms [9]. The active form of RNR III, α_2, contains the active site radical and the binding site for allosteric effectors. The β_2 subunit contains a small iron-sulfur-containing protein that is responsible for the formation of the glycyl radical [9]. Class III RNRs are anaerobic but are similar to other classes of RNR in that its R1 subunit contains the substrate-binding domain.

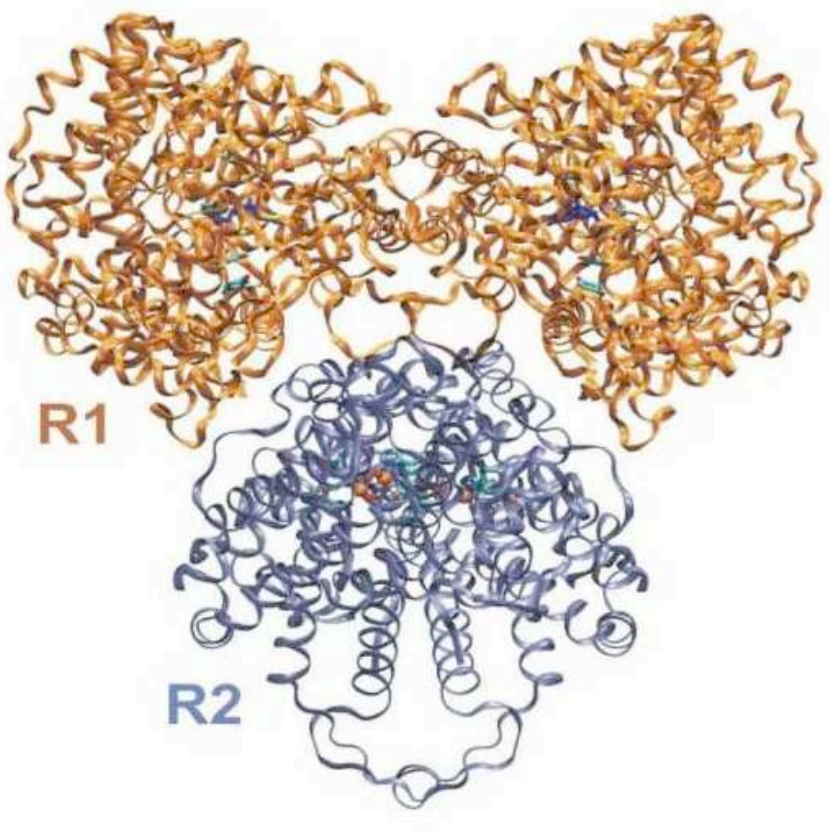

Figure 1. **RNR Structure.** Structural model of the R1/R2 holoenzyme of E.coli ribonucleotide reductase (Figure from M. Högbom, University of Stockholm; http://mvl.chem.tu-berlin.de/ak_hildebrandt/lendzian/projects/Ribonucleotide_Reductase.html)

1.2.1. Class I RNR

Class 1 RNR is an $\alpha_2\beta_2$ tetramer consisting of a large (R1, α_2) and small subunit (R2, β_2) [1-3]. Both subunits interact to create an active heterodimeric tetramer [2]. Each α monomer has a binding site for substrate and allosteric effectors; the first site is responsible for substrate specificity as well as maintenance of dNTP pools while a second site has both ATP and dATP binding ability and is also responsible for maintaining a balance between dNTP and NTP levels (reduction of NDP) [2]. R1 also contains several redox-active cysteine residues. Each β monomer of R2 contains a tyrosyl free radical that is induced by a binuclear iron complex. The tyrosyl free radical, required for catalytic activity, is also a

cofactor for NDP reduction. Its catalytic activity depends on its interaction with iron and on the formation of the complex between R1 and R2 [2].

1.2.2. Structure and Function of yeast RNR Class I Subunits

In *S. cerevisiae* two genes, *RNR1* and *RNR3*, encode R1 (α_2) and are located in the cytoplasm during the normal cell cycle. Each subunit of R1 is 85.5kDa. Human and yeast *RNR1* homolog share 66% sequence identity and 83% sequence similarity [10]. RNR1 is essential for mitotic viability and inducible by DNA damage while *RNR3* is non-essential and is highly inducible by DNA damage. It has been shown that *RNR3* on a high copy number plasmid can suppress the lethality of *RNR1* mutations [11]. Each R1 monomer contains: a main helical domain that has 220N-terminal residues, a large ten-stranded α/β barrel structure and a small five stranded α/β structure that have 480 and 70 residues respectively [11-13]. Each alpha and beta subunit contains five parallel strands and four connecting helices [13, 14].

The small subunit, R2 (β_2) is located in the cytoplasm but close to the nucleus during a normal cell cycle. *RNR2* and *RNR4* encode the small subunit of ribonucleotide reductase [3]. Both *RNR2* and *RNR4* are essential for mitotic viability and are inducible by DNA damage. Their amino acid sequences share 47% identity [1, 3, 6]. The N-terminus of *RNR2* is 50 amino acids longer than that of *RNR4*. As aforementioned, R2 contains a diferric iron center as well as a stable tyrosyl radical [6]. The radical is buried in a deeply hydrophobic region and is stabilized by the proximal iron center. The two μ-oxo linked central irons are organized via four carboxylates and two histidines [12, 15]. Upon reduction, some of the carboxylate-ligands change conformation due to the loss of the oxybridge, two bound water molecules and to the decrease from 4 to 6 of the coordination

number [12, 15]. The iron center thus becomes available to dioxygen for subsequent steps required for radical generation [12, 15].

1.2.3. Holoenzyme and Allosteric Regulation

There exists a crystal structure for class II RNR in which the all key structural features of the catalytic and regulatory domains of oligomeric RNRs are retained. The dimer interface responsible for effector binding in class I RNR is preserved through a single 130-residue insertion in the class II structure [16]. It is thought that the C terminus of R2 associates with that of R1.

The class I RNR R1 subunit is allosterically regulated with activating, ATP, and deactivating, dATP, effectors as shown below in Fig. 2. When dATP or ATP is bound to any allosteric sites, the enzyme accepts UDP and CDP into the active site. ADP enters the active site once dGTP is bound to an allosteric site and when dTTP is allosterically bound, GDP enters the catalytic site [16]. Conformation changes affecting the dimer interface of class II RNRs for example, confer substrate specificity for the active site [17]. Lower levels of dNTPs cause increase in RNR activity while the reverse holds true. End product binding also lowers and/or inhibits RNR activity for all classes of RNR [18].

RNR may also be regulated in a checkpoint dependent manner. In response to DNA damage, Mec1 and Rad53 protein kinases induce RNR activity and recruitment of DNA repair proteins [19-22]. These kinases are responsible for removal of Sml1, the protein inhibitor of RNR. Sml1 protein levels are lowest during S phase. These low levels are associated with post-transcriptional regulation and protein phosphorylation. These events require Mec1 and Rad53. Failure to remove Sml1 results in incomplete DNA replication, defective mitochondrial DNA propagation, lower levels of dNTPs and subsequent cell

death [20-22]. Some inhibitors of RNR have shown to have anti-tumoral properties. Hydroxyurea, an antitumor agent for example, is commonly used as a weak RNR inhibitor [1, 2].

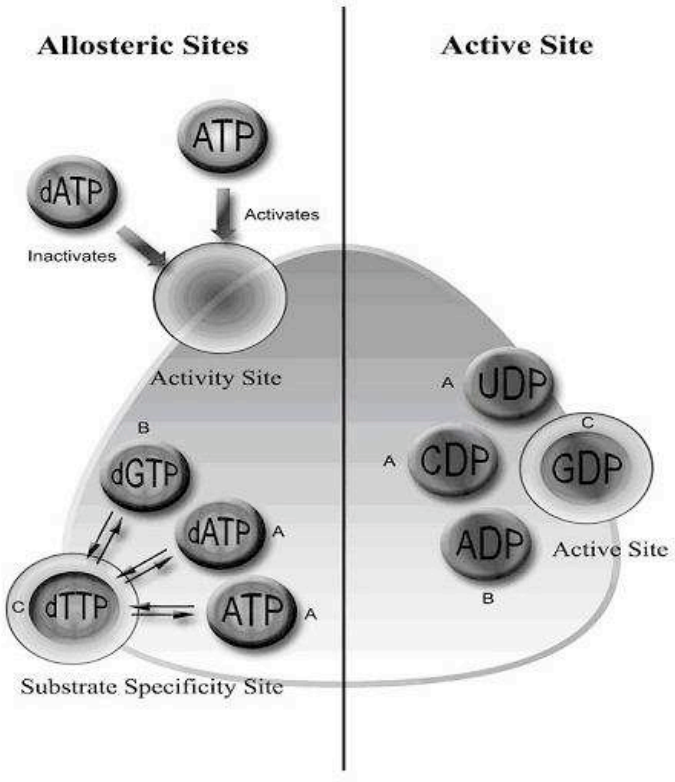

Figure 2. **Class I RNR Regulation**. RNR is activated when ATP is bound and deactivated when dATP is in the active site. Substrates can be reduced upon both activation and when the corresponding effectors are bound to the allosteric substrate specificity site. The following substrates UDP, CDP, ADP and GDP are converted to dNTPs [23].

1.2.4. Yeast RNR4

RNR small subunit protein Rnr4p encodes the small subunit β and has been shown to interact with Rnr2p via in vivo co-immunoprecipitation. [1, 2]. Rnr4p is inducible by hydroxyurea, a DNA damaging agent [1, 2]. This lethality can be overcome by overexpression of *RNR1* or *RNR3*. Overexpression of *RNR2* may complement rnr2 mutations but multiple copies of *RNR2* cannot compensate for rnr4 deletion mutants [1, 2].

Cells lacking Rnr4p are deficient in RNR activity, but the activity may be restored upon addition of exogeneous Rnr4p [2]. Rnr4p is not affected by tyrosyl free radical mutation: a phenylalanine substitution for tyrosine, Tyr 131 an R2 free radical, still complements the *rnr4Δ* mutant [2]. It is believed that Rnr4p lacks sequence elements for iron binding and therefore is not necessarily catalytically active but may play a structural role in the RNR complex. Sommerhalter and coworkers have shown an interaction that connects Rnr4 αA helix to Rnr4 α3 helix. This interaction stabilizes Rnr2 [24]. It has also been shown that the active site for Rnr4 in mouse has different interactions when compared to heterodimer Rnr4 thus supporting the theory that Rnr4 does not bind iron [24]. In addition the Rnr4 subunit lacks three iron-binding residues in *S. cerevisiae* [24].

Rnr4 is needed to activate Rnr2 in that the heterodimeric complex is required for maximum RNR activity [24]. It has been proposed that Rnr2 activity and its ability to control cofactor assembly is dependent on Rnr4 [24].

During the normal cell cycle of budding yeast, *RNR1* and *RNR3* are in the cytoplasm while *RNR2* and *RNR4* are in the nucleus [3, 25-27]. Under genotoxic stress *RNR2* and *RNR4* are displaced to the cytoplasm, which suggests a possible DNA damage checkpoint system. RNR activity is dependent on the heterodimeric complex formed by Rnr2 and Rnr4 that also suggests that they must also be within the same subcellular compartment for maximum enzyme efficiency [3]. A possible theory is that the R1 and R2 subcellular location control dNTP levels and that dNTP synthesis preferentially occurs in the cytoplasm. Perhaps the RNR activity and the levels of its holo-enzyme are regulated by this redistribution under genotoxic stress [3]. It is also important to note that for cells deficient in Mec1, Rad53 and Dun1, the Rnr2 and Rnr4 are not able to relocate to the

cytoplasm under DNA damaging conditions (HU and MMS) [3]. Consequently the co localization of Rnr2 and Rnr4 to the cytoplasm depends on the Mec1⁄Rad53⁄Dun1 pathway. Yao and colleagues also wanted to know if other cell cycle phases affected the redistribution of Rnr2 and Rnr4 and subsequently arrested the cell, undergoing genotoxic stress, in different cycles. The subunits can only relocalize outside the S phase and the only situation in which they cannot is when the Mec1⁄Rad53⁄Dun1 pathway is not fully efficient [3].

1.3. DNA Damage and Repair Pathways in Yeast

1.3.1. DNA Damage and Repair

DNA damage is a common occurrence either by environmental factors or by metabolic processes within a cell. Unrepaired lesions increase chances of tumor formation as cell functions are delayed or blocked. Modifications commonly occur at the helical level in which the damages, non-native chemical bonds or bulky adducts, distort the primary structure of the double-helix [28, 29].

Damages are characterized as either being via exogenous or endogenous. Endogenous sources include metabolic products and free radicals such as reactive oxygen species produced via oxidative deamination. Exogenous sources include ionization radiation, hydrolysis, thermal disruption, man made mutagenic compounds and carcinogenic compounds. There are several types of endogenous DNA damage: oxidation of bases, alkylation of bases, hydrolysis of bases and mismatch of bases [28, 29]. Exogenous damage types include pyrimidine dimers via UV crosslinkage, breaks in DNA via ionizing radiation, increased rate of depurination and single strand breaks via thermal disruption [28, 29].

Under genotoxic stress there are several DNA damage mechanisms that may come into play as shown in Fig. 3. It is important to note that cells can directly reverse damages such as thymine dimers (cyclobutyl dimer) caused by UV irradiation, methylation of guanine bases, and certain methylation of cytosine and adenine bases [28, 29]. DNA single strand breaks (SSB) can be repaired by using the non-defective strand as a template to correct the defective strand. Excision repair mechanisms for SSB excise the damaged nucleotide to be replaced with the correct nucleotide complementary to the non-damaged strand [28, 29]. These mechanisms include base excision repair (BER), mismatch repair (MMR) and nucleotide excision repair (NER). Double strand breaks (DSB), another type of DNA damage, can be repaired by either non-homologous end joining (NHEJ) or by recombination [29, 30]. This type of damage is hazardous to cells because they can ultimately lead up to genomic rearrangements. NHEJ uses a DNA ligase to repair the damage while recombination uses a template for repair [29, 30]. There also exists a post replication repair mechanism (PPR), translesion synthesis, in which bypass polymerases are able to replicate past the damaged DNA [28, 29]. This type of repair pathway is effective for DSB and adducts causing a stall in the replication fork [28, 29].

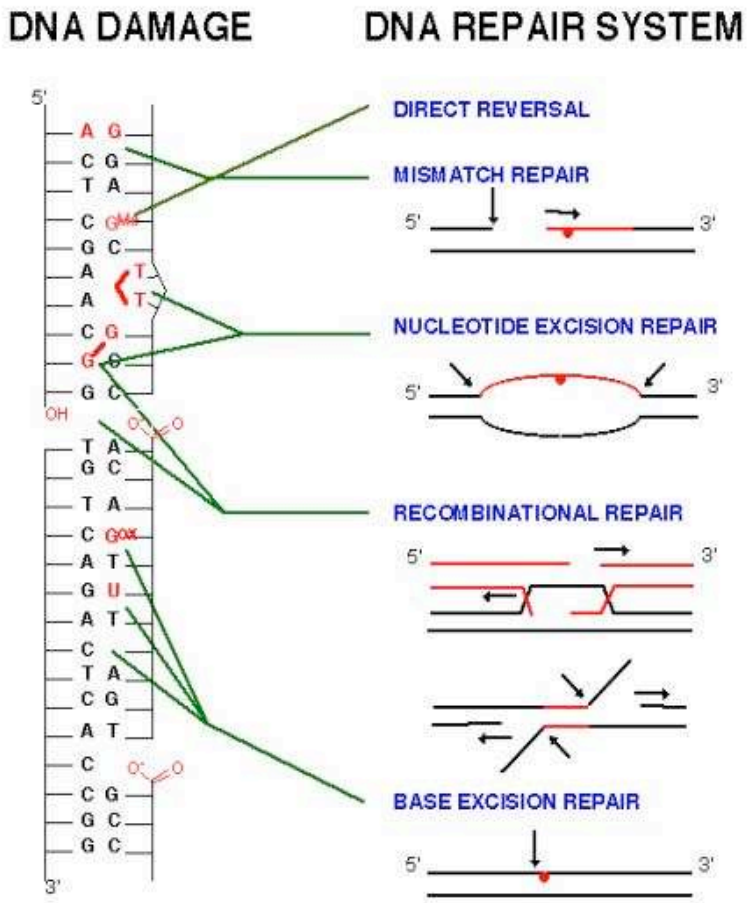

Figure 3. **DNA damage and repair**. Scheme of DNA repair systems for a wide range of DNA modifications. Each system recognizes a specific type of DNA damage, removes the damage and replaces it with a normal piece of DNA. (Figure from The DNA Interest Group www.bbrp.llnl.gov/repair/html/overview.html)

1.3.2. Base Excision Repair

BER is responsible for reparation of single strand breaks, of a single nucleotide damage, caused by oxidation, alkylation, hydrolysis or deamination [28, 31-33]. The repair mechanism is also shown in Fig. 4. BER utilizes two classes of enzymes in correcting nonbulky damage to bases; DNA gycosylases and AP endonucleases [31-33]. The DNA glycosylases are responsible for initiating BER by catalyzing the hydrolysis of the N-glycosyl bond linking damaged bases to the sugar phosphate backbone [33]. The latter

generates an apurinic/apyridimic site or an abasic site in DNA. These sites are also termed AP and are processed via an AP endonuclease [28, 33]. The 5' site upstream of the AP site is subsequently nicked by BER protein Apn1, an AP endonuclease, thus creating a free 3' hydroxyl and 5' deoxyribose phosphate [28]. Processing of AP sites by AP endonuclease occurs via one of two subpathways: short-patch or long-patch [28]. The short patch involves addition of a single nucleotide while long-patch includes DNA synthesis of multiple nucleotides [28, 32, 33]. Following processing, the hydroxyl and deoxyribose phosphate moieties are removed by DNA deoxyribophosphodiesterase [28]. The exonuclease activity of DNA polymerase Pol I extends the 3'hydroxyl to fill in the resulting gap and the DNA is sealed via a DNA ligase [28, 34].

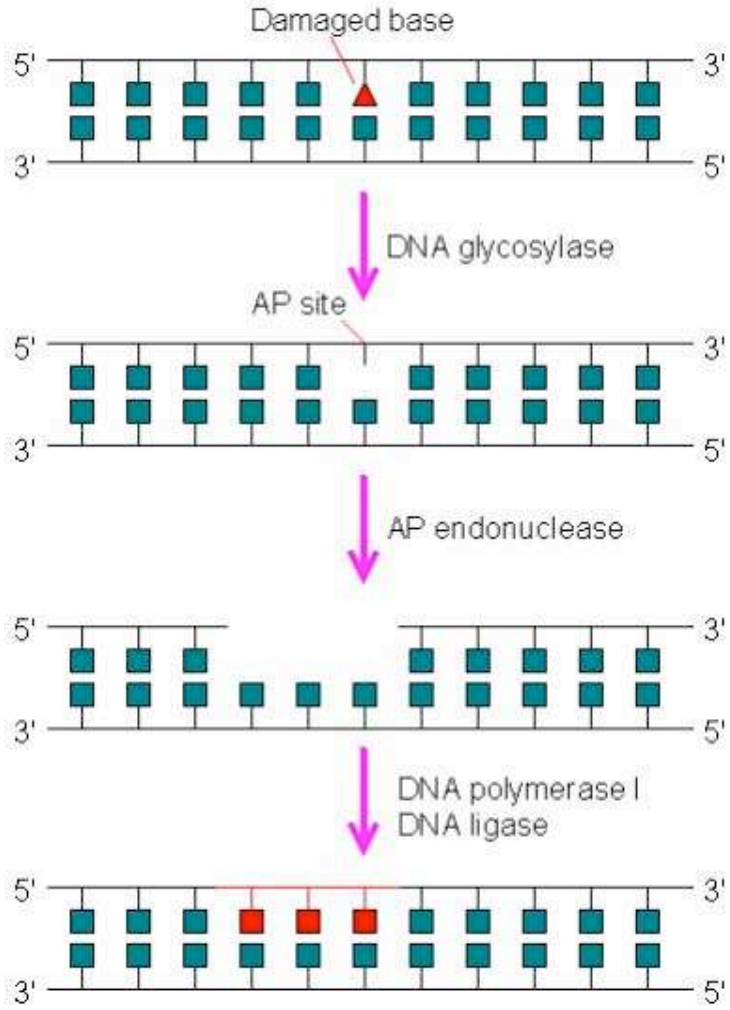

Figure 4. **Base Excision Repair**. During BER, the DNA glycosylase enzyme breaks the sugar bond and AP endonuclease subsequently nicks the site. DNA polymerase is responsible for adding in a new base using the 3'-5' strand as a template and the ligase seals the gap. (Figure from Beth A. Montelone, Ph.D.Division of Biology, Kansas State University 1998; http://www-personal.ksu.edu/bethmont/mutdes.html)

1.3.3. Mismatch Repair

MMR is a repair pathway for SSBs that recognizes the incorporation of a mismatched base during DNA replication and recombination [28, 29, 35, 36]. MMR involves two types of repair: long patch which can be up to a few kilobases in length and short patch which includes lengths of up to 10 nucleotides [28, 36].

There are 5 proteins involved in the MMR pathway of *S. cerevisiae*: Msh2, Msh3, Msh6, Msh1 and Pms1 as shown in Fig. 5 [35, 36]. Msh6 recognizes the lesion and binds to the DNA lesion while in complex with Msh2 in an ATP-dependent manner. The Msh2-Msh6 heterodimer recognizes base-base mismatches and insertion/deletion mispairs. The mispair consists of 1 or 2 unpaired nucleotides on one strand [36]. The heterodimer also recognizes, to a lower extent, larger inertion/deletion mispairs [36]. Msh2 and Msh3 also form a heterodimer that recognizes insertion/deletion mismatches of 2 to 10 nucleotides, weakly recognizes insertion/deletion mispairs, and has a very low affinity for single nucleotide insertion/deletion mismatches [36]. Once Msh2-Msh6 is bound to the lesion, the heterodimer Mlh1-Pms1 is recruited in an ATP-dependant fashion. In human cells, it is important to note that Mlh1 forms a heterodimer with Pms2. The Mlh1-Pms2 is more abundant than the Mlh1-Pms1 heterodimer [36]. Following recruitment of the mismatch heterodimers, Msh2 causes a conformational change in DNA in the form of a loop [28, 36]. The Msh2-Msh6 activates the Msh1-Pms1 endonuclease activity in an ATP-dependent manner [35, 36]. The endonuclease incises the discontinuous strand of DNA and yields a 5' terminus that serves as an entry site for Exo1 [36]. The presence Msh2-Msh6 activates and increases the processing mechanism of Exo1 hydrolysis in an ATP-dependent manner [36]. Exo1 is an exonuclease responsible for excision and subsequent removal of the loop [36]. The excision is followed by gap recognition and filling via RCF. The gap repair is completed via DNA polymerase and ligase.

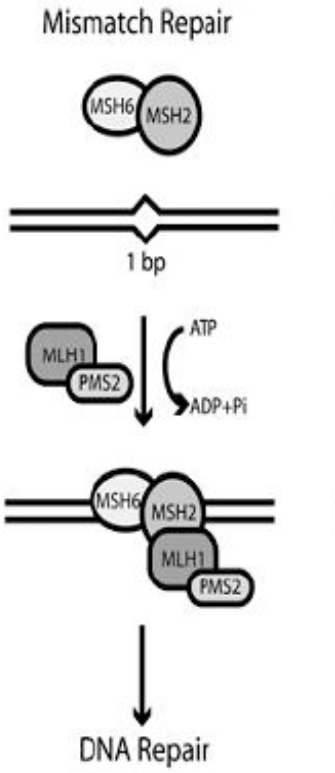

Figure 5. **Mismatch Repair**. Upon exposure to DNA damaging agents, the msh2-msh6 complex recognizes the damaged site and subsequently interacts with the mlh1-pms2 complex to signal cell cycle arrest. Following this arrest the cell can either result in apoptosis or repair of the damaged sites [37].

1.3.4. Nucleotide Excision Repair

NER like BER excises damages in DNA however NER repairs larger gap sizes of around 30 base pairs. NER as previously mentioned in section 1.8 and shown in Fig. 6, repairs bulky adducts and SSBs. Sources of bulky adducts include UV light and carcinogenic compounds such as 4-NQO. Bulky adducts include cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts [28]. NER can be subdivided into 2 repair pathways that recognize different types of helical damage: global genomic NER and transcription coupled NER. NER proteins include Rad1, 2, 3, 4, 10, 14, 25, Ssl1, Tbf1, 2 and 3 [28, 29, 38]. Rad14, a DNA damage and metalloprotein, recognizes and binds to the lesion [29].

The Rad1/Rad10 complex and Rad2 are endonucleases responsible for nicking the 5' and 3' sides of the lesion respectively. Removal of the short oligonucleotide occurs via DNA helicases Rad3 or Rad25. The resulting single strand gap is filled in via a DNA polymerase. The latter is regulated via TFIIH [38]. RPA protects the undamaged strand from degradation while PCNA assists with DNA polymerase activity. The gap is then sealed via a DNA ligase.

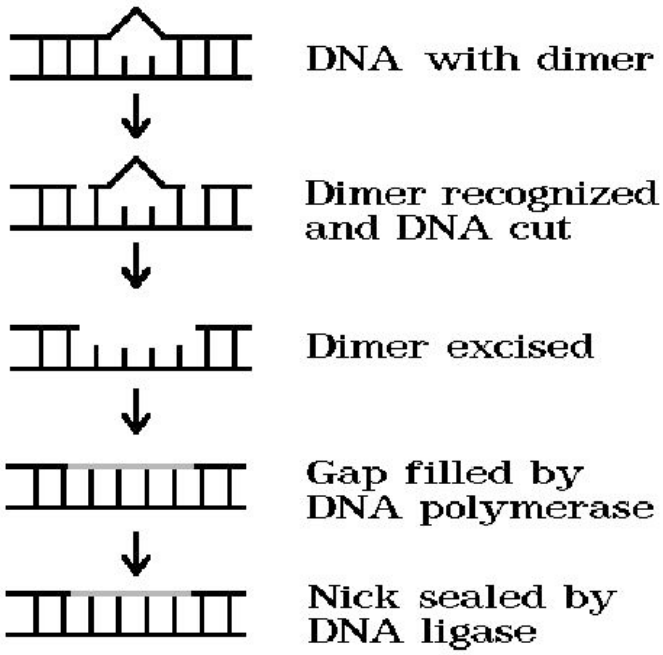

Figure 6. **Nucleotide Excision Repair**. Illustration of the steps involved in nucleotide excision repair. The dimer (damage), upon recognition, is cut and subsequently excised. The gap is filled via DNA polymerase and sealed via DNA ligase. (Figure from www.phys.ksu.edu/gene/excisio3.html)

1.3.5. Homologous Recombination

Homologous recombination, HR, is one of two mechanisms involved in DSB repair [28]. Such lesions may cause a collapse in the replication fork. Recombination repair proteins include Rad50, 51, 52, 54, 55, 57 and 59, Mre2, Mre11 and Xrs2 and are shown in Fig. 7 [28, 29].

HR requires Mre11/Rad50/Xrs2 complex to bind to the ends of the DSB and the following 5' ends flanking the break are resected by an unknown exonuclease. On the 3' end, there are long single-stranded DNA tails. Rad51, a recombinase, binds to these tails thus forming a nucleoprotein filament [28, 29]. The HR machinery then assists this filament in finding a partner, either on the sister chromatid or on another chromosome, in order to form a heteroduplex "joint molecule" [28]. The latter step is termed strand invasion. The invading strand is extended via DNA polymerases and branch migration [28]. The genetic information is then restored following strand exchange, ligation and resolution of Holliday junctions [28].

Single-strand annealing, SSA, is another form of HR repair [28]. Rad2 is the protein responsible for finding homologous pairing partners [28]. The flap on the 3' resected ends is then removed via an endonuclease called FEN-1 and subsequent ligation occurs [28].

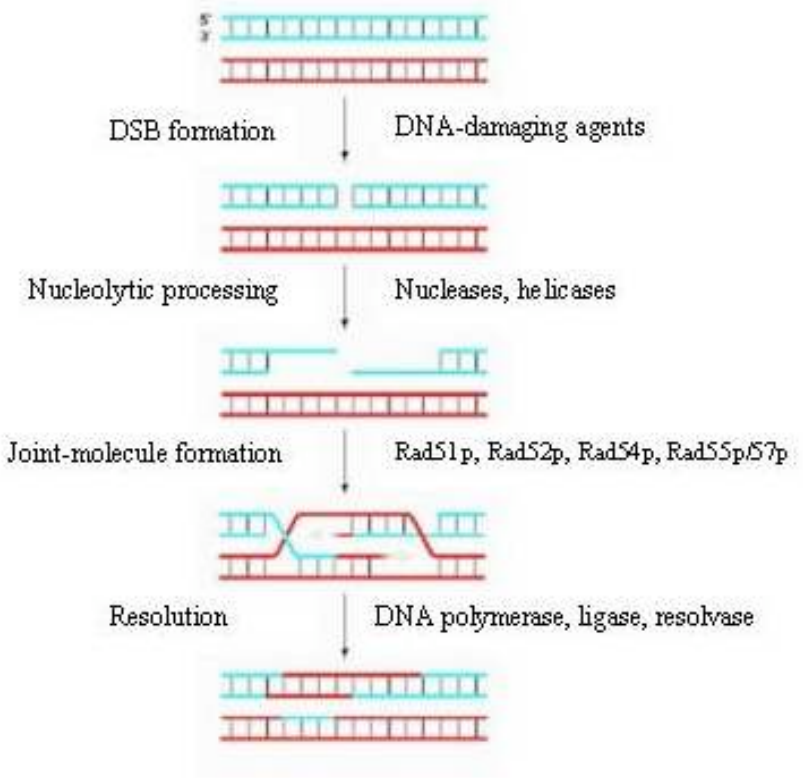

Figure 7. **Homologous Recombination**. DNA damaging agents cause DNA polymerase to replicate further downstream thus causing a gap in the DNA. The gap is filled by genetic recombination via strand invasion, strand exchange and branch migration with a daughter molecule. The gap is sealed via DNA polymerase and ligase. [39].

1.3.6. Translesion Synthesis

Translesion is a post replication repair (PRR) pathway that can bypass lesions in DNA that prevent DNA synthesis via the replication fork and is shown in Fig. 8 [28, 29, 40, 41]. PRR converts DNS damage-induced single-stranded gaps into large molecular weight DNA, but does not remove the blocking lesions [40, 41]. The error-prone and error-free bypass or translesion polymerases are able to skip over chemically modified bases [28, 29, 40, 41].

There are two families of translesion polymerases: the Y-family polymerases include η, ι, and κ while Pol ζ is a B-family polymerase. Yeast Rev3 and Rev7, complex to

form Pol ζ [41]. It has been shown that Rad30 (Pol η) also facilitates translesion replication, but in different pathway than Rev3 and Rev7 [41].

In a brief overview of PRR, the cell's replicative polymerase is unable to overcome blocking lesions in DNA and thus dissociates from the template. It is then replaced by another replicative polymerase also known as a bypass polymerase. The bypass polymerase adds 1 or 2 nucleotides to the strand opposing the lesion thus allowing for replication to continue [41]. The translesion polymerase then dissociates from the strand allowing for the cells highly processive polymerase to reassemble and continue DNA replication [41].

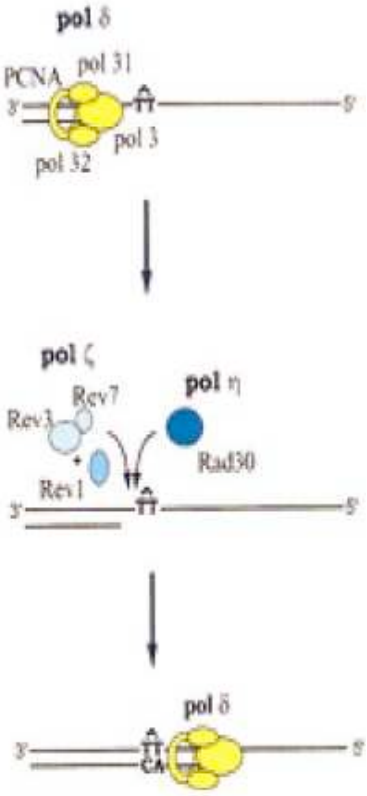

Figure 8. **Post Replication Repair**. Illustration of the steps and proteins involved in post-replication repair or bypass repair mechanisms. Pol δ is unable to continue replication upon encountering a blocking lesion and dissociates from the DNA. This polymerase is replaced by the 2 bypass polymerases pol ζ (Rev1, Rev3 and Rev7) and pol η (Rad30) that allow replication to continue. Upon passing the block, this bypass complex dissociates so that pol δ can continue replicating DNA. [41].

1.4. Research Objective

Our lab has previously shown that *rnr4*Δ mutants are sensitive to a class of DNA damaging agents but hyperresistant to the carcinogen 4-nitroquinoline-1-oxide (4-NQO). We have previously used 4-NQO to discover yeast mutants that are sensitive to the drug and these mutants have been characterized. 4-NQO is commonly used to study DNA repair response to damage and much is known about the mechanism of repair. Upon entering a cell, the drug becomes activated and subsequently binds to adenine and guanine to form bulky lesions. In a normal cell, such lesions are repaired via nucleotide excision repair (NER) in which NER repair proteins rad1/10 and rad2 excise the lesion for subsequent completion of DNA repair.

*rnr4*Δ resistance would be of importance to study since RNR plays a central role in regulating dNTP levels required for DNA replication. RNR is a crucial target for anticancer and antiviral therapy in rapidly dividing cancer cells and such a study would expand on DNA repair mechanisms that protect the cell. In addition, it would be of interest to block RNR assembly in order to initially diminish NTP levels required for cell growth, division and proliferation and to prevent the spreading of cancer. *rnr4*Δ is resistant to such blocks in assembly and thus provides NTPs required for cell growth. We intend to probe further in its ability to overcome drug treatment and target its repair pathway to prevent cell proliferation.

This drug hyperresistance may be attributed to either defects or arrests in any of the many DNA repair pathways. Our objective is to determine which pathway is involved in preventing these 4-NQO induced lesions by studying the following DNA repair pathways: nucleotide excision repair, mismatch repair, recombination and translesion synthesis. We

want to target and knock out the potential repair pathway allowing *rnr4*Δ cells to proliferate thus rendering the cells sensitive to future drug therapy.

2. Materials and Methods

2.1. Strains and media

The wild-type parental strains used were *S. cerevisiaie* BY4741. The collection of nonessential haploid *rnr4*Δ strains, derived from the *S. cerevisiaie* BY4741, was obtained from EUROSCARF (Frankfurt, Germany) (described in Table 1). Standard YPD (1% yeast extract, 2% peptone, 2% dextrose) growth media was used as patch colonies and cultural liquid.

Table 1. Yeast strains used in this study.

Strain	Construction	Source
BY4741	Mat a, his3-1, leu2-0,met15-0,ura3-0	C. Roberts
BY4741(*rnr4Δ*)	Isogenic to BY4741	X. Yang
BY4741(*msh6Δ*)	Isogenic to BY4741, except msh6::HIS3	This study
BY4741(*msh6Δrnr4Δ*)	Isogenic to BY4741, except msh6::HIS3	This study
BY4741(*rad1Δ*)	Isogenic to BY4741	This study
BY4741(*rad1Δrnr4Δ*)	Isogenic to BY4741	This study
BY4741(*rad51Δ*)	Isogenic to BY4741, except rad51::HIS3	This study
BY4741(*rad51Δrnr4Δ*)	Isogenic to BY4741, except rad51::HIS3	This study
747 (parent)		
747 (*rev3Δ*)		
BY4741(*mrc1Δ*)	Isogenic to BY4741	This study

2.2. Chemicals and Equipment

Growth culture reagents, yeast extracts, peptone and agar, were from Difco (Detroit, MI). 4-NQO, MMS and hydrogen peroxide were from Sigma (Saint Louis, MO). BLM A5 trihydrochloride was from Calbiochem (La Jolla, CA). Pfu DNA polymerase was from Stratagene (La Jolla, CA). Primers (Tables 2 and 3) were from Invitrogen (Carlsbad, CA). Running buffer condition: 2.5 mM Tris pH 7.0, 19.2 mM Glycine and 0.1% SDS. Nitrocellulose membrane (Hybond-C+, Amersham). Transfer/washing buffer (TBS: 25 mM Tris pH 7.0, 19.2 mM Glycine and 20% methanol). TBS-T (TBS + 0.1% Tween). Blocking buffer (5% non-fat dry milk in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% Tween 20). Anti-rabbit IgG-HRP conjugate (Sigma). UVC lamp (Fisher). Shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). PCR machine: PTC-100^{TM} Pragrammable Thermal Controller MJ research INC. MINI bead beater (BioSpec Products, Bartlesville,OK). Yeast extraction buffer (50 mM Tris pH 7.0, 30 mM KCl, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF and 1 mM DTT). Spheroplast buffer (1 M sorbitol, 10 mM $NaHPO_4$ pH 7.0, 10 mM EDTA). Lyticase (Sigma). Beta-mercaptoethanol (Fisher). TE/Li solution (10 mM tris, 1 mM EDTA pH 8.0, 0.1 mM LiCL). YPD (1% yeast extract, 2% peptone, 2% dextrose). YPDa (YPD + 2% agar). 40% PEG solution (Te/Li + 40% PEG)

Table 2. PCR Primers for *RNR4* Gene Amplification.

Name	Sequence
pYEX-A4-F1 (forward)	5'-TTCGATGATGAAGATACCCCACCAAACCCAAAAAAAG AGATCGAAATGGAAGCACATAACCAATTTTTGAAG-3'
PYEX-A4-R1 (reverse)	5'-TTCAGTATCTACGATTCATAGATCTCTGCAGGTC GACGGATCCCTTAGAAGTCATCATCAAAGTTAATTTCCTTGG-3'

Table 3. PCR Primers for *MSH6* Gene Amplification.

Name	Sequence
9 F2 MSH6-V-HINDIII-F1	GCA GCA TGC TCT CCA AGC TTA AAT AAA TAG ATG
9 F3 MSH6-V-BAMHI-R1	GGT TTT CTA TAA TAT ATG GAT CCT CCA TG

2.3. Chlonogenic Assays

Strains were tested on selective plates before use for survival and mutagenesis assays. Each strain was patched out on a control YPD agar plate and selective plates. The chlonogenic assays were carried out as described in [42].

Cultures were grown overnight at 30ºC in YPD medium. Cultures were then diluted to an optical density of 0.4 (2 x 10^6 cells) at 600 nm in fresh YPD and incubated to an optical density of 0.8-1.0 at 600 nm. Aliquots of the cultures were treated with MMS (ranging from 0 to 0.25%) for 1 hour, or 4-NQO (ranging from 0 to 6 μg/ml) for 1 hour, or hydrogen peroxide (ranging from 0 to 3%) for 1 hour at 30ºC in an incubator shaker. Each aliquot was diluted in sterilized water to 10^{-4} or to 10^{-5} and were plated on YPD agar plate. Colonies were counted after 48 to 72 hours of growth at 30ºC.

Cultures were grown overnight at 30ºC in YPD medium. Cultures were then diluted to an optical density of 0.4 (2 x 10^6 cells) at 600 nm in fresh YPD and incubated to an optical density of 0.8-1.0 at 600 nm. Each aliquot was diluted in sterilized water to 10^{-4} or to 10^{-5} and were plated on YPD agar plate. The plates were then treated with UVC (0.1 J/s/m^2, 0.2 J/s/m^2 and 0.4 J/s/m^2 with different time courses from 0 to 60 seconds and from

1 minute to 7 minutes) at room temperature. Plates were placed in a box to prevent light exposure. Colonies were counted after 48 to 72 hours of growth at 30ºC.

2.4. Mutagenesis Assays

The mutation assays were carried out as described in [43]. Single yeast colonies were grown overnight to stationary phase at 30ºC in YPD medium. The cells were diluted in fresh YPD to an optical density of 0.4 (2 x 10^6 cells) at 600 nm and then incubated to an optical density of 0.8-1.0 at 600 nm. Cells were treated with 4-NQO (0.1 μg/ml or 1.0 μg/ml) or MMS (0.15%) for 1 hour at 30ºC. The cells were washed and resuspended in water with a density of 10^7 cells/ml. Treated and untreated cells were plated at the appropriate dilution onto complete medium containing L-canavanine (25 mg/ml) but lacking arginine for Can^r mutant count and complete medium but lacking arginine for viable cell count. Colonies were counted after 72 to 120 hours at 30ºC. The frequency of Can^r mutants in each cell culture was calculated by dividing the Can^r mutant count by the viable cell count.

2.5. Spot Test and Gradient Plate Assay

Spot test and gradient plate experiments were carried out as described by [42]. Cultures were grown overnight at 30ºC in YPD medium and were subcultured in fresh YPD. The cultures were grown at 30ºC until the optical density was 1.0 at 600 nm. The following dilutions were performed in sterilized water: 1:1, 1:10, 1:50, 1:100, 1:200, 1:500 and 1:1000. YPD agar plates were prepared accordingly; the first plate with no drug, the second plate with 1 mM of hydroxyurea and a third plate with 10 mM of hydroxyurea. 2.5

μl of each dilution was spotted on each plate in a linear fashion. Each plate was analyzed for drug sensitivity after 24 hours.

The gradient plate assay was performed as previously described [29]. Cells were grown overnight and were replicated as a thin line along a drug gradient plate. The cells were analyzed for drug sensitivity after 48 hours of growth at 30ºC. The distance of growth of each strain is expressed as a percentage of the parental strain.

2.6. Preparation of Yeast Genomic DNA

The preparation of yeast genomic DNA was carried out as per [44]. Single colonies of BY4741 strains were grown overnight in YPD medium at 30ºC. The cells were then centrifuged at 1000 rpm for 5 minutes and washed twice with sterilized water. Cells were centrifuged and suspended in spheroplast buffer (1 M sorbitol, 10 mM$NaPO_4$ pH 7.0, 10 mM EDTA). The cells were incubated at 37ºC upon addition of 5μl of 20 μg/ml liticase (Sigma) and 3 μl of beta-mercaptoethanol (Fisher) for 30 minutes. Following incubation, the cells were spun down for 5 seconds at 12,000 rpm and resuspended into a second buffer (50 mM EDTA, 0.3% SDS, 5 μl protease K 20 mg/ml) for a 30 minute incubation at 65ºC. 20 ml of a 5 M potassium acetic acid solution was added to the sample for incubation on ice. The sample was centrifuged at for 5 seconds at 12,000 rpm. The supernatant was extracted via phenol and chloroform. Each extraction involved a 5 second centrifugation at 12,000 rpm 4ºC. The supernatant was precipitated with a 3 fold volume of 95% ethanol and stored at -80ºC for 15 minutes. The DNA was centrifuged for 15 minutes at 13,000 rpm and washed in 70% ethanol. The genomic DNA was resuspended in 20 μl of TE for PCR

amplification with the primers presented in Tables 2 and 3 and the program described in section 2.7.

2.7. PCR Program

PCR machine: PTC-100^{TM} Programmable Thermal Controller MJ research INC.

Master Mixture: DNA or genomic DNA 10-50 ng, 5 µl of 10X taq buffer, 0.5 µl of upstream oligo (1 µg/µl), 0.5 µl of downstream oligo (1 µg/µl), 2 µl of 10 mM dNTP, 6 µl of 25 mM $MgCl_2$, 1 µl of TAQ DNA polymerase and add sterilized water up to 50 µl.

PCR designed program:

1. 95ºC 5 minutes
2. 80ºC 6 seconds
3. 94ºC 50 seconds
4. 55ºC 1 hour (-1º/cycle)
5. 72ºC 4 minutes 30 seconds
6. 9 times repeat of step 3-5. Each cycle decreases by 1ºC in step 4.
7. 94ºC 40 seconds
8. 50ºC 1 hour
9. 72ºC 4 minutes 30 seconds
10. 20 times repeat from step 7.
11. 72ºC 10 minutes
12. 4ºC o/n

2.8. Single and Double Mutant Creation (Transformation)

The transformation protocol was carried out as per [44] and Table 4. Wild-type and *rnr*4Δ cells were grown overnight in YPD medium and subcultured in fresh YPD at 30ºC. 1 ml of cells were washed twice with 5 ml of sterilized water and spun down for 5 minutes at 1000 rpm 4ºC. Cells were resuspended in 1 ml of sterilized water and transferred to an eppendorf tube for a 5 second centrifugation at 6000 rpm. The pellet was washed in 800 µl of TE/Li and resuspended in 40 µl of TE/Li. The following samples were then prepared and incubated at 30ºC for at least 30 minutes.

Table 4. Transformation Protocol.

	BY4741		**BY4741 (*rnr4*Δ)**	
Sample	1	2	3	4
Cell	30 µl	30 µl	30 µl	30 µl
Carrier DNA	5 µl	5 µl	5 µl	5 µl
Fragment	-	5 µl	-	5 µl
PEG solution	150 µl	150 µl	150 µl	150 µl

The samples underwent heat shock (42ºC) for 15 minutes and were centrifuged for 5 seconds at 6,000 rpm. The pellet was resuspended in 100 µl of sterilized water and was plated on selective plates. Single colonies from plates of samples 2 and 4 were analyzed following a growth period of 72 to 120 hours. Analysis involved phenotype testing, PCR amplification and chlonogenic assays.

2.9. Western Blot (Rad53)

The western blot experiment was carried out as per (www.assaydesigns.com/corp/protocol-GCPR-WB.pdf). Yeast cells were grown overnight in YPD medium, washed twice with sterilized water and centrifuged at 4ºC at 4000 rpm with a ss-24 rotor. The pellet was resuspended in yeast extraction buffer (50 mM Tris pH 7.0, 30 mM KCl, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF and 1 mM DTT) and homogenized via a MINI bead beater for 20 seconds at 5000 rpm. Cells were beat for 6 rounds with 1-minute intervals on ice. Protein was collected after removal of cellular debris via centrifugation at 9000 x g for 10 minutes. A Bradford assay, as described in [45] and by the BIORAD company, was used for protein quantification.

20 and 40 microgram of whole cell lysate per lane was loaded onto an SDS-PAGE mini gel. The gel was run for one hour at 120V/20 mA until the dye reached close to the end of the gel. The running buffer condition was as follows: 2.5 mM Tris pH 7.0, 19.2 mM Glycine and 0.1% SDS. The proteins were then transferred to a Hybond-C+ nitrocellulose membrane at 100V/250 mA for 1 hour. Transfer buffer condition: 25 mM Tris pH 7.0, 19.2 mM Glycine and 20% methanol. Following the transfer the blot was blocked with a blocking buffer.

The blot was then incubated with anti-Rad53, the primary antibody (diluted to 1:1000 in blocking buffer), for 45 minutes in blocking buffer (TBS-T + 5% milk) at room temperature. The blot was washed 3 times for 5 minutes with TBS-T under gentle agitation. The secondary antibody (anti-rabbit diluted 1:15000 in TBS-T) was used for 30 minutes and the blot was washed 3 times for 5 minutes for subsequent development.

3. Results

3.1. *rnr4Δ* hyperresistance to 4-NQO

In order to determine the drug phenotypes of *rnr4*Δ we tested the parent BY4741 and mutant with 4-NQO, MMS, H_2O_2, and UVC. We wanted to determine if the mutant showed the same drug sensitivity to the DNA damaging agents as a parental strain.

Exponentially growing cells were treated with MMS, H_2O_2, UVC and 4-NQO. Cells were scored for fractional survivors as described in chapter 3. The mutant is resistant to 4-NQO, as shown in Fig. 9, while it is sensitive to the other DNA damaging agents. The survival curves for MMS, H_2O_2 and UVC are shown in Figures 10, 11 and 12. It has been shown in the literature that 4-NQO can generate intracellular oxidative stress and these induced lesions are processed via the NER pathway [42]. Although 4-NQO has been classified under "UV-mimetic", the sensitivity of the *rnr4*Δ to UVC clearly shows that the strains resistance to 4-NQO is not due to the "UV-mimetic" [46].

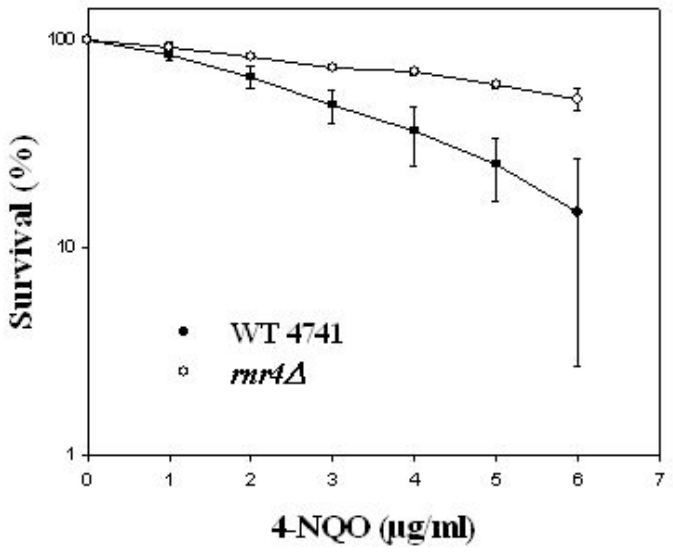

Figure 9. **4-NQO Survival Curve Assay.** Log phase strains were treated with increasing concentrations of 4-NQO for 1 hour at 30ºC. *rnr4*Δ strains (o) were resistant to 4-NQO with respect to the parent WT4741 strain (●). Results were expressed as the mean ± SD from at least three separate experiments.

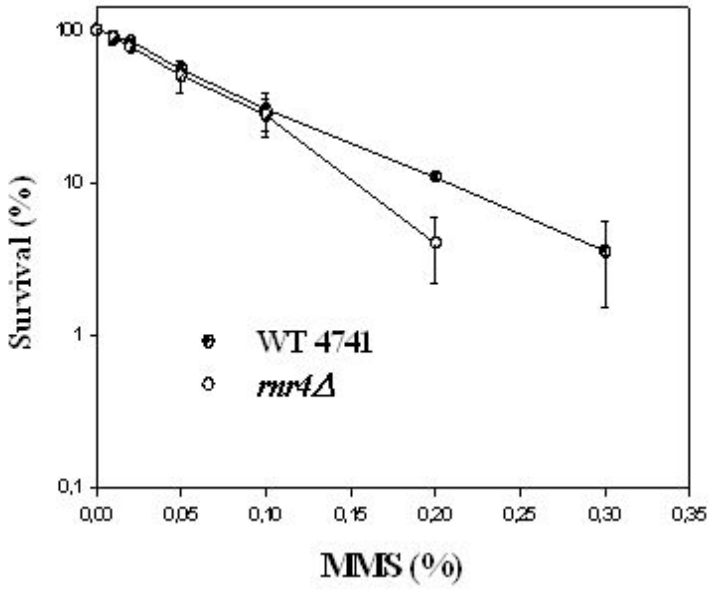

Figure 10. **MMS Survival Curve Assay**. Log phase strains were treated with increasing concentrations of MMS for 1 hour at 30°C. *rnr4*Δ strains (o) were sensitive to MMS with respect to the parents WT4741 strain (●). Results were expressed as the mean ± SD from at least three separate experiments.

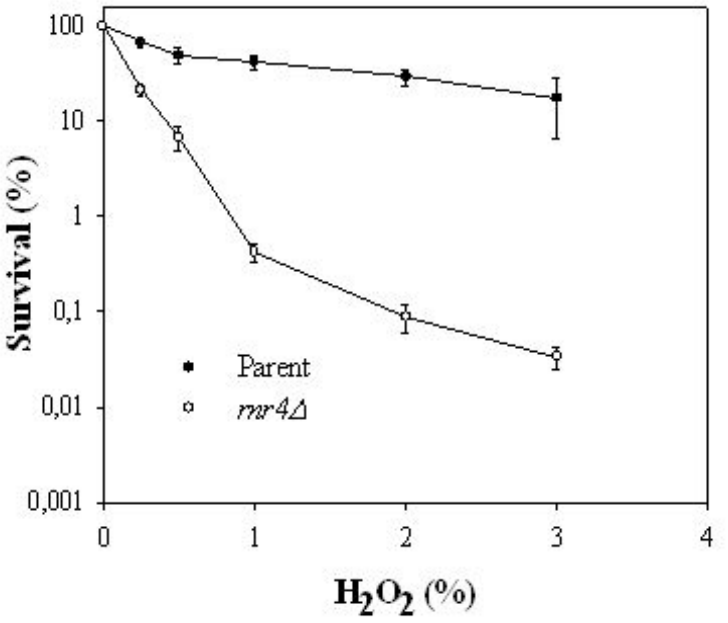

Figure 11. **H_2O_2 Survival Curve Assay.** Log phase strains were treated with increasing concentrations of H_2O_2 for 1 hour at 30ºC. *rnr4*Δ strains (o) were sensitive to H_2O_2 with respect to the parent WT4741 strain (●). Results were expressed as the mean ± SD from three separate experiments.

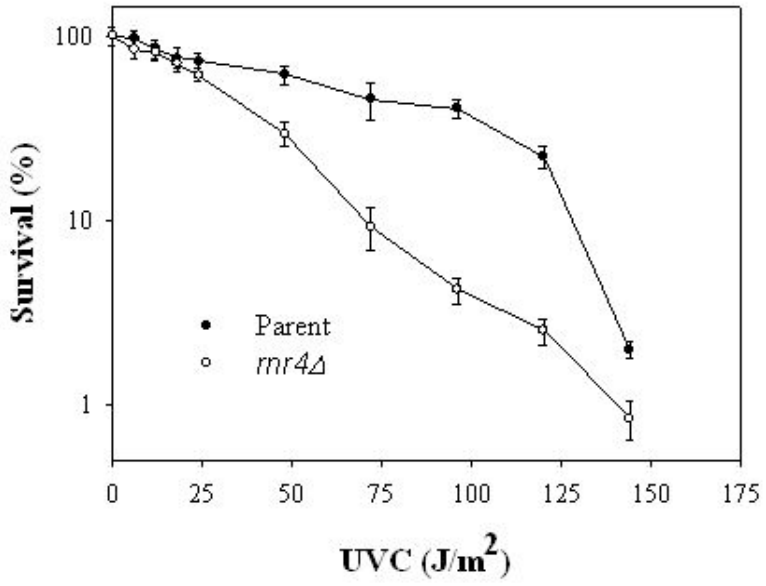

Figure 12. **UVC Survival Curve Assay**. Log phase strains were treated with a fixed dose of UVC for different time points at 30ºC. *rnr4*Δ strains (o) were sensitive to UVC with respect to the parent WT4741 strain (●). Results were expressed as the mean ± SD from three separate experiments.

3.2. Mutation Analysis

Since *rnr4Δ* is resistant to 4-NQO and 4-NQO is known to induce mutations, we wanted to know if this resistance was due to low levels of mutation so we decided to measure the forward mutation frequency. It would be expected that under 4-NQO induced lesions, the parent cells would show hypermutable phenotypes. The opposite would hold true for the mutant if the Rnr4 plays a role in the repair of 4-NQO-induced lesions. We did a mutation assay in which we have challenged parent and *rnr4*Δ cells with and without 0.1 μg/ml 4NQO. We scored for forward mutation at a specific locus called CAN1. Cells that have a mutated CAN1 gene become resistant to canavanine, analog of arginine. The forward mutation frequency was measured on –arg plates containing 25 μg/ml canavanine for both the wild type and mutant strains as described in the materials and methods.

Under normal growth condition, the mutant and parent strains showed a similar trend with respect to the mutation rate (Table 5 and Fig. 13). Upon 4-NQO treatment for 1 hour at 30ºC, the parent strain showed an approximately 20 to 30 fold increase in mutation rate with respect to the *rnr4Δ*. The results show that *rnr4*Δ 4-NQO resistance is due to low levels of mutation and that Rnr4 plays an important role in causing 4-NQO induced lesions in a wild type strain.

Table 5. Mutation analysis of can^r mutation in *rnr4Δ*.

Strain	**4-NQO (1 µg/µl)**	**Frequency (10^{-8})**	**Fold**	**Fold difference Parent vs *rnr4*Δ**
Parent	-	247.0 +/- 165	30.28	24.62
Parent	+	7480.9 +/- 1120		
*rnr4*Δ	-	298.4 +/- 83	1.23	
*rnr4*Δ	+	367.4 +/- 70		
Parent	-	160.3 +/- 35.8	53.02	24.10
Parent	+	8499.0 +/- 2190		
*rnr4*Δ	-	194.5 +/- 62	2.20	
*rnr4*Δ	+	427.9 +/- 141		

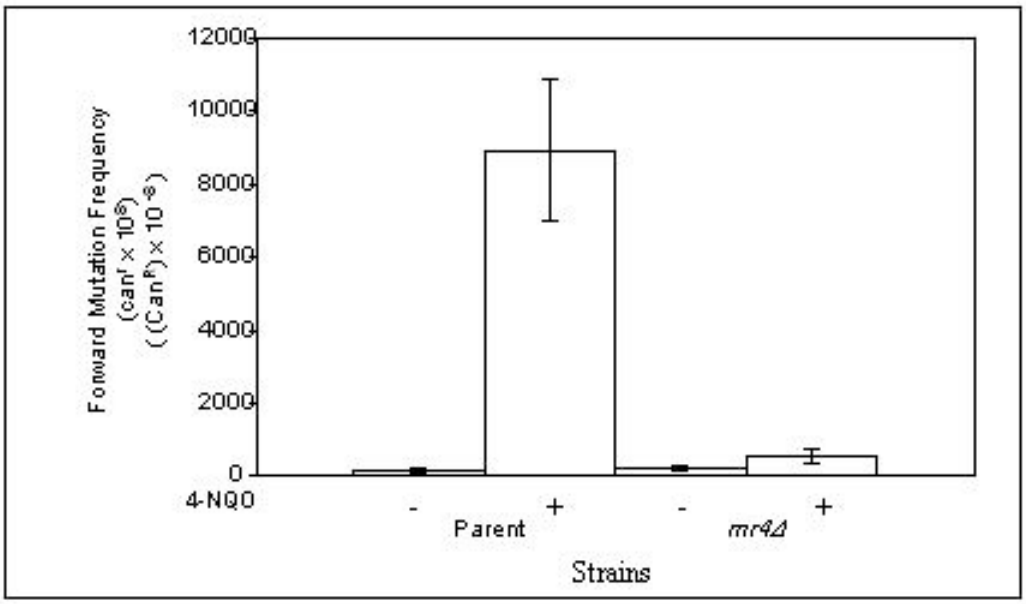

Figure 13. **Mutation Analysis for *rnr4*Δ.** Log phase strains were treated and untreated with a fixed concentration of 4-NQO for 1 hour at 30ºC. Untreated *rnr4*Δ strains showed the same level of mutation frequency as the untreated parent strain. The parent strain however showed a 20 to 30-fold increase in mutation upon 4-NQO treatment while the mutant showed a 1 to 5 fold increase. Results were expressed as the mean ± SD from at least three separate experiments.

3.3. Chlonogenic Assays for NER mutants

Based on these preliminary observations, we hypothesized that under low levels of dNTPs as in an *rnr4*Δ a more efficient repair pathway is recruited to prevent 4-NQO induced mutations. It has been shown that *rnr4*Δ mutants are deficient for ribonucleotide reductase activity thus producing lower levels of dNTP required for DNA replication [2]. In order to determine which pathway was involved in preventing these 4-NQO induced lesions each DNA repair pathway had to be studied. We decided to first make single and combination knockouts for the NER pathway. NER, as previously mentioned, has been a common cellular pathway responsible for processing UVC and 4-NQO induced lesions.

We tested the following strains wild type, *rad1*Δ, *rnr4*Δ, *rad1*Δ*rnr4*Δ with MMS, H_2O_2, UVC and 4-NQO in order to determine if the combination mutants would show the same drug resistance to 4-NQO and drug sensitivity to the other DNA damaging agents as the *rnr4*Δ mutant. Exponentially growing cells were treated with MMS, H_2O_2, UVC and 4-NQO. Cells were scored for fractional survivors as described in chapter 3.

With respect to MMS (Fig. 14), H_2O_2 (Fig. 15) and UVC (Fig. 16) the combination mutant *rad1*Δ*rnr4*Δ showed similar drug sensitivity as an *rnr4*Δ. It is of interest to note that the double knockout did not show resistance to 4-NQO but an increased level of sensitivity with respect to the wild type. However *rad1*Δ*rnr4*Δ strain was more resistant to 4-NQO treatment when compared to *rad1*Δ as shown in Fig. 17. The latter preliminary results suggest that the cell does not mediate 4-NQO resistance through the NER pathway.

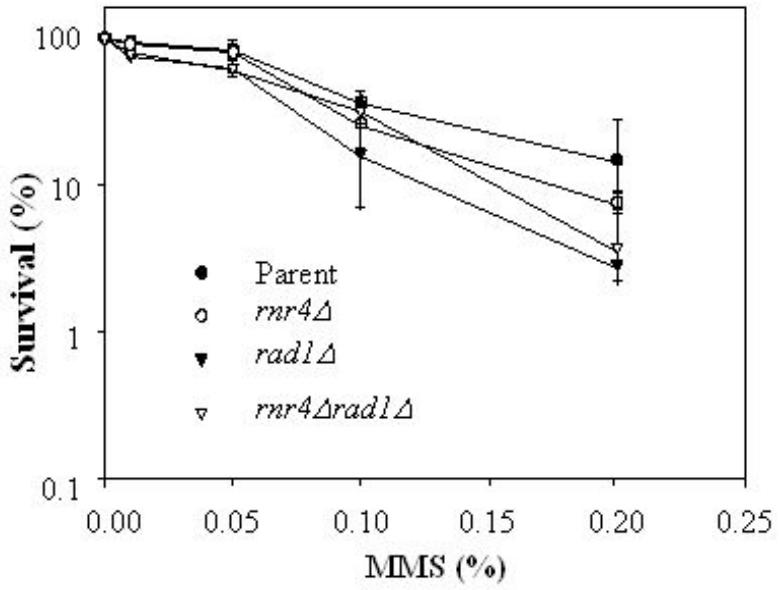

Figure 14. **MMS Survival Curve for NER Mutants**. Log phase strains were treated with increasing concentrations of MMS for 1 hour at 30ºC. *rad1*Δ, *rnr4*Δ, and *rad1*Δ *rnr4*Δ strains (▼), (o) and (∇) respectively, were sensitive to MMS with respect to the parent WT4741 strain (●). *rad1*Δ showed more drug sensitivity compared to all the other strains tested. Results were expressed as the mean ± SD from at least three separate experiments.

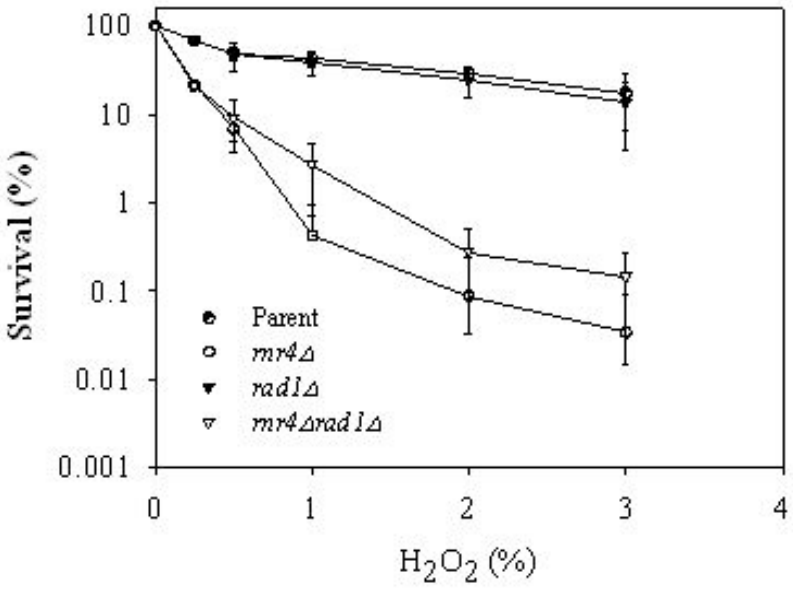

Figure 15. **H_2O_2 Survival Curve for NER Mutants**. Log phase strains were treated with increasing concentrations of H_2O_2 for 1 hour at 30ºC. *rnr4*Δ and *rad1*Δ *rnr4*Δ strains (o) and (∇) respectively, were sensitive to H_2O_2 with respect to the parent WT4741 strain (●). *rad1*Δ (▼) showed similar drug phenotype in comparison to the wild type. Results were expressed as the mean ± SD from three separate experiments.

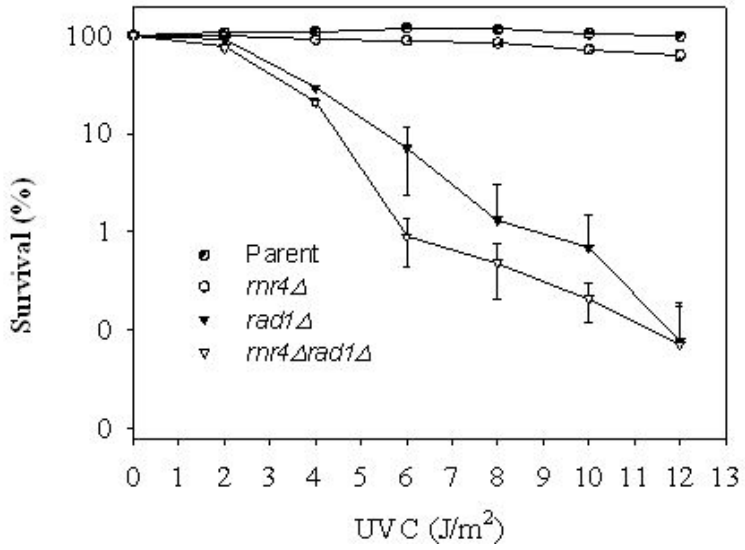

Figure 16. **UVC Survival Curve for NER Mutants**. Log phase strains were treated with a fixed dose of UVC for different time points at 30ºC. *rnr4Δ* and *rad1Δrnr4Δ* strains (o) and (∇) respectively, were sensitive to UVC with respect to the parent WT4741 strain (●). *rad1*Δ (▼) showed similar drug phenotype in comparison to the wild type. Results were expressed as the mean ± SD from three separate experiments.

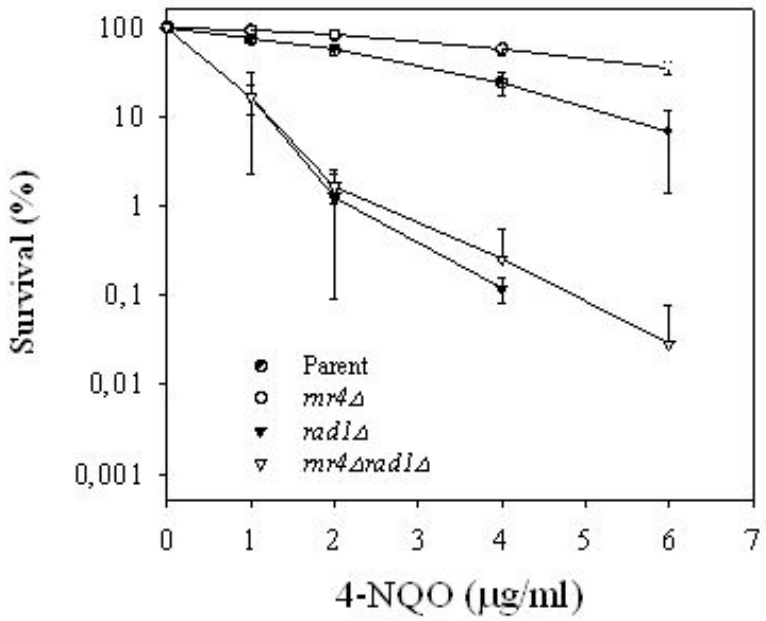

Figure 17. **4-NQO Survival Curve for NER Mutants.** Log phase strains were treated with increasing concentrations of 4-NQO for 1 hour at 30ºC. *rad1Δ* and *rad1Δrnr4Δ* strains (▼) and (∇) respectively, were sensitive to 4-NQO with respect to the parent WT4741 strain (●). *rnr4*Δ (o) maintained its drug resistance in comparison to the wild type. Results were expressed as the mean ± SD from three separate experiments.

3.4. NER Mutation Analysis

It would be expected that under 4-NQO induced lesions, the combination mutant would show hypermutable phenotypes at a higher frequency then that of a wild type strain as the double mutant shows a higher level of 4-NQO sensitivity. We performed another mutation assay in which we have challenged all 4 strains with and without 0.1 µg/ml 4-NQO. We scored for forward mutation at a specific locus called CAN1 and used plates containing 25 µg/ml canavanine.

Under normal growth condition, the mutant and parent strains showed a similar trend with respect to the mutation rate (Fig. 18). The untreated *rad1*Δ and *rad1*Δ*rnr4Δ* strains showed increased levels of mutation frequency with respect to the untreated parent and *rnr4*Δ strain. Upon 4-NQO treatment, *rad1*Δ and *rad1*Δ*rnr4*Δ showed a 3 fold and 2.5

fold increase in mutation frequency respectively when compared to the wild type. More importantly, the results clearly indicate that rnr4 prevented some level of the mutations thus causing the slight resistance to 4-NQO. Knocking out Rad1 clearly did not completely abolish 4-NQO resistance in and *rnr4*Δ. Other repair pathways must also be tested before being ruled out in mediating 4-NQO resistance.

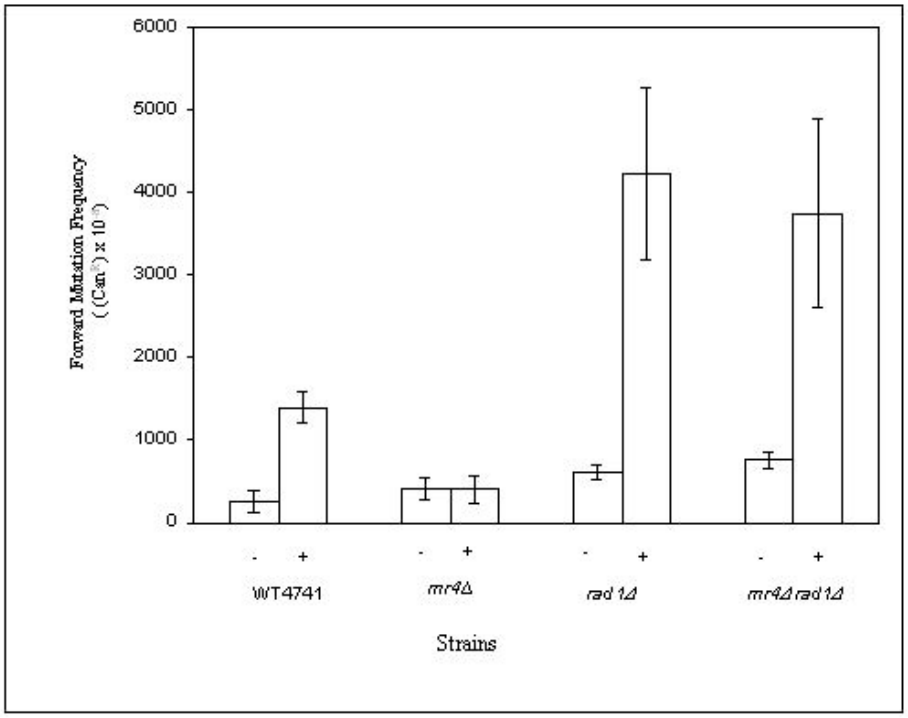

Figure 18. **NER Mutation Analysis.** Log phase strains were treated and untreated with a fixed concentration of 4-NQO for 1 hour at 30ºC. Untreated *rad1*Δ*rnr4Δ* strain showed an increased level of mutation frequency compared to the untreated parent and *rnr4*Δ strain. Upon 4-NQO treatment, *rad1*Δ and *rad1*Δ*rnr4*Δ showed a 3 fold and 2.5 fold increase in mutation frequency when compared to the wild type. Results were expressed as the mean $\pm$ SD from at least three separate experiments.

3.5. MMR Chlonogenic Assays

As previously mentioned, a more efficient DNA repair pathway is recruited to prevent 4-NQO induced mutations under low levels of dNTPs that exist in *rnr4*Δ mutants.

The second pathway we decided to study was the mismatch repair for which we proceeded by making single and combination knockouts.

We tested the following strains wild type, *msh6*Δ, *rnr4*Δ, *msh6*Δ*rnr4*Δ with MMS, and 4-NQO in order to determine their drug phenotypes. We wanted to determine if the combination mutant showed the same drug resistance to 4-NQO and drug sensitivity to the other DNA damaging agents as the *rnr4*Δ mutant. Exponentially growing cells were treated with MMS and 4-NQO. Cells were scored for fractional survivors as described in chapter 3.

With respect to MMS, the combination mutant *msh6*Δ*rnr4*Δ showed similar drug sensitivity as an *rnr4*Δ as shown in Fig. 19. The double knockout did show resistance to 4-NQO with respect to the wild type, but showed a slightly lower level of resistance when compared to *rnr4*Δ as seen in Fig. 20. The results suggest that since the *rnr4*Δ 4-NQO resistance was not abolished with *msh6*Δ, MMR may be ruled out as a pathway mediating 4-NQO resistance.

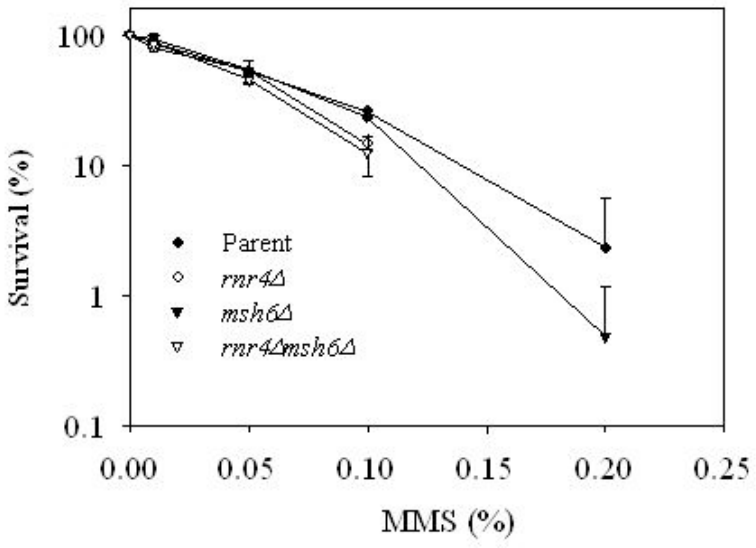

Figure 19. **MMS Survival Curve for MMR Mutants.** Log phase strains were treated with increasing concentrations of MMS for 1 hour at 30ºC. *msh6*Δ,

rnr4Δ and *msh6Δrnr4Δ* strains (▼), (o) and (∇) respectively, were sensitive to MMS with respect to the parent WT4741 strain (●). *rnr4Δ* (o) and *msh6Δrnr4Δ* (∇) cells were treated with 0.15% and 0.20% MMS but showed no survival for this set of experiments. Results were expressed as the mean ± SD from at least three separate experiments.

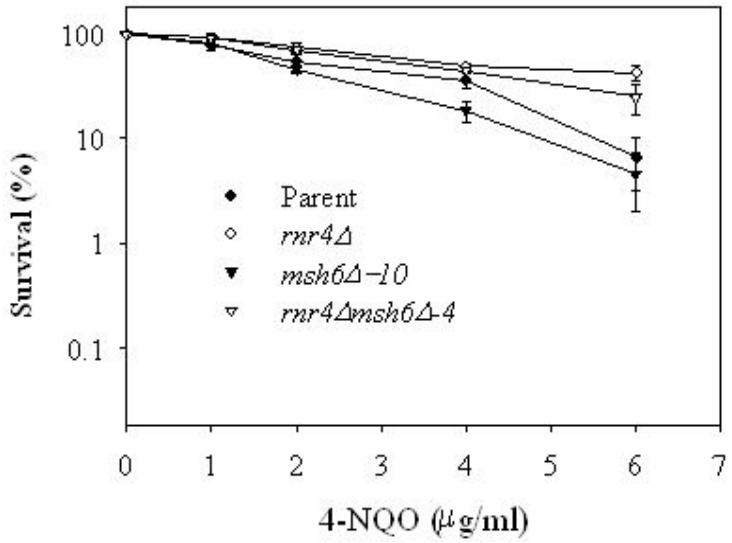

Figure 20. **4-NQO Survival Curve for MMR Mutants.** Log phase strains were treated with increasing concentrations of 4-NQO for 1 hour at 30ºC. *msh6*Δ (▼) showed similar sensitivity pattern to MMS as the wild type (●). *rnr4Δ* and *msh6Δrnr4Δ,* (o) and (∇) respectively, showed almost the same level of resistance to 4-NQO. Results were expressed as the mean ± SD from at least three separate experiments.

3.6. MMR Mutation Analysis

It would be expected that under 4-NQO induced lesions, the combination mutant would show a lower mutation frequency then that of a wild type strain as the double mutant shows a higher level of 4-NQO resistance. We performed another mutation assay in which we have challenged all 4 strains with and without 0.1 μg/ml 4-NQO. We scored for forward mutation at a specific locus called CAN1 and used plates containing 25 μg/ml canavanine.

Under normal growth condition, the mutant and parent strains showed a similar trend with respect to the mutation rate (Fig. 21). Untreated *msh6*Δ strain showed a

relatively similar level of mutation frequency compared to the untreated parent strain. *msh6*Δ*rnr4*Δ untreated showed a 2 to 3 fold frequency in comparison to *rnr4*Δ and the wild type respectively. Upon 4-NQO treatment, *msh6*Δ showed a 10 fold increase in mutation as expected since the strain showed sensitivity in the 4-NQO Chlonogenic assay. Since *msh6*Δ*rnr4*Δ showed lower 4-NQO resistance compared to *rnr4Δ,* it was expected to show higher levels of mutation frequency than *rnr4*Δ upon 4-NQO treatment. *msh6*Δ*rnr4*Δ showed a 2 fold increase in mutation frequency when compared to *rnr4*Δ. Thus MMR can be ruled out as a repair pathway since knocking out Msh6 does not abolish *rnr4*Δ resistance to 4-NQO.

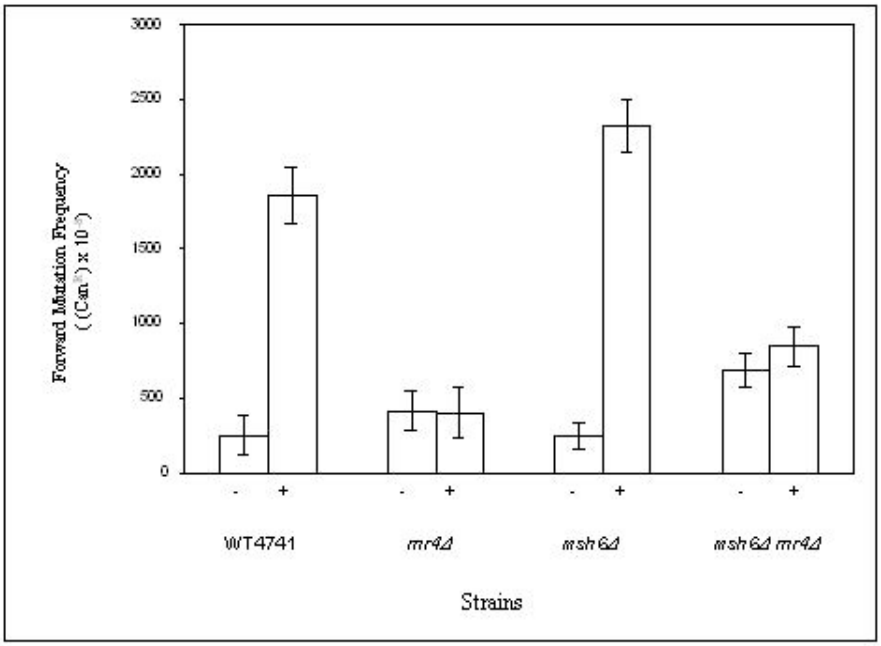

Figure 21. **MMR Mutation Analysis**. Log phase strains were treated and untreated with a fixed concentration of 4-NQO for 1 hour at 30ºC. Untreated *msh6*Δ strain showed a relatively similar level of mutation frequency compared to the untreated parent strain. *msh6*Δ*rnr4*Δ untreated showed a 2 to 3 fold frequency in comparison to *rnr4*Δ and the wild type respectively. Upon 4-NQO treatment, *msh6*Δ showed a 10 fold increase in mutation while *msh6*Δ*rnr4*Δ showed a slight increase in mutation. Results were expressed as the mean $\pm$ SD from at least three separate experiments.

3.7. Recombination Pathway

The next target was the recombination pathway through which we attempted to make single and combination mutants of *rad51*Δ and *rad51*Δ*rnr4*Δ. We wanted to investigate if the double knockout affected cell proliferation and survival in the presence of 4-NQO. The attempt proved futile as the double knockout appeared to be lethal to the cell. We attempted several transformations quite unsuccessfully. Zheng and colleagues have shown that deletion of Rad53 in yeast is lethal, but may be overcome by the following events: overexpression of RNR genes, increasing levels of dNTPs, deletion of Sml1 an RNR inhibitor and by removal of the RNR transcription repressor Crt1 [21, 47-49].

3.8. Regulatory Pathway

Mrc1 is a mediator protein that activates Rad53. Mrc1 follows the replication fork and binds to sequences with replication blocks. A knockout of Mrc1 would thus slow down replication as does a knockout of Rnr4. *rnr4*Δ causes a cell to have a lower level of dNTPs thus slowing down replication. We therefore wanted to test the drug phenotype for cells lacking Mrc1. In order to assess the role of the mediator protein in dNTP accumulation, we tested whether 4-NQO treatment would diminish the size of dNTP pools and affect the cells survival in wild type and *mrc1*Δ strains. We also performed an MMS survival assay for both strains.

*mrc1*Δ cells showed drug sensitivity upon treatment with MMS with respect to the parent strain as shown in Fig. 22. When the *mrc1*Δ cells were treated with 4-NQO as shown in Fig. 23, they were resistant to drug treatment with respect to the wild type. Since

knocking out Mrc1 did not abolish the cells resistant to 4-NQO, the mediator protein is not responsible for *rnr4*Δ hyperresistance to 4-NQO.

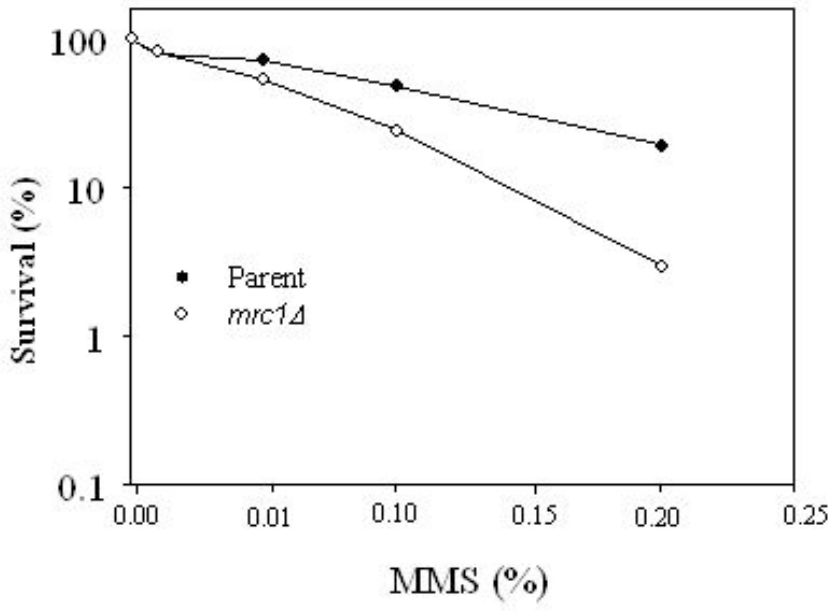

Figure 22. **MMS Survival Curve for *mrc1Δ*.** Log phase strains were treated with increasing concentrations of MMS for 1 hour at 30ºC. *mrc1Δ* (o) was sensitive to MMS with respect to the parent strain (●). Results were expressed as the mean ± SD from at least three separate experiments.

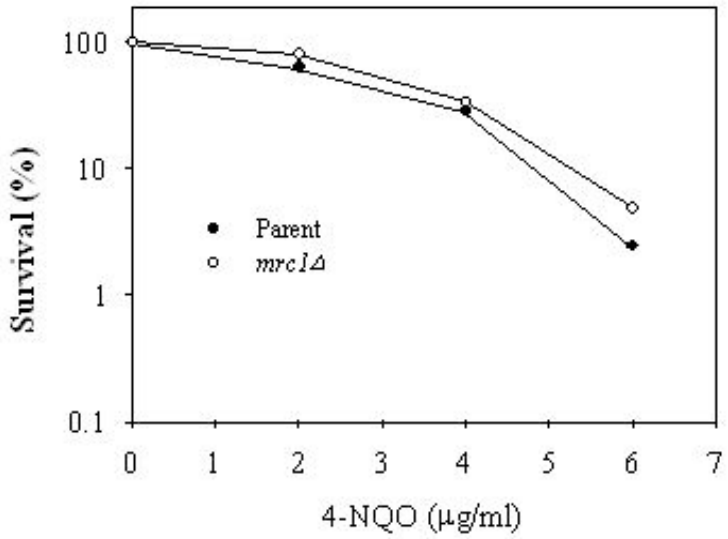

Figure 23. **4-NQO Survival Curve for *mrc1Δ*.** Log phase strains were treated with increasing concentrations of 4-NQO for 1 hour at 30ºC. *mrc1*Δ (o) showed a slightly higher level of resistance in comparison to the wild type (●). Results were expressed as the mean ± SD from at least three separate experiments

3.9. Translesion Synthesis

We tested the following strains wild type and *rev3*Δ with MMS (data not shown) and 4-NQO in order to determine if the combination mutants would show the same drug resistance to 4-NQO and drug sensitivity to the other DNA damaging agents as the *rnr4*Δ mutant. Rev3 as previously mentioned is a bypass polymerase involved in post replication repair. Exponentially growing cells were treated with MMS, H_2O_2, UVC and 4-NQO. Cells were scored for fractional survivors as described in chapter 3.

Even though *rev3Δ* shows reduced resistance to 4-NQO treatment with respect to the wild type (Fig. 24), a double knockout, *rnr4*Δ*rev3*Δ will have to be made in order to determine whether bypass polymerases play role in conferring 4-NQO resistance.

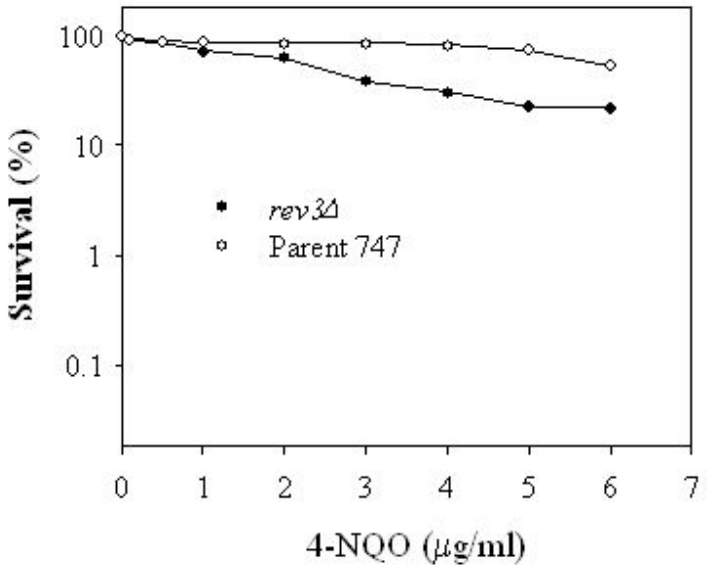

Figure 24**. 4-NQO Survival Curve for Bypass Polymerase**. Log phase strains were treated with increasing concentrations of 4-NQO for 1 hour at 30ºC. *rev3*Δ (●) showed resistance in comparison to the parent (o). Results were expressed as the mean ± SD from at least three separate experiments

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4. Discussion

We have previously used 4-NQO to find yeast mutants that are sensitive to the drug and these mutants have been characterized (Fig. 17, Fig. 20) [29, 42]. We have recently discovered strains that are resistant to 4-NQO one of them deleted for the RNR4 gene. The *rnr4Δ* strain was isolated in a genome widescreen for mutants that were sensitive to bleomycin (Zhao, X., unpublished data). Its 4-NQO resistance was discovered upon cross-examination with a number of DNA damaging agents. It is important to elucidate the mechanisms conferring cellular resistance since much is not known concerning Rnr4 and we would subsequently be able to expand on DNA repair mechanisms that protect the cell. Our findings show that *rnr4*Δ is hyperresistant to 4-NQO and not to other DNA damaging agents such as bleomycin, hydrogen peroxide, UVC and MMS. We chose to work with *rnr4*Δ because this mutant showed a sharper resistance to 4-NQO when compared to that of an *rnr1*Δ as shown by a former student (Zhao, X., unpublished data). In addition, consistent with this resistance, *rnr4*Δ showed a sharp reduction in 4-NQO-induced mutations as compared to the parent.

We wanted to determine the cause of 4-NQO resistance [50-53]. We know that 4-NQO is metabolized by a series of enzymes upon entering a cell in order to be activated. Once activated, it binds to A and G to form a bulky adduct that causes a block for RNA polymerase [50-53]. The bulky lesion may be removed by a number of repair pathways [50-53]. We therefore tested various repair pathways in order to determine which one was responsible for protecting the cell upon 4-NQO treatments. Initially, we were able to rule out BER since a former graduate student performed both chlonogenic and mutation assays with *apn1Δrnr4Δ* (Zhao, X., unpublished data). *apn1Δrnr4Δ* showed resistance when

treated with 4-NQO (Zhao, X., unpublished data). We were also able to rule out, following chlonogenic and mutation assays, NER, MMR, and recombinational repair as possible pathways mediating the cell's resistance to 4-NQO.

We hypothesized that since rnr4 is knocked-out, it is possible that the NER pathway is upregulated since NER is a dominant pathway for 4-NQO bulky lesion repair. Our findings show that a *rad1Δrnr4Δ* mutant does not abolish 4-NQO resistance (Fig. 17). Studies have shown that even though 4-NQO mimics UV damage, parallel responses are not always observed [54]. In fact, studies show that mutants that were sensitive for UV and γ-irradiation are not sensitive to 4-NQO and that there is a specific pathway controlling 4-NQO resistance [54].

We subsequently tested *msh6Δrnr4Δ* and *rad51Δrnr4Δ* since the cell possibly induce repair via MMR or recombination. The MMR knockout showed no loss of resistance but the recombination mutant was lethal in an *rnr4Δ* background (Fig. 20). The yeast genome database, YGD, provides findings that show *rad51Δrnr4Δ* is a synthetic lethal. This data suggests that recombination plays an essential role cell survival but not in repairing 4-NQO-induced DNA lesions. Moreover, the database also shows that the following recombination proteins are synthetic lethals in an *rnr4Δ* background: Mre11, Rad50, Rad51, Rad52 and Xrs2. We may conclude that knocking out these proteins is not only being lethal in an *rnr4*Δ , but they would also be unable to confer 4-NQO resistance.

We also decided to focus on bypass polymerases that are recruited through a post-replication repair pathway. We show that the cell's resistance has been partially abolished in a *rev3Δ* mutant with respect to the wild type (Fig. 24). This pathway would be of interest to pursue since a combinational knockout was not tested (*rev3Δrnr4Δ*). Our mutant *rnr4Δ* shows very slow growth; cells slow down for several reasons one being diminished pools

of dNTPs. However, one may postulate that this slow growth is due to the inactivation or slowing down of high fidelity DNA polymerase and to the recruitment of a single or multiple bypass polymerases. Studies have shown that high fidelity polymerase strongly opposes the bypass mechanism [41]. As previously mentioned, we know there exists 2 independent bypass mechanisms: one involving the formation of a complex (Rev1, Rev3 and Rev7) and the other involving Rad30, a single bypass polymerase. Sabouri and colleagues have shown that deletion of genes encoding for *REV1* and *REV3*, but not *RAD30*, resulted in 4-NQO sensitivity [55]. They show that increasing levels of dNTPs would abolish cell sensitivity. In addition, under DNA damage (4-NQO treatment) resulting in increased levels of dNTPs, the translesion polymerase is able to bypass the lesion at a rate of 19 to 27% [55]. These results indicate that translesion polymerases may play an important role in 4-NQO cellular response. Further investigation, chlonogenic and mutation assays, is thus required involving the following combinational mutants: *rev1Δrnr4Δ*, *rev3Δrnr4Δ* and *rev1Δrev3Δrnr4Δ*. In addition, even if the deletion of the Rad30 protein does not implicate 4-NQO resistance, it should be tested since Rad30 shows limited structural homology with Rev3 [41]. Rad30 also has a human homolog thus giving strong evidence that the bypass polymerase is conserved from bacteria to humans [41].

Our findings show the possibility of bypass polymerase being recruited during the cell's slow growth, but this slow growth may also allow for recruitment of other repair proteins. We therefore targeted other suspects such as Mrc1 and Rad53. As previously mentioned, Mrc1 is a mediator protein of replication and activates Rad53. Mrc1 follows the replication fork and binds to sequences with replication blocks. Since *rnr4*Δ slows down replication due to low dNTP levels, a knockout for Mrc1 would also accordingly show slow cell growth. We wanted to show that the slow growth of *rnr4*Δ and 4-NQO resistance was

not due to a slowdown in replication. Our results show that *mrc1*Δ shows no increased resistance to 4-NQO treatments in comparison to the parent thus ruling out its possible role in drug resistance (Fig. 23).

We then targeted Rad53, a DNA damage checkpoint protein. In response to DNA damage the cell's Rad53 activity is dramatically increased [56, 57]. Huang and colleagues have made several unsuccessful attempts to probe for levels of Rad53 in a wild type and *rnr4*Δ treated with 4-NQO in a time-dependent manner however Sidorova and colleagues were able to show a phosphorylation difference after 4-NQO treatment [48, 56-59]. Sidorova has shown changes in phosphorylation levels in a fresh batch of cells treated with 4-NQO and in cells containing HA-tagged Rad53 [56, 57]. We have made several unsuccessful attempts in probing for Rad53 in both wild type and *rnr4Δ* treated with 4-NQO. This could be due to the specificity of the protocol demanding for very accurate antibody dilutions or to using cell extracts from a previously performed chlonogenic assay. The yeast genome database shows that it is a synthetic lethal in *rnr4*Δ. Since this deletion is a proven lethal it would logically follow that this protein would not be involved in mediating 4-NQO resistance. Since Huang's unpublished data shows that *rad53Δdun1Δ* is also lethal in yeast and that the YGD also show many other types of checkpoint proteins are synthetic lethals in an *rnr4*Δ (Table 6), they would not be involved in mediating resistance.

Table 6. YGD Checkpoint Proteins.

Protein	Description	Experiment Type
Ddc1	DNA damage checkpoint protein	Synthetic Lethality
Dun1	Cell-cycle checkpoint serine-threonine kinase required for DNA damage-induced transcription of certain target genes	Synthetic Lethality Synthetic Growth Defect
Mec1	Genome integrity checkpoint protein and PI kinase superfamily member	Synthetic Lethality
Mec3	DNA damage and meiotic pachytene Checkpoint protein	Synthetic Lethality
Mrc1	S-phase checkpoint protein found at replication forks	Synthetic Growth Defect
Rad17	Checkpoint protein	Synthetic Lethality
Rad24	Checkpoint protein	Synthetic Lethality
Rad53	Protein kinase	Synthetic Lethality
Rad9	DNA-damage checkpoint protein	Synthetic Lethality

Our results raised many challenging possibilities since there appears to be so many possible proteins involved in conferring *rnr4*Δ resistance to 4-NQO. Further investigation is required concerning bypass polymerases. Although the YGD gives rise to many target proteins involved in repair processes and checkpoint pathways, it does not present us with any bypass polymerases. Lis and colleagues studied replicative polymerases by disrupting the proofreading activities of polδ and polε in *rnr4*Δ [60]. Both showed increased mutation but a larger effect was seen with polδ. They however tested with EMS an alkylating agent [60]. We may assume then that proofreading reduces Rnr4 contribution to mutation and that polδ seems to both upregulate dNTP levels within a cell and to activate translesion synthesis. They also show that knocking out Rev3 had no effect on the mutation frequency, but we must note that EMS does not create the same type of lesion as does 4-NQO. They

also show that Fyv6 plays a role in a subset of mutations induced by Rnr4 [60]. Fyv6, function required for yeast viability protein 6, is involved in telomere length regulation [60]. It is not clear what role Fyv6 plays in the cell, but they believe that it may interact with thioredoxin to subsequently regulate RNR activity [60]. It would be of interest to perform 4-NQO clonogenic assays with *fyv6*Δ and *fyv6Δrnr4Δ*.

In addition, we have only targeted one of many proteins mediating replication in the cell. Aside from Mrc1, the following proteins Csm3, Dcc1, Get1 and Tof1 also show synthetic growth defect in an *rnr4Δ*. The N-terminal acetyltransferases NatA (Ard1, Nat1), a nucleosome assembly factor (Asf1), post replication repair proteins (Rad18, Rad5), a ubiquitin-conjugating enzyme (Rad6) and a transcriptional modulator involved in regulation of meiosis (Wtm1) are all synthetic lethals in an *rnr4Δ* that have very distinct functions in a cell.

In hindsight, we must assume that there are many proteins involved in 4-NQO resistance since there are numerous proteins involved in repair mechanisms and since there are many yeast proteins yet to be discovered the list is quite unending. The best approach would be to first eliminate proteins that are synthetic lethals in an *rnr4Δ* and to eliminate proteins involved in the BER, NER, MMR and recombinational repair mechanisms. The second step would be to target proteins conferring 4-NQO resistance (ex. Rev1, Rev3, Fyv6) and knock-out each of these proteins in an *rnr4Δ.* We would the be able to perform both a 4-NQO and control (with MMS) chlonogenic assayand to perform a mutation assay in order to test for proteins unable to produce mutations following induction with 4-NQO. It would be interesting to determine whether defects in the repair process or proteins conferring 4-NQO resistance in *rnr4Δ* will have an effect on both the fidelity of DNA replication and repair and on the viability of cells upon 4-NQO treatment (genotoxic stress).

5. Conclusions and Perspectives

Here we have shown that *rnr4*Δ cells are sensitive to various DNA damaging agents such as methyl methane sulfonate, bleomycin, hydrogen peroxide, and ultraviolet radiation (UVC 254 nm). We have also shown that *rnr4*Δ is resistant to 4-NQO and consistently shows a sharp reduction in 4-NQO-induced mutations as compared to the parent. We wanted to determine which pathway was able to confer resistance to 4-NQO and thus targeted numerous DNA repair mechnisms. We were able to rule out BER, NER, MMR and recombinational repair. Deletion of the *REV3* gene partially abolished 4-NQO resistance in *rnr4*Δ. These results suggest that Rev3 and possibly other translesion polymerases (Rev1, Rev7, Rad30) could play a role in the repair of bulky DNA lesions under low levels of dNTPs. Future studies should focus on creating combinational mutants and performing the corresponding chlonogenic and mutation assays to verify drug resistance to 4-NQO. Additional studies should also include a Rad53 kinase assay involving the immunoprecipitation of untagged or HA-tagged Rad53 out of yeast cell extracts with rabbit polyclonal antibody to Rad53 or 12CA5 mouse monoclonal antibodies to the HA epitope as described by Sidorova and colleagues. These experiments should provide further insight into the *rnr4Δ* cell's resistance to 4-NQO.

Yeast is an ideal model system to deduce biological processes in mammalian cells. It is easier to manipulate genetically in terms of multiple mutagenesis and stability. Many yeast proteins share remarkable homology to human proteins. This conservation would allow one to make predictions with respect to the functional mechanisms of human proteins based on yeast proteins. Interestingly, cisplatin, creates bulky DNA lesions as does 4-NQO [61-63]. Cisplatin, a platinum-based chemotherapeutic drug, is used to treat various types of cancers and is responsible for creating intra and interstrand crosslinks between DNA

bases [61-63]. Cisplatin resistance is mediated via ribosomal proteins and elongation factors [61-63]. Studies have shown the cell's sensitivity is restored when you block phosphorylation and kinase phosphorylation in cisplatin resistant cells [61-63].

A recent study showed that malignant tumors developed in mice drinking water containing 4-NQO and that there was early activation of Akt mTOR [64]. mTOR is a serine/threonine kinase that regulates cell growth, proliferation, motility, survival, protein synthesis and transcription [64]. mTOR is the mammalian target of rapamycin, an immunosuppressant used to prevent rejection in organ transplantation and has anti-proliferative properties [64]. Czerninski and colleagues have shown that rapamycin treatment halts malignant conversion of precancerous lesions [64]. Interestingly, both cisplatin and rapamycin, involve phosphorylation pathways that would be of interest to follow up with *rnr4*Δ.

In terms of cancer research, it is important to understand DNA repair pathways in order to identify and target factors that confer resistance to anticancer drugs. Effective treatment of cancer not only requires development of new chemotherapeutic agents, but also development of strategies that will modulate repair capacity to render cells sensitive or by overcoming their resistance to cancer treatments. The work presented here provides valuable insight regarding both cellular response to drug resistance and regarding the drug mechanisms of other chemotherapeutic agents. This would lead to the creation of more efficient drugs, improvements in drug therapy and the development of new therapeutic methods [60, 65].

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