Analysis of Yeast Genes Influencing the Lethality of DNA Damage Related Checkpoint Mutants

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ABSTRACT

Kim, Eunmi., Analysis of Yeast Genes Influenceing The Lethality of DNA Damage Related Checkpoint Mutants. Doctor of Philosophy (Biomedical Sciences), May, 2009, 195 pages, 34 figures, 5 tables, 128 references, 2 titles.

The purpose of this study was to determine the functions of Hug1 and Srl3. It has been reported that HUG1 or SRL3 deletion rescues the lethality of a DNA damage checkpoint gene deleted mutant, mec1Δ. It is known that the lethality of mec1Δ can be rescued by high dNTP levels. To elucidate the functions of these proteins, the phenotypes of hug1Δ and srl3Δuvs, as well as the transcript profile of hug1Δ were analyzed. Novel phenotypes of hug1Δ were uncovered: resistance to oxidative stress or heat shock, earlier arrest in G1/G0 phase, defect in hydroxyurea-induced filamentation, and slow growth in response to combined stresses of hydroxyurea and reduced dextrose content. These phenotypes correlate with a transcription profile that indicated altered stress responses in hug1Δ as compared to WT. We assumed that the reason for many constitutively expressed stress-related transcripts is a higher dNTP level in hug1Δ compared to WT. The similarities in the phenotypes of dif1Δ and sml1Δ to those of hug1Δ support the assumption. The phenotypes of dif1Δ and sml1Δ were studied since Dif1 and Sml1 are known inhibitors of ribonucleotide reductase activity. Furthermore, Dif1, Sml1, and Hug1 are considered proteins that evolved from the same ancestor protein.

Initially, Srl3 was a protein of special interest because the commercially available srl3Δ mutant causes high spontaneous mutation rates and sensitivity to UV light.
However, during the course of this study, it was found that the two phenotypes originated from a second, unrelated mutation in srl3Δ strain. Through complementation test and sequencing, this mutation was identified as a nonsense mutation of MMS2, a gene involved in post-replication repair.
ACKNOWLEDGMENTS

I would like to give special thanks to my major professor, Dr. Wolfram Siede. He was very kind and patient with me, my research, and writing, as well as, did his best to support me. He gave me the opportunity to train in his lab and become a scientist. I inspired by his creativeness as well as meek personality during education. I would like to thank the members of my graduate committee, Dr. Robert Wordinger, Dr. Dan Dimitrijevich, Dr. Rafael Alvarez-Gonzalez and Dr. Jerry Simecka for their guidance and supports for the completion of dissertation and degree.

I would like to thank my lab member, Donald Rozario, for his assistance in the experiments for filamentous phenotype and in grouping transcript profiles. I would like to thank my friend, Shaun Logan, for her help in correcting English. I would like to thank my friends, Myriam Iglewski, Pil Jo Kim, Sung-Yong Whang, and Shaoak Maresh for sharing their wisdom and worm heart. I would like to thank all other friends who supported me by prayers and kindness.

I would like to express appreciation to my parents, Dong-yun Kim and Kahee Kim, for their love and support throughout my life and for their patience to see me become a scientist. I would like to thank my sisters and brother for their love and supports as well. They did their role as siblings if siblings are for the hard times.
TABLE OF CONTENTS

Chapter                                                                 Page

LIST OF FIGURES -------------------------------------------------ix
LIST OF TABLES --------------------------------------------------xii
LIST OF ABBREVIATIONS ------------------------------------------xiii

I. INTRODUCTION

Purpose of Study ------------------------------------------1
Hug1 -----------------------------------------------------3
Srl3 ------------------------------------------------------5
Mec1 ------------------------------------------------------6
Rad53 -----------------------------------------------------9
Regulation of deoxyribonucleotide triphosphate (dNTP) pools------------------10
DNA damage tolerance (DDT) pathway: translesion synthesis (TLS),
post replication repair (PRR), and checkpoint proteins-------------------16
Growth of Culture ----------------------------------------24
Filamentation in S. cerevisiae -----------------------------28
II. MATERIALS & METHODS

List of *S. cerevisiae* strains used ......................................................... 33

Measurement of spontaneous mutation rates ............................................ 36

Polymerase chain reaction (PCR) procedure ................................................. 38

Isolation of yeast chromosomal DNA for PCR ........................................... 38

Lithium acetate (LiAc) transformation ....................................................... 39

Method for transferring of a yeast gene deletion------------------------------- 40

Observation of cell cycle progression from *G*\(_1\) phase after
ultraviolet (UV) irradiation ........................................................................ 40

Observation of cell cycle progression from *S* phase after UV irradiation------- 41

Observation of cell cycle progression from *G*\(_2\)/M phase after UV irradiation---- 41

Collecting samples for the observation of cell cycle progression from
*G*\(_1\) phase after 4-Nitoquinoline 1-oxide (4-NQO) treatment .................. 42

Collecting samples for the observation of cell cycle progression from
*G*\(_1\)/*G*\(_0\) phase after 4-NQO or UV treatment ................................. 42

Preparation of samples for FACS analysis to detect cell cycle
distribution by DNA content................................................................. 43

Preparation of samples for FACS analysis to detect Green fluorescence
protein (GFP) levels ............................................................................. 44

Measurement of the levels of *Rnr3* and *Rnr4* following HU treatment ...... 44

Isolation of *uvs* mutation from *srl3Δuvs* ............................................. 45
Sensitivity tests .................................................................................................................. 45
Western blotting ................................................................................................................ 46
Filamentation and growth under conditions of slowed DNA synthesis ............. 47
Isolation of total RNA ...................................................................................................... 47
Analysis by gene arrays and comparison profiles ...................................................... 48
Cluster analysis within transcription profiles through group assignment......... 49
Obtaining % of stress responding and filamentation related genes
from comparison transcript profiles ............................................................................. 49

III. RESULTS

1. Studies on the role of Hug1 in DNA damage and stress responses ............. 51

   The expression of Hug1 after HU or 4-NQO treatment ........................................ 52

   The influence of HUG1 deletion on the cell cycle progression and
   on the cell cycle recovery from DNA damage checkpoint arrest .................. 52

   The influence of HUG1 deletion on the protein levels of Rnr3 and Rnr4 .... 64

   The sensitivity of hug1Δ to UV, 4-NQO, or t-BH in cultures of
   different age ............................................................................................................ 72

   The sensitivity of hug1Δ to high temperature (heat shock) in cultures of
   different age ............................................................................................................ 73

   The influence of HUG1 deletion on G1/G0 arrest ................................................ 77
Chapter 2: Studies on the (suspected) role of Srl3 in DNA damage responses and spontaneous mutagenesis.

Spontaneous mutation rate and UV sensitivity of srl3Δ (=srl3Δuvs) and those of dNTP level regulating gene deleted mutants

Cell cycle progression, checkpoint arrest, and cell cycle recovery from DNA damage checkpoint arrest of srl3Δ (=srl3Δuvs)

The sensitivity of srl3Δuvs to UV in different cell cycle phases

The sensitivity of srl3Δuvs to different DNA damaging agents

Separation of uvs mutation from srl3Δ background mutation and the characterization of ‘uvs’ (uvs mutant) phenotype

Epistasis studies with ‘uvs’ (uvs mutant)

Studies on the allelism of UVS
IV. DISCUSSION

1. Studies on the role of Hug1 in DNA damage and stress responses

A role of Hug1 in the recovery from checkpoint arrests in cell cycle phases other than $G_1$ or $G_1/G_0$ phase?

Why are there differences between GFP fluorescence peaks of Rnr3 and those of Rnr4 (Figure 9)?

The resistance of $hug1\Delta$ to heat shock and to t-BH can be explained by the transcript profile of $hug1\Delta$ and stress tolerance mechanisms.

The $hug1\Delta$/WT (2% dextrose) comparison profile does not match with published environment stress response (ESR) profile, fermentation stress response profile, $mec1\Delta$, $tup1\Delta$, and $pho80\Delta$ profile.

Early arrest of $hug1\Delta$ in $G_1/G_0$ may indicate $hug1\Delta$ has more activation of stress responses.

How does Hug1 influence the transcript levels of 10% of genome?

It is likely that $hug1\Delta$ increases dNTP pool, and the increased dNTP pool changes transcript profile.

Conclusions and future studies for Hug1.
2. Studies on the (suspected) role of Srl3 in DNA damage responses and spontaneous mutagenesis

Overview of Mms2 family

The epistatic or synergistic relationship of Mms2 to Rad5, Rad50, and Rad52

There is a possibility that Srl3 influences the recovery from checkpoint arrest

DNA damage tolerance (DDT) pathway is required for bypassing UV, streptonigrin, and MMS induced DNA damage

The high spontaneous mutation rate of ‘uvs’ may be due to Polζ

A nonsense mutation occurred in MMS2 gene of the ‘uvs’ strain

Is it still possible that Srl3 has a role in increasing dNTP pool?

Conclusions and future studies for Mms2 and Srl3

V. REFERENCES

vi
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The RNR and dNTP pool</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>DNA damage tolerance (DDT): translesion synthesis (TLS) and post-replication repair (PRR)</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>Molecules involved in the DDT pathway and their functions</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>Growth curve of typical yeast culture</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>Signal cascades for filamentation</td>
<td>29</td>
</tr>
<tr>
<td>6</td>
<td>Hug1 expression after HU (100 mM or 200 mM) or 4-NQO (0.1 ug/ml) Treatment</td>
<td>53</td>
</tr>
<tr>
<td>7</td>
<td>The effect of HUG1 deletion on cell cycle progression of G1 synchronized cells with/without DNA damage checkpoint arrest</td>
<td>56</td>
</tr>
<tr>
<td>8</td>
<td>The effect of HUG1 deletion on the cell cycle progression of G1/G0 phase synchronized cells with/without DNA damage checkpoint arrest</td>
<td>60</td>
</tr>
<tr>
<td>9</td>
<td>The effect of HUG1 deletion on the protein levels of Rnr3 and Rnr4 during recovery from HU treatment</td>
<td>66</td>
</tr>
<tr>
<td>10</td>
<td>The effect of HUG1 deletion on the UV, 4-NQO, or t-BH sensitivities of differently aged cultures</td>
<td>70</td>
</tr>
</tbody>
</table>
Figure 11. The effect of HUG1 deletion on the heat shock (52°C) sensitivities of differently aged cultures---------------------------------75

Figure 12. The cell cycle phases of 1 day cultured WT and hug1Δ-----------------------------78

Figure 13. The effect of HUG1 deletion on HU (slowed DNA synthesis) induced filamentation------------------------------------------------------------------------81

Figure 14. HUG1 deletion causes slow growth when cells are plated on 100 mM HU and 0% dextrose containing YPD plate-----------------------------83

Figure 15. The effect of ECM7 deletion on HU (slowed DNA synthesis) induced filamentation------------------------------------------------------------------------85

Figure 16. The number of genes whose transcripts are modulated by HUG1 deletion and/or HU treatment,----------------------------------------------------------88

Figure 17. The potential functional significance of transcripts modulated by HUG1 deletion--------------------------------------------------------92

Figure 18. The HU induced filamentation is defected by DIF1, SML1, and HUG1 deletion-----------------------------------------------------------103

Figure 19. The hug1Δ, difΔ, and sml1Δ are sensitive on low dextrose and 100 mM HU containing YPD plate-----------------------------------------------105

Figure 20. The surviving fractions of WT, hug1Δ, dif1Δ, and sml1Δ after heat shock (52°C)-----------------------------------------------107

Figure 21. The UV sensitivities of srl3Δuvs and RNR subunit deleted mutants--------114
Figure 22. The cell cycle arrest and progression of WT and srl3Δuvs with or without UV irradiation----------------------------------------119

Figure 23. The UV sensitivities of G1, S, and G2/M phase arrested srl3Δuvs--------122

Figure 24. The sensitivities of srl3Δuvs to UV, methyl-methane sulfonate (MMS), and streptonigrin-------------------------------------------------124

Figure 25. The comparison of UV sensitivities for WT, srl3Δuvs (BY4741), srl3Δ (BY4741), and srl3Δ (BY4742)--------------------------------127

Figure 26. Separation of uvs mutation and SRL3 deletion from the srl3Δuvs strain----130

Figure 27. Definitions used in the epistasis study: epistatic, synergistic, and additive interactions------------------------------------133

Figure 28. Epistasis studies between ‘uvs’ and rad5Δ, as well as, ‘uvs’ and rad50Δ----136

Figure 29. Epistasis studies between ‘uvs’ and rad52Δ--------------------------------140

Figure 30. Studies on the allelism of UVS------------------------------------------144

Figure 31. DNA sequence of MMS2 gene isolated from srl3Δuvs------------------------146

Figure 32. The domains and sequences of Hug1 and other dNTP regulating proteins are aligned----------------------------------------157

Figure 33. The amino acid sequence alignment of catalytic domains of ubiquitin-conjugating enzymes-----------------------------------173

Figure 34. Evolutionary relationships among ubiquitin-conjugating enzymes--------175
LIST OF TABLES

Table 1. The percentage of filamentation related genes among genes whose
transcript levels are increased above +4 (+log₂2) or decreased
below -4 (−log₂2) in each comparison profile-----------------------------97

Table 2. The relative transcript levels of ECM7 in WT and hug1Δ with or
without HU treatment, when the level of its transcript in WT
without HU treatment is considered 1-------------------------------------99

Table 3. The grouping result of genes whose transcript levels changed more
than ± log₂1.5 (± 2.83) by HUG1 deletion or HU treatment-----------------100

Table 4. The ratio of spontaneous mutation rate of srl3Δuvs to WT, and those of
dNTP level regulating gene deleted mutants, srl3Δ, and ‘uvs’ to WT--------112

Table 5. The grouping result of hug1Δ HU/WT HU (0.2% dextrose)
comparison profile---------------------------------------------------------159
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>Ac-4-HAQO</td>
<td>4-acetoxyaminoquinoline 1-oxide</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase-promoting complex</td>
</tr>
<tr>
<td>ara-CMP</td>
<td>analog of dCMP</td>
</tr>
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<td>Asf1</td>
<td>anti-silencing function 1</td>
</tr>
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<td>A-T</td>
<td>ataxia-telangiectasia</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia-telangiectasia mutated</td>
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<td>ATP</td>
<td>adenosine-5’-triphosphate</td>
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<td>ATR</td>
<td>ataxia-telangiectasia &amp; Rad3 related</td>
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<td>bud site selection 8</td>
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<td>C</td>
<td>cys (cysteine)</td>
</tr>
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<td>chromatin assembly factor 1</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>can-</td>
<td>canavanine absent</td>
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<td>can+</td>
<td>canavanine containing</td>
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</table>

xi
<table>
<thead>
<tr>
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<th>Full Name</th>
</tr>
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<td>CPD</td>
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<td>Crt1</td>
<td>calreticulin 1</td>
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<tr>
<td>Csm2</td>
<td>chromosome segregation in meiosis 2</td>
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<td>CSM-Arg</td>
<td>complete supplement mixture minus arginine</td>
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<td>Cys</td>
<td>cysteine</td>
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<td>dATP</td>
<td>deoxiadenosine-5’-triphosphate</td>
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<td>Dbf4</td>
<td>dumbbell forming 4</td>
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<tr>
<td>dCMP</td>
<td>deoxycytidine monophosphate</td>
</tr>
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<td>Definition</td>
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<tr>
<td>dCTP</td>
<td>deoxycytidine triphosphate</td>
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<td>DNA damage checkpoint 2</td>
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<td>Dbf4-dependent kinase</td>
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<td>DNA damage tolerance</td>
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<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
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<td>Dif1</td>
<td>damage-regulated import facilitator</td>
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<td>deoxyribonucleic acid</td>
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<td>dNTP</td>
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<td>DSB</td>
<td>double strand break</td>
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<td>dsDNA</td>
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<td>deoxythymidine triphosphate</td>
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<td>Dun1</td>
<td>DNA-damage uninducible</td>
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<td>E1</td>
<td>ubiquitin-activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>ubiquitin ligase</td>
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<tr>
<td>Ecm7</td>
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<td>EDTA</td>
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<td>ESR</td>
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</tr>
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<td>exonuclease 1</td>
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<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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</table>
FHA    forkhead-associated
FRE    filamentous responsive element
GCR    gross chromosomal rearrangement
GFP    green fluorescence protein
Hir1/Hir2 histone regulation 1/ histone regulation 2
His    histidin
HR     homologous recombination
HSPs   heat shock proteins
HU     hydroxyurea
Hug1   hydroxyurea and UV and gamma radiation induced
ID     identity
IgG    immunoglobulin G
IMDH   β-isopropylmalate dehydrogenase
IR     ionizing radiation
K164   lysine (Lys) at 164
 kan    kanamycin
Kss1   kinase suppressor of Sst2 mutations
Leu    leucin
LiAc   lithium acetate
Lys    lysine
MAPK   mitogen-activated protein kinase
MATα   mating type α
MATa   mating type a
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<td>MluI-box Binding Protein</td>
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<td>MCM</td>
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<td>mouse embryonic fibroblasts</td>
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<td>Mig1</td>
<td>multicopy Inhibitor of GAL gene expression</td>
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</tr>
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</tr>
<tr>
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<td>meiotic recombination 11</td>
</tr>
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</tr>
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<td>multicopy suppressor of SNF1 mutation 2</td>
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<td>Pcls</td>
<td>Pho85 cyclin</td>
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<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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</tr>
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Rad6  radiation sensitive 6
Rad9  radiation sensitive 9
Rev1  reversionless 1
Rev3  revertibility 3
Rev7  revertibility 7
RFC  replication factor C
Rim15  regulator of IME2
RING  really interesting new gene
RNA  ribonucleic acid
RNR  ribonucleotide reductase
Rnr1  ribonucleotide reductase 1
Rnr2  ribonucleotide reductase 2
Rnr3  ribonucleotide reductase 3
Rnr4  ribonucleotide reductase 4
Rox1  repressor of hypoxic genes
RPA  replication protein A
SAG  serial analysis of gene expression
SCDs  SQ/TQ cluster domains
Sch9  suppressor of chitin synthase disruption
SDS  sodium dodecyl sulfate
Siz1  SAP and MIZ-finger domain 1
Sml1  suppressor of Mec1 lethality

xvii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Spa2</td>
<td>spindle pole antigen 2</td>
</tr>
<tr>
<td>SQ</td>
<td>serine-glycine</td>
</tr>
<tr>
<td>Srl3</td>
<td>suppressor of rad53</td>
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<tr>
<td>Srs2</td>
<td>suppressor of rad six 2</td>
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<tr>
<td>SSA</td>
<td>synthesis-dependent strand annealing</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single stranded DNA</td>
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<tr>
<td>Ssn6</td>
<td>suppressor of Snf1</td>
</tr>
<tr>
<td>Ste12</td>
<td>sterile 12</td>
</tr>
<tr>
<td>Ste20</td>
<td>sterile 20</td>
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<tr>
<td>SUMO</td>
<td>small ubiquitin-like modifier</td>
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<tr>
<td>Swe1</td>
<td>Saccharomyces Wee1</td>
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<tr>
<td>SWI/SNF</td>
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</tr>
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<td>Swi4</td>
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<td>TAP</td>
<td>tandem affinity purification</td>
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<tr>
<td>t-BH</td>
<td>tert-Butyl hydroperoxide</td>
</tr>
<tr>
<td>Tca</td>
<td>tri-chloroactic acid</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>tricarboxylic acid cycle</td>
</tr>
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<td>TE</td>
<td>Tris/EDTA</td>
</tr>
<tr>
<td>TEB</td>
<td>Tris/EDTA/SDS</td>
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<td>transposon enhancement control 1</td>
</tr>
<tr>
<td>Tel1</td>
<td>telomere maintenance</td>
</tr>
<tr>
<td>Abbreviation</td>
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<td>Thr</td>
<td>threonine</td>
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<tr>
<td>Thr-225</td>
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<tr>
<td>TLS</td>
<td>translesion synthesis</td>
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<tr>
<td>Tof1</td>
<td>topoisomerase I-interacting factor 1</td>
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<tr>
<td>TOR</td>
<td>target of rapamycin</td>
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<td>trypytophan</td>
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<td>whiskey 5</td>
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<tr>
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<td>wild type</td>
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<td>WD repeat containing transcriptional modulator 1</td>
</tr>
<tr>
<td>Xrs2</td>
<td>X-ray sensitive</td>
</tr>
<tr>
<td>YNB</td>
<td>yeast nitrogen base</td>
</tr>
<tr>
<td>YPD</td>
<td>yeast extract/peptone/dextrose</td>
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I. INTRODUCTION

Purpose of Study

The purpose of this study is to determine the function of two budding yeast (S. cerevisiae) proteins, Srl3 (protein) and Hug1, which are related to the lethality of DNA damage or replication fork arrest checkpoint kinase deleted mutants, mec1Δ (deletion mutant) and rad53Δ. The overexpression of SRL3 (gene) suppresses the lethality of mec1Δ and that of rad53Δ (31). The deletion of HUG1 rescues mec1Δ lethality (8).

Yeast, which is a simple eukaryotic organism, is a good tool for understanding mechanisms in higher eukaryotes. It contains 16 chromosomes and about 6000 open reading frames (ORF) [63]. Because checkpoint proteins and cell cycle pathways are evolutionarily well conserved, yeast has often been used for checkpoint studies (18, 55). Therefore, the studies on Hug1 and Srl3 can extend the understanding of the roles of DNA damage or replication fork arrest checkpoint proteins in higher eukaryotes.

Checkpoint proteins sense damage or malfunctions in a cell and amplify the damage signals to activate protection, repair mechanisms, and apoptosis. Among checkpoint proteins, DNA damage checkpoint proteins are activated specifically by DNA damage or replication fork arrest (2, 94). The signal cascades for DNA damage or replication fork arrest induce cell cycle arrest and other cellular reactions in order to protect cells from further damage and assist in repairing the damage (18). Therefore,
defects of DNA damage checkpoint lead to genome instability, causing mutations related to cancer and other diseases (30, 80). In humans, the most important DNA damage checkpoint proteins are Ataxia-Telangiectasia mutated (ATM), and Ataxia-Telangiectasia (A-T) & Rad3 related (ATR) [2]. The mutations in these proteins cause A-T and Seckel syndrome, respectively (64). It has been observed that A-T patients have immunological deficiencies, disabling ataxia, higher incidence of cancer, cerebellar atrophy and early death (85).

The homologue of ATM in S. cerevisiae (budding yeast) is Tel1. Human ATR is homologous to the budding yeast protein, Mec1 (54). They both sense DNA damage. ATM senses double strand breaks and ATR senses single stranded DNA tracts (2). The important downstream kinases of ATM (Tel1) and ATR (Mec1) are Chk1 (Chk1) and Chk2 (Rad53), which are directly phosphorylated by ATM (Tel1) and ATR (Mec1). Activated Chk1 (Chk1) and Chk2 (Rad53) amplify and transmit signals for DNA damage or replication fork arrest to trigger protection and repair reactions (18).

Mec1 and Rad53 have several roles other than recognizing DNA damage and replication fork arrest, and triggering signal cascades for protection and repair mechanisms. Many functions are still being discovered. What is known will be described in the following sections: ‘Mec1’ and ‘Rad53’.

It is generally understood that the lethality of mec1Δ is caused by the inability of the replisome to progress across certain parts of the chromosome. Cha RS and Kleckner N (2002), measured the replication of mec1Δ, and found that mec1Δ cannot finish replication. They named the replication progression difficulty regions ‘replication slow zones’ (22) but the zones are not defined. They suggested that Mec1 gives impulses of
deoxynucleoside triphosphate (dNTP) when the replisome is arrested, to make replication possible across the ‘replication slow zones’. This opinion is supported by the fact that mutants which increase the dNTP pool rescue mec1Δ and rad53Δ lethality. dNTPs are precursors of DNA. However, some reports indicate that many other proteins of unknown function are involved in rescuing the lethality of mec1Δ and rad53Δ (8, 31). Therefore, studying those proteins whose functions are unknown but influence the lethality of DNA damage checkpoint mutants, might give some clues for better understanding DNA damage checkpoints as well as cell death.

**Hug1**

Hug1 is composed of 68 amino acids and its molecular weight is 7,514 Da. The yeast genome web site ([www.yeastgenome.org](http://www.yeastgenome.org)) shows that Hug1 directly interacts with general transcriptional co-repressor Tup1, which forms a complex with four Ssn6 and is recruited to the promoters by DNA binding proteins such as: Crt1, Mig1, and Rox1 (42). Basrai MA, et al. (1999), extensively studied Hug1. The descriptions of Hug1 in this paragraph are the results of their studies. Hug1 transcriptional expression is only detectable when there is DNA damage or replication fork arrest. This is proved by measuring the mRNA level of HUG1 before and after treatment of UV, γ-irradiation, hydroxyurea (HU). However, the transcriptional expression is not limited to a specific cell cycle. Transcriptional expression of HUG1 is under the control of Crt1, a transcriptional repressor downstream of Mec1-Rad53-Dun1. When there is DNA damage or replication fork arrest, the Mec1-Rad53-Dun1 signal cascade phosphorylates and removes Crt1 from the promoters of HUG1. The signal transduction for transcriptional
inductions of RNR2, RNR3, RNR4, and CRT1 are the same as HUG1. They are all under the control of Crt1, a transcriptional repressor. Basrai MA, et al. (1999), could not determine the exact function of Hug1, but they found out that Hug1 influences the phenotypes of mec1 and dun1 mutants: first, hug1Δ rescues the lethality of mec1Δ, second, hug1Δ suppresses the HU sensitivity of dun1Δ, and third, the overexpression of HUG1 enhances the HU sensitivity of mec1Δsml1-1 (strain contains MEC1 deletion and SML1 mutation). Based on these phenotypes and transcriptional regulation of the HUG1 gene, Basrai MA, et al (1999), have lead to the hypotheses that Hug1 may have a role in the transcriptional recovery from checkpoint arrests, and that Hug1 regulate ribonucleotide reductase (RNR) to reduce dNTP pools (8).

During screening the substrates of all kinases in S. cerevisiae, Ptacek J, et al. (2005), identified Hug1 as a substrate of Pho85/Pho80 kinase complex. It was a proteomic study to find out the substrates of all kinases. Pho85 is a cyclin dependent kinase (CDK) and has 50% similarity to Cdc28 (70). Pho85 can form complexes with 10 different Pho85 cyclins (Pcls), and each complex targets different substrates (19). Pho85 is required for G₁ progression, resistance to osmotic stress, polarity of actin and endocytosis, to name a few (49). Pho80 is one of the 10 cyclins (49). Pho85/Pho80 complex prevents extremely sensitive cellular reaction to environmental stresses such as Ca²⁺, osmotic stress, and nutritional stress (70). Pho85/Pho80 complex inhibits phosphate starvation induced gene expression by inhibiting transcription factor, Pho4 (70). Through the inhibition of Pho4, Pho85/Pho80 complex has a role in the vacuole inheritance and in the resistance to aminoglycoside antibiotics (82, 118). Vacuoles store poly-phosphates which are the source of phosphate during phosphate starvation.
Pho85/Pho80 has a role in G\textsubscript{1} phase adaptation through inhibiting Pho4 and Swi5 transcription factors which prevent S phase entry (123). When cells cannot repair some damage even after G\textsubscript{1} (or G\textsubscript{2}) checkpoint arrest, they often restart cell cycle progression with the damage. This is called G\textsubscript{1} (or G\textsubscript{2}) phase adaptation. Pho85/Pho80 inhibits Rim15 to prevent G\textsubscript{0} entry in parallel with other stress responding pathways (112, 126).

**Srl3**

Srl3 stands for ‘suppressor of rad53\textsubscript{Δ} lethality’. Desany BA, et al. (1998), overexpressed cDNA library in rad53\textsubscript{Δ} or mec1\textsubscript{Δ} cells, and found genes whose overexpression rescues the lethality of rad53\textsubscript{Δ} \& mec1\textsubscript{Δ}. Srl3 is one of them; overexpression of SRL3 rescues rad53\textsubscript{Δ} or mec1\textsubscript{Δ} lethality (31). Yeast genome web site ([www.yeastgenome.org](http://www.yeastgenome.org)) describes Srl3 as a cytoplasmic protein and potential substrate of Cdc28.

Srl3 became interesting since it was observed in our lab that srl3\textsubscript{Δ} has high spontaneous mutability and is sensitive to UV. However, in the middle of the project to determine the function of Srl3, we realized that the interesting two phenotypes (spontaneously mutable to a high degree and UV sensitivity) of the srl3\textsubscript{Δ} strain do not originate from SRL3 deletion but from a second mutation (we named the mutated gene as UVS). Later, we could identify the second mutated gene (UVS) as MMS2.
Mec1

The protein, Mec1 is an essential DNA damage and replication fork arrest checkpoint kinase in *S. cerevisiae*. It is homologous to Rad3 in *S. pombe* (fission yeast) and to ATR in humans (32, 80, 91). It belongs to the serine-threonine kinases and contains a phosphatidylinositol 3-kinase (PI3K) domain as C-terminal catalytic motif.

Among DNA damage and replication fork arrest checkpoint proteins, Mec1 is one of the first proteins recognizing DNA damage and replication fork arrest by sensing single stranded DNA (ssDNA) [81]. Therefore, it is also called a DNA damage sensing protein. Ddc2 forms a complex with Mec1 and recruits Mec1 to replication protein A (RPA) which coats ssDNA (73). Before the damage is sensed by Mec1, ssDNA is generated by DNA damage repair proteins such as nucleotide excision repair (NER) proteins or Tel1/Mre11-Rad50-Xrs2 (MRX) complex (12, 104). Replication fork arrest also generates ssDNA. The ssDNA is generated because of the uncoupling of MCM replicative helicase and DNA polymerase during replication fork stall (17). After Mec1 is recruited to RPA, it is activated and passes the DNA damage signals to downstream proteins (104). The signal cascades lead to DNA damage responses, such as checkpoint arrest, activation of repair proteins, induction of transcription, increase of dNTP pool, inhibition of late origin firing, and stabilization of replication forks, through activating downstream DNA damage checkpoint kinases, Rad53 and Chk1 (81).

The signal cascade of Mec1-Rad53 checkpoint activation triggers cell cycle arrest in all the cell cycle phases: in G₁ to S phase transition, in S phase, and in G₂ to M phase transition (93, 100). It was proved earlier that Mec1 has a role in DNA damage induced G₁ arrest. This was observed by defects in G₁ arrest in mec1 mutant after UV and γ-
irradiation (101). Activation of Rad53 causes G₁ arrest partially by directly phosphorylating and inhibiting Swi6, a transcriptional inducer of Cln1 and Cln2. Inhibiting transcription of Cln1 and Cln2 arrests cells in G₁ phase, because the increase of Cln1 and Cln2 determines G₁ to S transition (100). The involvement of Mec1-Rad53 signal cascade in intra S phase checkpoint is also evident by delayed S phase process of mec1 mutant or rad53 mutant after methyl methanesulfonate (MMS) treatment (85). The inhibition of late origin firing (99) and slowed replication fork movement (61) are the reasons for slowed S phase progression in S. cerevisiae. The inhibition of late origin firing depends on checkpoints, but the slowed replication progressions are independent on the checkpoints (61). However, in fission yeast, it is proved that early origin firing and slowed replication movements are the reasons for the slowed S phase after DNA damage. These inhibitions are through DNA damage checkpoint activation. The Rad3-Cdc1 (Mec1-Rad53) inhibits S phase Cdc25 phosphatase which is an activator of Cdc2 (Cdc28) [61]. It is assumed that inactivated Cdc2 may influence the proteins involved in the maintenance of replication stability and/or in the bypass of DNA lesions to slow down the replication forks (61). For G₂/M arrest, Mec1-Rad53 signaling prevents metaphase entry through Cdc28-cyclin B complex and spindle elongation machinery (60). Cdc28 activation is essential for spindle pole body separation, an early step for the formation of the mitotic spindle (60). Mec1-Rad53 checkpoint proteins prevent transition from M to G₂ phase by mainly regulating spindle elongation (60). This is different from normal cell cycle regulation which depends on degradation of cyclins (including Cdc28-cyclin B) through anaphase promoting complex (APC) [60, 94].
A deletion of MEC1 is lethal. It is known that the lethality of mec1Δ is suppressed by high dNTP pools (24, 31, 50). As mentioned, Cha RS and Kleckner N (2002), found out that the lethality of mec1Δ is due to the profound defect in the ability to finish chromosomal replication. This is due to the ‘replication slow zones’, in which the replication apparatus has difficulty in progressing though and causing replication fork arrest (22). Additionally, they think that Mec1 has a role in giving dNTP pulses in these replication difficulty regions to stimulate the progression of replication fork and makes replication apparatus possible to finish replication (22). There are other opinions about the reasons for the lethality of mec1Δ and rad53Δ. They said that the main reasons for mec1Δ and rad53Δ lethality are unstabilized replication forks and incapable of replication resumption (81, 95). Nedelcheva-Veleva MN, et al. (2006), suggested that Mec1 has a role in regulating the speed of progress of replication fork by synchronizing the unwinding of chromosome and the processing of replication fork during DNA synthesis, to bring about the stabilization of replication fork. Their suggestion is based on the facts that Mec1 slows down replication fork advancement by phosphorylating minichromosome maintenance (MCM) and Mrc1/Tof1/Csm2 (MTC) complex, and that Mec1 stimulates the replisome process by giving dNTP pulses (22, 47). MCM is a helicase and unwinds dsDNA for replication fork advancement. MTC complex interacts with MCM and restrains the replisome progression (47). Mec1 not only influences MCM, MTC complex, and dNTP pulse, but it also stabilizes Polδ and Polε in the replisome to help the resumption of replication (29).

The stability of the replication fork is important for reducing double strand breaks (DSB), which often cause cell death (64, 66). Mec1 has some roles in managing DSB (93,
Mec1 stimulates meiotic recombination via phosphorylating RPA, and triggers non-homologous end joining (NHEJ) via phosphorylating histone H2A in conjunction with Tel1 (93). DSB is ultimately repaired by homologous recombination (HR) or NHEJ (93). Additionally, Mec1 takes part in the recruitment of the Rev1-Polζ complex (the translesion synthesis (TLS) polymerases), to DSB sites (46). Mec1 also plays a role in telomere maintenance with Tel1 by phosphorylating Cdc13, a telomere binding and telomerase recruiting protein (108).

Rad53

The protein, Rad53 is the ortholog of mammalian Chk2, an essential serine-threonine checkpoint kinase (12, 53). The deletion of RAD53 is lethal, and rad53 point mutants grow slowly, lose chromosomes, and dissociate MCM helicase (32).

The Rad53 has many functions. Rad53 amplifies the Mec1/Tel1 checkpoint signals to downstream proteins and also has Mec1/Tel1 independent roles. As a mediator of Mec1/Tel1 activation, fully activated Rad53 results in cell cycle arrest, transcriptional induction of repair genes, activation of repair proteins, an increase in dNTP pool, stabilization of stalled replication forks, and inhibition of late origin firing (32, 73). These effects are accomplished through the modification of Rad53 downstream proteins such as: Dun1, transcriptional factors like Swi6, and repair proteins (94, 127).

The Rad53 has roles independent of Mec1 for example, degradation of overflowing histones, stabilization of stalled replication fork, initiation of replication, and cytokinesis (32, 103). Unphosphorylated Rad53 down regulates excess histones posttranscriptionally without Mec1 or histone chaperones (CAF-1, Asf1 and Hir1/Hir2)
[43, 90]. Although unphosphorylated Rad53 physically interacts with Asf-1, it is not required for Rad53 mediated degradation of histones (96). Rad53 participates in stabilizing the replication fork independent of Mec1 by stabilizing MCM in the replication fork and preventing Exo-1 (exonuclease) dependent replication fork breakdown (30, 32, 95). For stabilization of replication fork, Mec1 and Rad53 both have independent functions (30, 32, 95). Rad53 takes part in initiation of DNA replication by interacting with Dbf4-dependent kinase (DDK), Cdc7-Ddf4, which activates pre-replication complex and is required for Cdc45 to load DNA polymerases and other replication proteins onto replication origins in coordination with CDK and MCM replication helicase (32). Smolka MB, et al. (2007), identified the forkhead-associated (FHA) domain of Rad53 interacting proteins. The proteins are Rad9, Mrc1, Asf1, Ptc2, and Swi6 and Swi6 associated proteins (Swi4, Mbp1, Whi5), Septins, and bud site selection proteins. Through their studies, they found out a new function of Rad53. Rad53 plays a role in cytokinesis by interacting with Septins, which form ring-like protein scaffolds at the bud neck.

**Regulation of deoxyribonucleotide triphosphate (dNTP) pools**

Increased dNTP pools suppress the lethality of MEC1 deletion and RAD53 deletion. This is proven by two facts. Deletion of negative dNTP pool regulator (Sml1, Dif1, or Crt1), or over-expression of positive dNTP pool regulator (Rnr1 or Rnr3) causes mec1Δ and rad53Δ strains to be viable (24, 31, 50, 68, 120). It is suggested that high levels of dNTP rescue the lethality of mec1Δ, since high dNTP pools compensate for the lack of dNTP pulse. It is thought that Mec1 gives dNTP pulses to stalled replication
apparatus due to ‘replication slow zones’, the natural structure of chromosome, in order to stimulate stalled replisomes across the zone. (22).

The level of dNTP is different in every cell cycle phase and increases after DNA damage. During the cell cycle, the level of dNTP is the lowest in the G₁ phase and the highest in the S phase (25). In response to DNA damage, the level of dNTP is increased 6 to 8 fold over that of logarithmically growing cultures in *S. cerevisiae* (26).

The level of dNTP is controlled in all cell cycle phases. The level of dNTP influences cell cycle arrest. A 35 fold increase in dNTP level delays cell cycle transition from G₁ to S phase by inhibiting activation of replication origins (25). Additionally, there is a report that large RNR subunit is degraded during cell cycle transition from S to G₂ phase. β subunit (small subunit) is degraded by anaphase-promoting complex (APC) in mouse cell (27).

Among dNTPs, the levels of the four nucleotides are asymmetric. The exact ratio of each dNTP cannot be defined, because there are high variations from one experiment to another, as well as among organisms. However, dGTP is commonly the least abundant among dNTPs, usually comprising 5% to 10% of the total dNTP pool. In synchronized S-phase HeLa cells, the ratio of the concentration of dATP:dTTP:dCTP:dGTP is 6:6:3:1 (75).

The excess of dNTPs increases misinsertion and frameshift mutations. It enforces the formation of non-Watson-Crick base pairs, and the omission of proofreading during DNA replication. The imbalance of dNTPs also causes misinsertion. However, it is not yet verified if imbalanced dNTPs cause frameshift mutations (75).
**Figure 1. The RNR and dNTP pool.** A. The structure and role of RNR. RNR is composed of two large subunits and two small subunits. Large subunits (Rnr1Rnr1, Rnr1Rnr3, or Rnr3Rnr3) regulate the balance and the amount of dNTPs through allosteric regulation. Small subunits (Rnr2Rnr4) initiate reduction of NTP by using the binuclear ferric iron center and tyrosyl free-radical. B. Signal cascades for RNR and dNTP regulation. RNR activity and levels (transcriptional regulation and localization) determine the levels of dNTPs. DNA damage or replication fork arrest triggers Mec1/Rad53/Dun1 kinase cascades. Activated Dun1 kinase stimulates dNTP increase through phosphorylating Sml1, Crt1, Wtm1, and Dif1. Phosphorylation causes degradation or inactivation. Sml1 binds to the large subunit of RNR and inhibits RNR activity. Crt1 binds to the promoters of RNR2, RNR3, RNR4, and represses their transcription. Dif1 localizes Rnr2 and Rnr4 subunits into the nucleus to reduce the level of RNR holoenzyme. Wtm1 anchors Rnr2 and Rrn4 in the nucleus. Modified from Mol Cell. 2008 32:70-80 and Genes Dev. 1998 12(18):2956-70.
A.

Ribonucleotide reductase

B.

- Mec1
  - Rad53
  - Dun1
    - Sml1: RNR activity
    - Crt1: RNR transcription
    - Dif1: Rnr2, Rnr4 cytoplasmic localization

\[ \text{dNTP} \]
The level of dNTPs and the balance among dNTPs are controlled by RNR. RNR converts nucleotide triphosphate (NTP) to dNTP and determines the level and balance of dNTPs. There is an abundance of NTPs compared to dNTPs in the cell, so the level of NTPs is not the limiting factor for the level of dNTPs. There is no salvage synthesis of dNTPs in budding yeast, because it does not have salvage kinases which phosphorylate deoxyribonucleosides (92). However, in mammals, there are several kinases for the salvage pathway (92).

The RNR is composed of four subunits (Figure 1). The four genes encoding RNR subunits in *S. cerevisiae* are RNR1, RNR2, RNR3, and RNR4. RNR1 and RNR3 encode large RNR subunits. RNR2 and RNR4 encode small subunits. *S. cerevisiae* has several RNRs composed of different subunits. The combination of large subunits can be Rnr1·Rnr1, Rnr1·Rnr3, and Rnr3·Rnr3. Rnr1 is always expressed, but Rnr3 presents undetectable levels in unchallenged cells. Even though the overall amount of Rnr3 is never more than one-tenth of that of Rnr1, Rnr3 is expressed more than 100 fold of the level of unchallenged cells when there is DNA damage (35). Comparably, the increase of Rnr1 after DNA damage is 3 to 5 fold. The role of Rnr3 is suggested to help the production of dNTPs limited by the level and activity of Rnr1 when there is DNA damage (24, 35). Rnr1·Rnr3 has higher activity than Rnr1·Rnr1, whereas Rnr3·Rnr3 has less than 1% of the activity of Rnr1·Rnr1 (24). There are three combinations of small subunits: Rnr2·Rnr2, Rnr2·Rnr4, and Rnr4·Rnr4. The active form is only Rnr2·Rnr4 (23). Rnr2 holds tyrosyl free radical which starts reduction of NDP to dNDP. The role of Rnr4 in the small subunit complex is to correctly fold and stabilize Rnr2 (23). To cause replication fork arrest, HU is usually used. HU lowers dNTP levels by quenching the free
radical on the small subunit of RNR, and low dNTP levels inhibit the progression of replication and induce replication fork arrest (35).

The level of dNTPs is determined by three factors in *S. cerevisiae*: the activity of RNR, transcription of RNR subunits, and localization of RNR subunits (25). Sml1 in *S. cerevisiae* and Spd1 in *S. pombe* are inhibitors of RNR activity. They both bind to and inhibit the large subunit of RNR (25). The expression of RNR2, RNR3, and RNR4 are regulated by the transcriptional inhibitor, Crt1. Crt1 represses the transcription of RNR subunit genes by binding to the promoters of the genes, and recruiting the general transcription co-repressor complex (Ssn6/Tup1) to the promoters. Crt1 binds to Ssn6 (50, 125). Localization of RNR small subunits, Rnr2 and Rnr4, also contributes the level of dNTPs. Rnr2 and Rnr4 are present in the nucleus but are translocalized to the cytosol where Rnr3 and Rnr1 are present when dNTP induction is required (58, 75). Dif1 imports Rnr2 and Rnr4 into the nucleus to reduce the level of RNR holoenzyme, and Wtm1 anchors them in the nucleus (68, 120). After DNA damage, all the three RNR repressor mechanisms (Sml1, Crt1, and Dif1) are removed by Mec1-Rad53-Dun1 kinase cascade (Figure 1) [31, 128, 68, 120]. Dun1 phosphorylates Sml1, Crt1 and Dif1 and degrades or inactivates them (31, 68, 120, 125).

For the restoration of the RNR activity, it has to be reduced. Thioredoxin and glutaredoxin are donors of hydrogen to cysteines at the surface of RNR, and finally reduces active sites of RNR. Thioredoxin and glutaredoxin are reduced by NADPH (58, 79).
DNA damage tolerance (DDT): *translesion synthesis (TLS), post replication repair (PRR), and checkpoint proteins*

The lesion of DNA, such as the abasic site and covalently modified DNA, in the leading template strand prevents the progression of replication fork during replication of DNA (9, 57). The reason is that the lesion is not able to be replicated by replicative polymerases, Pol ε and Pol δ (114). These blockages of the replication process can cause the collapse of the replication fork which often induces cell death or genomic instability (4, 78, 118). Therefore, cells have evolved DNA damage tolerance (DDT) mechanisms which do not remove DNA damages in the template strand, but bypass the DNA lesions during replication to prevent replication fork collapse (4). DDT mechanisms include *translesion synthesis (TLS) and post replication repair (PRR)* pathways (Figure 2) [15, 38]. TLS has error-free and error-prone bypass ways, and PRR is an error-free bypass way (4). There are also reports that checkpoint proteins are involved in the DDT pathway (Figure 3) [7, 56].

The TLS and PRR pathways are initiated by ubiquitination of proliferating cell nuclear antigen (PCNA), which is encoded by POL30. As a sliding clamp and scaffold for replication apparatus, homotrimer PCNA surrounds DNA and helps the process of leading strand replication apparatus during replication (4). PCNA also is involved in cell cycle arrest and chromatin structure maintenance (4). When replication apparatus encounters DNA lesions, K164 on PCNA is covalently linked with mono-ubiquitin, Lys-63-linked poly-ubiquitin, or small ubiquitin-like modifier (SUMO). Mono-ubiquitinated PCNA signals TLS, poly-ubiquitinated PCNA triggers PRR, and SUMO-modified PCNA
Figure 2. DNA damage tolerance (DDT): translesion synthesis (TLS) and post-replication repair (PRR). TLS and PRR are two DNA damage bypass mechanisms that occur when the replication apparatus encounters abnormal DNA structures in the leading strand during replication. The bypass does not repair abnormal DNA structure, but prevents the collapse of replication fork which can cause GCR and cell death. TLS often induces mutations because TLS polymerases insert incorrect bases opposite the damaged bases in most cases. However, PRR is an error-free bypass mechanism because it uses the newly synthesized sister-chromatid (duplicate of lagging strand) as a template to bypass the damaged DNA, instead of using the damaged DNA template (leading strand), through template switching. Thus, defects in the PRR pathway cause high spontaneous mutation rate as well as sensitivity to DNA damaging agents. Modified from Cell Res. 2008 18(1):162-73.
prevents gross chromosomal rearrangement (GCR) by inhibiting recombination and supporting PRR (Figure 3) [14, 78, 87, 113].

Translesion synthesis (TLS) – Rad6 (ubiquitin-conjugating enzyme [E2]) and Rad18 (ubiquitin ligase [E3]) mono-ubiquitinate PCNA on K164 (Figure 3) [48, 110]. C-terminus of 76 amino acid long ubiquitin forms isopeptide bond with ε-amino group of lysine residue (K164) of PCNA (4). Rad6 has roles other than TLS. This is proved by the fact that rad6 mutant has slow growth, telomere elongation, protein degradation, and no sporulation phenotypes (6, 76).

Mono-ubiquitinated PCNA replaces replicative polymerases, Polδ and Polε with TLS polymerases (4, 69, 110), and bypasses the replication blockages. TLS polymerases containing reduced fidelity than Polδ and Polε are able to insert and extend nucleotides opposite to DNA lesions on the template strand. TLS polymerases often induce mutations because except for Polζ, all of them loose replicative sites and do not have proofreading 3’-5’ exonuclease activity (4). There is specificity of TLS polymerases to the type of DNA damage (4). Humans have Polη, Polτ, and Polκ for TLS polymerases (55, 69). Polτ only inserts a nucleotide, but Polκ extends as well as inserts the nucleotides opposite a damaged nucleotide (55, 69). Polη is encoded by RAD30 and bypasses UV induced cyclobutane TT dimer, cyclobutane TC dimer, and (6-4) photoproducts at CC without error (106). Humans also contain Polζ like yeast, but Polζ is unlikely participating in TLS in humans (55, 69). However, there is a possibility that Polζ functions as TLS polymerase in humans, because recently it was reported that deletion of Rev3L, a subunit of polζ, increases chromosomal instability in mouse embryonic fibroblasts (MEF) [119]. Yeast TLS polymerases are Polη, Polζ, and Rev1 (76, 106). Polζ is composed of
catalytic subunit (Rev3) and accessory subunit (Rev7), and can only extend nucleotides from the nucleotides inserted opposite DNA lesions by other polymerases such as Rev1 and Polη (69, 76, 106). Rev1 inserts one deoxycytidine monophosphate (dCMP) to the 3’ end of DNA (106).

Postreplication repair (PRR) - K164 of PCNA is poly-ubiquitinated by Rad5 (ubiquitin ligase [E3]) and Mms2-Ubc13 (ubiquitin-conjugating enzyme [E2]). They form a K63-linked poly-ubiquitin chain on mono-ubiquitinated K164, which is formed by Rad6 and Rad18 (4, 28). Mono-ubiquitination of PCNA by Rad6-Rad18 complex is a prerequisite for poly-ubiquitination of PCNA by Rad5 and Mms2-Ubc13 (28).

Rad5 structure contains a really interesting new gene (RING) finger domain responding to E3 activity and DNA-dependent 3’-5’ helicase domain, which is common to the SWI/SNF helicase family. Its RING finger domain is embedded in the helicase domain (28). Rad5 interacts with Ubc13 through its RING finger domain as normal E2 and E3 reactions (110). Rad5 recruits Ubc13-Mms2 to damaged DNA through its interaction with ssDNA, Rad18, and Ubc13 (110). Ubc13 and Mms2 are cytosolic proteins, but are translocated to nucleus when there is DNA damage (110). The helicase domain of Rad5 is important for forming chicken foot structure which is needed for the template switching after polyubiquitination of PCNA (9, 57).

Even though Ubc13 and Mms2 work together as a ubiquitin-conjugating enzyme complex for poly-ubiquitination of PCNA, they have different roles (16). Ubc13 is a true E2 but Mms2 does not have E2 activity. Mms2 has sequence homology to ubiquitin conjugating proteins, but lacks an active Cys (Cysteine) site of E2 (4, 14). However, Mms2 represses the TLS pathway to support the PRR pathway by inhibiting Polζ (13).
**Figure 3. Molecules involved in the DDT pathway and their functions.** When the replication apparatus encounters abnormal DNA structures, the DDT pathway bypasses the lesion possibly resulting in mutations. In the DDT pathway, there are TLS and PRR pathways as described in figure 2. In the TLS pathway, Rad6 (ubiquitin-ligase) and Rad18 (ubiquitin-conjugating enzyme) mono-ubiquitinate PCNA, and recruit TLS polymerases. The TLS polymerases insert several dNTPs opposite the damaged DNA sequences by replacing replication polymerases, Polδ and Polε. Following mono-ubiquitination of PCNA, it can be polyubiquitinated by Ubc13-Mms2 (ubiquitin-conjugating enzyme) and Rad5 (ubiquitin-ligase) to initiate the PRR pathway. Ubc13 is a true ubiquitin-conjugating enzyme, but Mms2 is not. However, Mms2 is equally as important as Ubc13, and directs the PRR pathway by inhibiting TLS polymerase. After polyubiquitination of PCNA, replication blockage is bypassed without mutations. It is because the PRR pathway uses newly synthesized sister chromatid (duplicate of lagging strand) as the template, instead of the DNA damage containing leading strand. Helicase activity of Rad5, Rad50, and other unknown mechanisms play a part in changing the template strands and replication. For the PRR pathway, sumoylation of PCNA inhibits homologous recombination (HR) to prevent GCR. Ubc9 (SUMO-conjugating enzyme) and Siz1 (SUMO ligase) sumoylate PCNA. Sumoylated PCNA recruits a helicase, Srs2, which disrupts homologous recombination filaments and inhibits HR. Modified from Cell Res. 2008 18(1):162-173. Review.
Poly-ubiquitination of PCNA initiates PRR, but little is known about the later step of PRR (4). The PRR pathway uses the newly synthesized sister lagging strand as a template instead of the damaged leading strand. This is called template switching. It is assumed that template switching is accomplished by chicken foot intermediate, which is formed by fork reversal mediated by Rad5 helicase and annealing activity (Figure 2) [9, 57]. A DNA lesion in leading DNA strand blocks the replication and causes the gap between leading and lagging DNA strands. At this point, Rad5 helicase domain unwinds replication fork backwardly and allows template switching without exposing ssDNA, which will cause DNA damage checkpoint activation (9, 57). In this later stage of the PRR pathway, Rad50 which is part of the Mre11-Rad50-Xrs2 (MRX) complex is required (28). It is known that the MRX complex participates in DNA double strand break repair and Rad50 tethers the two severed dsDNA ends (5).

During PRR, SUMO-modified PCNA inhibits recombination to prevent the gross chromosomal rearrangements (16, 78, 114). The K164 residue of PCNA, which is the site for ubiquitination, is also sumoylated with Ubc9 and Siz1. Sumoylated PCNA recruits a 3’ to 5’ helicase, Srs2, which disrupts Rad51 nucleoprotein filaments and prevents recombination (Figure 3) [87, 113].

**Checkpoint proteins** - It is reported that DNA damage checkpoint proteins are involved in the DDT pathway (Figure 3) [7, 56]. Barbour L, et al. (2006), suggested that the Rad18 pathway, which represents the DDT pathway, is composed of Rad9 (DNA damage checkpoint protein), Mms2 (ubiquitin-conjugating enzyme involved in error-free PRR), and Rev3 (a subunit of Polζ which has a role in error-prone TLS) pathways in *S. cerevisiae*. They added the Rad9 pathway to DDT and suggested that checkpoint arrest
may help the PRR pathway to bypass the lesion. This suggestion is based on the following results: rad9Δ is epistatic to rad18Δ only during short exposure of MMS but synergistic to mms2Δ and rev3Δ regardless of the exposure time to MMS, and spontaneous/induced mutation rates of mms2Δ are half reduced by mms2Δrad9Δ, the double mutant (7). Additionally, Kai M, et al. (2007), proved in fission yeast that phosphorylation of Rad9 (Ddc1 in S. cerevisiae) in Thr-255 inhibits Rad51 dependent recombination by stimulating the error-free PRR pathway, and that Rad9 interacts with Mms2 directly. Ddc1 is a DNA damage sensor and activates Mec1.

**Growth of culture**

The growth of yeast culture depends on fermentation and respiration using available carbohydrates. Growth of culture is divided into four phases: exponential phase, diauxic shift, post-diauxic phase, and stationary phase (Figure 4). This is divided by growth rate. The fast growth of cells in the exponential phase slows down after diauxic shift. Finally, during stationary phase, there is no population growth (116).

The phases of culture are related to the glucose content in the medium and energy metabolism of yeast which determines the growth rate. In the exponential phase, yeasts perform fermentation using the abundant glucose in the medium. Fermentation is a glycolysis process and produces ethanol. Glycolysis is an anaerobic catabolic mechanism in which glucose is degraded to pyruvate. Pyruvate is changed to ethanol and ethanol is excluded back to the media. At diauxic shift, glucose is exhausted in the medium and cells start to do aerobic respiration through the TCA cycle in addition to fermentation
Figure 4. Growth curve of typical yeast culture. As cultures get old, their growth rate changes. Depending on the changes in growth rate, the culture is divided into four phases: exponential phase, diauxic shift, post-diauxic phase, and stationary phase. In the exponential phase, there is an abundance of glucose in the media. Yeast generate energy by fermentation and start storing glycogen at this phase. Fermentation produces ATP and ethanol through glycolysis. After diauxic shift, glucose is limited and yeasts start respiration (TCA cycle, the aerobic catabolic process) using ethanol, as well as, perform fermentation using stored glycogen. In diauxic shift, trehalose accumulation begins. Fermentation stops in stationary phase when stored carbon sources (glycogen and trehalose) are depleted. However, respiration continues by using ethanol, the by product of fermentation. From Microbiol Rev. 1993 57(2):383-401. Review.
Yeast can do fermentation even in this stage, because it can convert stored glycogen (polysaccharide) and trehalose (disaccharide) to glucose (37). The glycogen accumulation starts immediately after culturing and peaks at diauxic shift. While, trehalose accumulation begins at the diauxic shift and continues until stationary phase (116). At late G1 (just before Start), glycogen and trehalose are suddenly metabolized to glucose by cAMP pulse, and this sudden flow of glucose triggers respiration as well as fermentation at early S phase, to have energy for the cell cycle (cell growth). After the cell cycle, the remnant energy is stored as glycogen/trehalose in the cytosol (37). When stored carbohydrates (glycogen/trehalose) are exhausted, fermentation is halted, but respiration continues by using ethanol, the byproduct of fermentation (37).

Stationary phase cells have a lot of stresses compared to exponentially growing phase cells. They face starvation (lack of carbon, nitrogen, sulfur, phosphorus, etc.), lack of oxygen, and oxidative stress. Nutrients and oxygen are exhausted and oxidative stress is generated because of respiration. Therefore, stationary phase cells are different from other phase cells.

Stationary phase cells are nonproliferating, G0 cells. Stationary phase cells are characterized to have thick and less porous cell walls, more round and numerous mitochondria, accumulation of proteases in various cellular compartments, accumulation of electron-dense materials in vacuoles, characteristically folded chromosomes, accumulation of trehalose and glycogen, abundant lipid vesicles, decrease in phospholipid synthesis, increase in triacylglycerol synthesis, tremendous reduction of mRNA and protein synthesis, and resistance to heat shock stress (11, 116).
There are proteomic studies on different stages of cultures. Trabalzini L, et al. (2003), observed that 54 proteins (have roles in protein synthesis and RNA turnover, carbohydrate metabolism related proteins) are increased and 33 proteins (have roles in protein folding/cell stress, carbohydrate metabolism, protein synthesis, and degradation) are induced in two day cultures compared to the log phase in the wine strain of *S. cerevisiae*. Kusch H, et al. (2008), did a similar study using *Candida albicans*, a conditional pathogenic fungus in humans. These results explain that there are increased environmental stresses and lack/altered carbon sources in older cultures.

**Filamentation in *S. cerevisiae***

It has been noted that *S. cerevisiae* changes its morphology from ovoid to filamentous, and *vice versa* depending on environmental conditions. Filamentation, which is characterized by elongated, branched, and several unipolar attached cells, is observed in both diploid and haploid cells (122).

The budding patterns are different between oval and filamentous forms, and they decide the polarity of filamentation. In oval growth (yeast-form), the haploids form a new bud near the site where the mother has been budded from its mother. This is called axial budding. Ax11 has pivotal roles for it. Conversely, diploid oval cells form a new bud opposite the site where the mother was budded in a previous generation. This is called bipolar growth (20). Interestingly, these budding patterns disappear in filamentous growth. Inspite of the absence of specific budding patterns, filamentous growth requires several yeast-form budding site selection (cell polarity) proteins such as: Bud8, Bud5, Spa2, Pea2, and Bud6 (121).
**Figure 5. Signal cascades for filamentation.** Nitrogen starvation or HU treatment (HU treatment causes slowed DNA synthesis and replication fork arrest) induces filamentation through these signal cascades. Cdc42 is the converging point of signal cascades induced by both stimulants. Cdc42 induces filamentation through mitogen-activated protein kinase (MAPK) and other unknown mechanisms. Nitrogen starvation activates Ras which is a general stress responding GTPase. Ras activates Cdc42 directly and also through cyclic AMP (cAMP)/protein kinase A (PKA). cAMP activates PKA, and PKA activates Swe1. Swe1 activates MAPK. Swe1 and MAPK induce the activation of Cdc42 by inhibiting mitotic cyclin-dependent kinase (CDK-Clb2/Cdc28). Swe1 phosphorylates and inhibits Cdc28 and causes G2 delay. Cdc42 also activates MAPK (Kss1) by forming a complex with Ste20. MAPK activates transcription factors, Ste12/Tec1, which bind to FRE (filamentous responsive element) and induce transcription of genes involved in filamentation. HU treatment activates Cdc42 through Mec1/Rad53 DNA damage checkpoints. Mec1/Rad53 directly and indirectly (through Swe1) activates Cdc42. However, Swe1 is not necessary for slowed DNA synthesis induced filamentation. HU treatment induced Cdc42 activation causes filamentation through unknown pathways. The dotted line represents an unnecessary pathway. Modified from Yeast. 2005 22(13):1069-77.
Filamentation of yeasts can be a potential model system for eukaryotic differentiation, because first, filamentation and differentiation share mitogen-activated protein kinase (MAPK) and cyclic adenosine monophosphate (cAMP) pathways (52), and second, filamentation is formed through inhibition of CDK (Clb2/Cdc28) which is a conserved cell cycle protein complex (122). Additionally, Cdc42 which is required for all filament inducing pathways plays a part in differentiation of higher eukaryotes as well (122).

It is said that filamentation is needed to forage nutrients and avoid toxins because it is induced by lack of nitrogen, short of glucose, isoamyl alcohol (and other by-products of fermentation [\textit{n}-butanol, \textit{n}-hexanol, ethanol]), mild heat stress (37°C), osmotic stress (1 M NaCl), mating pheromones, and the presence of compounds that affect the organization of lipids of the cell membrane or the glucan structure of the cell wall (21, 72). Consistent with the concept that filamentation is needed to forage for nutrition, endocytosis related genes are required for the formation of filamentation after nitrogen starvation, HU treatment, and butanol treatment. Endocytosis is a means for taking in nutrients from the environment (121).

There are additional reports that filamentation can be formed by slowed down DNA synthesis (54). When \textit{S. cerevisiae} is treated with several agents that slow down DNA synthesis, such as HU, ara-CMP (analog of dCMP), and MMS, yeast forms filamentations through important DNA damage checkpoint proteins, Mec1/Rad53 (54).

Nitrogen starvation, isoamyl alcohol, and slowed DNA synthesis commonly induce filamentation, but the signal cascades for them to trigger the filamentation are different (121). However, Wu, X. and Jiang YW (2005), connected all the known
filamentation inducing pathways (Figure 5). This figure shows that Cdc42 is the converging point for all filamentation inducing signal cascades. Mec1/Rad53, Ras2, and Clb2/Cdc28 are connected to Cdc42, and cAMP/PKA is connected to Cdc42 and MAPK.

A Rho-type GTPase, Cdc42, is an important protein for actin-dependent morphogenesis (52). It is the upstream protein for bud site selection (polarization), shmoo (mating-projection) formation, cytokinesis, as well as filamentation (52). Cdc42 induces filamentation through MAPK activation and the unknown mechanisms (121). MAPK activation induced filamentation is as follows: Cdc42 activates Ste20/14-3-3 and Ste20/14-3-3 triggers activation of the MAPK cascade (Ste11/Ste7/Kss1). Finally, Kss1 (MAP kinase) activates transcription factors Ste12 and Tec1, which transcribe filamentous responsive element (FRE) containing genes (72). HU treatment which causes replication fork arrest and slowed DNA synthesis does not induce filamentation through MAPK but Cdc42 (54). Because slowed DNA synthesis induces filamentation through unknown mechanisms, further studies of this pathway are necessary.
II. MATERIALS & METHODS

List of S. cerevisiae strains used

The yeast strains used are derived from BY4741, BY4742, BY4744, BY4744-3A or BY4744-3C genetic backgrounds. The strains of BY4741, BY4742, BY4744-3A and BY4744-3C backgrounds are haploids, and strains of BY4744 background are diploids. The strains of BY4744 background are constructed by mating BY4741 and BY4742 strains in our laboratory. It is named BY4744 to distinguish it from the commercially available diploid strains of BY4743, which are also constructed by mating BY4741 and BY4742 strains. The haploids of BY4744-3A and BY4744-3C backgrounds are obtained from the diploid strains of BY4744.

Each deletion strain is constructed by replacing specific genes with kanMX4. KanMX4 gives resistance to geneticin (G418) and is inserted for selection of gene deletion. The symbol, :: represents a gene insertion, and Δ the deletion of a gene. Therefore, AΔ::B means A gene is deleted by replacing A gene with B gene. RNR3-GFP strain represents a GFP tagged RNR3 gene containing strain. Each background has different auxotrophic markers for selection. Auxotrophy is generated by the deletions of the specific amino acids synthesizing enzymes. For example, leu2Δ0 cannot produce leucine because LEU2 encoding β-isopropylmalate dehydrogenase (IMDH), which catalyzes the third step in the leucine biosynthesis pathway, is completely deleted. MATα
represents mating type α, and MATα mating type α. The strains used here were purchased from Open Biosystem or constructed in our laboratory.

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**Measurement of spontaneous mutation rates**

Nine parallel cultures were made by inoculating 2000 cells in 4 ml YPD (yeast extract [1%], peptone [2%], dextrose [2%]) liquid medium. The cultures were incubated in a 30°C shaker at 75 rpm until the titer of the culture reached at least $1 \times 10^8$ cells/ml. It took two to three days depending on the type of strain inoculated. From each of the nine cultures, $2 \times 10^7$ cells were taken and plated on nine canavanine containing plates (can+ plates, the concentration of canavanine was 60 mg/l). For standardization, 100 µl from a $10^{-5}$ time culture dilution was plated on a canavanine minus plate (can- plate). The composition of can- plate was agar (1.4%), dextrose (2%), $1 \times$ yeast nitrogen base (YNB: w/o amino acid, w/o ammonium sulfate, w/o carbohydrate), and $1 \times$ complete supplement mixture minus arginine (CSM-Arg) [Sunrise science]. The plates were incubated at 30°C until formation of colonies. Colonies were formed within three to four days. Colony numbers from each can+ plates and can- plate were determined. Colony numbers from nine can+ plates were used to determine the median number and the following calculations were performed.

**Spontaneous Median Mutant Frequency**  =  

Median titer of colony forming cells on can+ plate 

/ Titer of colony forming cells on can- plate
(Titer = cell number [No.]/ml,

Median titer of colony forming cells on can+ plate

= [1000 × Counted median colony No. on can+ plate] / Plated volume in µl on can+ plate,

Titer of colony forming cells on can- plate = $10^6 \times$ Counted colony No. on can- plate )

Spontaneous Mutation Rate =

Spontaneous Mutational Events / Cell / Generation (M) = $m / 2cw$

( $m = r_0 / r_0/m$  
  $r_0$ : Median titer of colony forming cells on can+ plate  
  $r_0/m$ : Taken from table 3, Lea and Coulson, 1949,

c : Median titer of colony forming cells on can- plate,

w : Residual growth index, We did not consider w since our final goal was to obtain the ratio of spontaneous mutational rates, and w from all strains was considered the same. )

Ratio of Spontaneous Mutational Rate to WT = Mutant M / Wild Type M
**Polymerase chain reaction (PCR) procedure**

Polymerase chain reaction (PCR) was performed in a reaction mixture of 50 µl. The mixture was composed of yeast DNA (5 µl), forward primer (4 µM), reverse primer (4 µM), dNTP (0.3 mM), MgCl₂ (2, 3, or 4 mM), Taq DNA polymerase (2.5 U), and 10 × PCR buffer (5 µl). The denaturing temperature before the PCR cycle was 95°C and the duration was 5 minutes. The PCR cycle was set to a denaturing temperature (95°C) for 30 seconds, annealing temperature (52°C or 54°C) for 30 seconds, and extension/polymerization temperature (72°C) for 3 minutes. After 35 PCR cycles, there was an extension period for 3 minutes at 72°C. To verify PCR product, gel electrophoresis was performed using agarose gel (0.6%) containing ethidium bromide (EtBr [0.2%]).

**Isolation of yeast chromosomal DNA for PCR**

A loopful of cells was suspended with 180 µl of 50 mM Tris/50 mM ethylenedinitrilotetraacetic acid (EDTA), and 20 µl of 10% sodium dodecyl sulfate (SDS). Half of the suspension volume of zirkonium beads was added to the cell suspension. To break down the cell wall, the mixture was vortexed vigorously 3 times (30 seconds each). After addition of 300 µl of sterile water, DNA was extracted with 500 µl of phenol:chloroform (1:1) at 13,000 rpm for 5 minutes. The aqueous phase was transferred to a new tube and re-extracted with 500 µl of chloroform. After centrifugation, the aqueous phase was transferred to new tube. 50 µl of 3 M sodium acetate (pH 5.2) and 1 ml of 100% ethanol were added to the aqueous phase. DNA was precipitated for 10 minutes at 13,000 rpm, and the dried pellet was dissolved in 500 µl of Tris/EDTA (TE
[pH 8.0]). The solution was incubated at 37°C for 1 to 3 hours after 1 µl of RNase was added. Once again, DNA was extracted with 500 µl of chloroform by spinning for 5 minutes at 13,000 rpm. The aqueous phase which contained yeast chromosomal DNA was transferred to a new tube.

**Lithium acetate (LiAc) transformation**

Log phase culture was obtained by inoculating \(2.5 \times 10^8 (5 \times 10^6 \times 50)\) cells from overnight culture in a new 50 ml YPD, and incubating for 4 to 5 hours at 30°C. After incubation, cell number reached between \(6 \times 10^6\) cells/ml and \(1 \times 10^7\) cells/ml. Cells were harvested for 5 minutes at 3,000 rpm, and washed once with sterile water. Cells were resuspended with 1 ml of 100 mM LiAc and transferred to a new microcentrifuge tube. Cells were centrifuged at 14,000 rpm for 15 seconds and resuspended with 400 µl of 100 mM LiAc. 50 µl of cell suspension was taken and centrifuged. Polyethylene glycol (PEG [33.33 %]), LiAc (0.1 M), denatured Salmon-sperm DNA (0.278 µg/ml) and PCR product (0.1-10 µg) were added to the pellet. The total volume was adjusted to 360 µl with water. Cells were completely mixed by vortexing vigorously. The tube was incubated at 30°C for 30 minutes, and cells were heat shocked at 42°C for 30 minutes. Cells were centrifuged at 8,000 rpm for 15 seconds. For auxotrophic marker selection, cells were resuspended with 1 ml of sterile water, and 200 µl of cell suspension was plated on a CSM-specific amino acid(s). For G418 selection, after cells were incubated in liquid YPD for 5 to 6 hours, 200 ul of cell suspension was plated on the G418 (200 mg/l) YPD plate. This method is followed by Agatep R, et al. (1998)’s protocol.
**Method for transferring of a yeast gene deletion**

Total DNA was isolated from a gene knock out strain which had a deletion of a gene of interest. The ORF of the gene was replaced by kanMX4 which gives resistance to G418. Using the isolated DNA template and specific forward/reverse primers for the gene of interest, PCR was performed. The PCR product was transformed to a yeast strain by LiAc transformation to delete the gene of interest (previously described). After transformation, the cells transformed with the PCR product were selected using G418 selection plates. To check if the gene of interest is specifically replaced, DNA from G418 selected transformants was isolated, and PCR was performed using primers whose sequence is the further up-stream sequence of the forward primer sequence used for deletion, or the further down-stream sequence of the reverse primer sequence used for deletion. The knock out of a gene was verified by the size of the PCR product. Gel electrophoresis was performed using agarose gel (0.6%) containing EtBr (0.2%).

**Observation of cell cycle progression from G\textsubscript{1} phase after ultraviolet (UV) irradiation**

Log phase culture was obtained by inoculating $2.5 \times 10^8 \ (5 \times 10^6 \times 50)$ cells from overnight culture in 50 ml of YPD, and incubating for 4 to 5 hours at 30°C. After incubation, cell number would reach between $6 \times 10^6$ cells/ml and $1 \times 10^7$ cells/ml. 10 ml of cells were centrifuged at 3,000 rpm for 5 minutes, and resuspended with 10 ml of YPD. 50 µl of α-factor (1 mg/ml) was added. After cells were incubated at 30°C for 1 hour and 15 minutes, 50 µl of α-factor (1 mg/ml) was again added and the cells were incubated for another 30 minutes. After the formation of “shmoos” (the sign of G\textsubscript{1} arrest by
pheromone) were checked under the microscope, cells were centrifuged at 3,000 rpm for 5 minutes and washed once with sterile water. Cells were resuspended with 8 ml sterile water. 4 ml of cell suspension was transferred to a sterile petridish (diameter: 6 cm) containing a sterilized stir bar. Cells were irradiated with 254 nm UV (80 J/m²) while stirred. 4 ml of irradiated and 4 ml of non-irradiated cells were centrifuged and resuspended with 4 ml of YPD separately. The titer of cells was approximately $1 \times 10^7$ cells/ml. The percentage of budding cells ($[[\text{No. of budding cells/Total No. of counted cells}] \times 100]$) was determined under the microscope every 10 to 15 minutes during incubation at 30°C.

**Observation of cell cycle progression from S phase after UV irradiation**

Log phase culture was obtained as described above. 5 ml of cells were centrifuged for 5 minutes at 3,000 rpm, and resuspended with 5 ml of YPD containing HU (0.2 M). After a 2 hour and 30 minute incubation, cells were centrifuged and washed with sterile water. S phase arrested cells were resuspended with 1 ml of YPD. After 10 seconds of sonication, cells were streaked on YPD plates and UV irradiated or not. Before and during the incubation of the plates at 30°C, the percentage of microcolonies ($[[\text{No. of colonies composed of more than two cells among counted colonies/No. of total counted colonies}] \times 100]$) was determined under the microscope every hour.

**Observation of cell cycle progression from G2/M phase after UV irradiation**

Log phase culture was obtained as described above. 5 ml of cells were centrifuged for 5 minutes at 3,000 rpm and resuspended with 5 ml of YPD containing nocodazole (10
µg/ml). Cells were collected after 3 hours of incubation. Cells were washed once with sterile water and resuspended with 1 ml of YPD. Cells were streaked on YPD plates and UV irradiated. The percentage of microcolonies was determined at one hour intervals as described above.

Collecting samples for the observation of cell cycle progression from G₁ phase after 4-Nitroquinoline 1-oxide (4-NQO) treatment

Log phase culture was obtained as described above. 20 ml of cells were centrifuged for 5 minutes at 3,000 rpm and resuspended with 20 ml of YPD. The suspension was incubated for 1 hour and 15 minutes at 30°C, following the addition of 100 µL of α-factor (1 mg/ml). After the culture was divided in half, another 50 µl of α-factor (1 mg/ml) was added to the two cultures, and 100 µl of 4-NQO (100 µg/ml) or 100 µl of YPD (control) was added to the cultures. After the cultures were incubated for another hour (cells were G₁ arrested) at 30°C, they were centrifuged for 5 minutes at 3,000 rpm. Cells were washed with sterile water once and resuspended with YPD. Before and during incubation, 1 × 10⁷ cells were collected and fixed with 100% ethanol every hour and were analyzed by fluorescence activated cell sorting (FACS).

Collecting samples for the observation of cell cycle progression from G₁/G₀ phase after 4-NQO or UV treatment

The G₁/G₀ phase arrested WT and hug1Δ were obtained by culturing for 7 days. In order to observe cell cycle progression from G₁/G₀ arrest, 7 day cultures were diluted with YPD to 1 × 10⁷ cells/ml and incubated. Cells were collected at appropriate intervals
during incubation. To detect the progression of cell cycle from G₁/G₀ arrest, FACS analysis was performed with the collected samples. For 4-NQO treatment, 7 day cultures were treated with 4-NQO (4 µg/ml) for 30 minutes or 3 hours before dilution. After treatment, 4-NQO treated 7 day cultures were washed once with sterile water, diluted with YPD to yield a 1 × 10⁷ cells/ml titer, and then incubated until collection. For UV treatment, 3.75 × 10⁸ cells from 7 day cultured cells were centrifuged, resuspended with 15 ml of sterile water, and UV irradiated (80 J/m²) while being stirred with a sterilized magnetic bar. UV irradiated cells were centrifuged, resuspended with YPD at 1 × 10⁷ cells/ml, and incubated. Before and during incubation, 1 × 10⁷ cells were collected and fixed with 100% of ethanol every hour and were analyzed by FACS.

**Preparation of samples for FACS analysis to detect cell cycle distribution by DNA content**

Before or during incubation, 1 × 10⁷ cells were collected and washed once with sterile water. Cells were fixed by adding 100% ethanol to the pellet and thoroughly vortexing to avoid cell clumps. Cells were stored at 4°C. When all the samples were ready, cells were extensively vortexed and centrifuged for 1 minute at 14,000 rpm. After cells were washed one time with 1 ml of water, they were resuspended with 1 ml of 50 mM sodium citrate (pH 7.0). The sample solution was transferred to FACS tubes and 8 µl of 10 mg/ml DNase-free RNase A was added and followed by incubation for 1 hour at 50°C. After this stage, samples were stored overnight at 4°C. After addition of 25 µl of 10 mg/ml proteinase K, the suspension was incubated for 1 hour at 50°C, then 1 ml of 2 µg/ml propidium iodide was added. FACS tubes had to be protected from light after the
addition of propidium iodide. Just before FACS analysis, samples were sonicated and vortexed briefly. FACS was performed using Beckman coulter cytomics FC 5000 flow cytometry analyzer. The data was analyzed by CXP2.2 analysis software.

**Preparation of samples for FACS analysis to detect green fluorescence protein (GFP) levels**

Before or during incubation, $1 \times 10^7$ cells were collected and washed once with sterile water. Cells were fixed by adding 100% of ethanol to the pellet and vortexing thoroughly. Until the collection of all the samples was complete, the fixed cells were stored at 4°C. Before FACS analysis, samples were sonicated and vortexed briefly.

**Measurement of the levels of Rnr3 and Rnr4 following HU treatment**

Overnight cultures of WT (without GFP tagged protein) and WT (with GFP tagged protein) and hug1Δ (with GFP tagged protein) were diluted to $5 \times 10^6$ cells/ml (total volume was 50 ml) and cultured until cells reached between $6 \times 10^6$ cells/ml and $1 \times 10^7$ cells/ml. The log phase cultures were treated with HU (100 mM) for 1 hour at 30°C. After treatment, cells were washed once, resuspended with 10 ml of YPD to $1 \times 10^7$ cells/ml, and incubated. Before and during incubation, $1 \times 10^7$ cells were collected for FACS analysis to detect the GFP level.
Isolation of uvs mutation from srl3Δuvs

srl3Δuvs (BY4741; MATa, his’, leu’, met’, ura’) and WT (BY4742; MATα, his’, leu’, lys’, ura’) were mated by cross-replica plating the streaks from both strains on met’ and lys’ synthetic plates (Figure 26). On the met’ and lys’ plate, only mated strains (diploid) could survive because BY4741 cannot survive without Met and BY4742 cannot survive without Lys. The diploids were streaked on the pre-sporeulation plate and incubated for 1 or 2 days. The diploids on the pre-sporeulation plate were re-streaked on the sporulation plate and incubated until tetrads could be observed. In order to help the sporulation, plate was kept at 4°C for 5 hours and incubated again at 30°C overnight. The pre-sporeulation plate was composed of peptone (2%), yeast extract (1%), dextrose (2%), and agar (1.4%). The compositions of the sporulation plate were potassium acetate (1%), His (0.77 g/l), Leu (0.69 g/l), Ura (0.77 g/l), and agar (1.4%). A loopful of tetrads was treated with zymolyase (1 mg/ml) for about 20 minutes at room temperature to digest the cell wall, and then the solution was diluted with sterile water. Tetrads were dissected under the microscope.

Sensitivity tests

As described in the figures, log phase cells, 1 day cultures, and 7 day cultures were used. For UV irradiation, cells were plated on YPD plates and irradiated with UV. The doses given to cells are designated in the figures. For streptonigrin, MMS, 4-NQO, and tert-Butyl hydroperoxide (t-BH) treatment, liquid culture was treated for 30 minutes, 30 minutes, 1 hour, and 3 hours, respectively. The concentrations given to cells are designated in the figures. Cells were washed once with sterile water, and then untreated
and treated cells were plated on separate YPD plates. For heat shock, differently diluted cells were heat shocked for several minutes at 52°C. The treatment times are designated in the figures. The same volumes of heat treated/untreated cell dilutions were then plated on YPD plates. Plates were incubated at 30°C until surviving cells formed colonies (about 3 to 4 days). Colonies in each plate were counted and the surviving fraction was calculated. Surviving fraction is defined as titer of colony forming cells with treatment divided by titer of colony forming cells without treatment (Surviving fraction = titer of colony forming cells with treatment / titer of colony forming cells without treatment).

**Western blotting**

Cell pellets were washed once with 20% TCA and resuspended with 200 µl of 20% TCA. Cells were lysed by vortexing with zirconium beads for 20 minutes at 4°C. Cell lysates were collected and centrifuged for 10 minutes at 3,000 rpm at room temperature. Pellets were resuspended with 200 µl of 1 × SDS loading buffer, and then 100 µl of 1 M Tris base was added. After 3 minutes of boiling and 5 minutes of centrifugation at 14,000 rpm, protein samples were loaded. Samples were run on a acrylamide gel (10%) at 175 V until they had passed through the stacking gel, then the voltage was increased to 200 V. Proteins were transferred to a nitrocellulose membrane using Semi-dry transfer method for 45 minutes at 15 V. Rabbit peroxidase anti-peroxidase soluble complex (Sigma) primary antibody (1:500 dilution) and Donkey anti-rabbit immunoglobulin G (IgG) secondary antibody (1:5000) were used to detect tandem affinity purification (TAP) tag. Membranes were treated with primary antibody overnight.
and with second antibody for 1 hour. Membranes were exposed to film after treatment with Immun-Star™ HRP Lumino/Enhancer (Bio Rad).

**Filamentation and growth under conditions of slowed DNA synthesis**

Filamentation was induced by plating logarithmic phase cells on YPD plates containing HU (100 mM). The YPD plates were composed of peptone (2%), yeast extract (1%), dextrose (0.2%), and agar (1.4%). The YPD plates not containing HU were used for control experiments. Filamentation was observed after 12 hours at 30°C with the light microscope at 10× objective. Pictures were taken with an Ocular lens camera connected to imaging software (Dinocapture).

**Isolation of total RNA**

Cells were harvested after 12 hours of incubation on YPD plates containing 0.2% dextrose, 2% dextrose, or 0.2% dextrose + 100 mM HU. For collection, cells were scraped using sterile water. Cells were centrifuged at 3,000 rpm for 5 minutes, washed one time with sterile water, and then stored at -80°C overnight. 2 × 10⁸ cells were resuspended in 400 µl of TEB (Tris-HCl [10 mM, pH 7.5], EDTA [10 mM, pH 8.0], SDS [0.5%]), and then 400 µl of 65°C heated acid phenol and zirkonium beads were added. Cells were lysed with a Bead beater 3 times (30 seconds each). The lysates were incubated at 65°C for 30 minutes. During the incubation, lysates were occasionally vortexed. After incubation on ice for 5 minutes, the lysates were centrifuged for 5 minutes at 15,000 rpm (4°C). The upper aqueous phase was re-extracted with 400 µl of acid phenol for 5 minutes and centrifuged again. The upper aqueous phase was re-
extracted with 400 µl of chloroform and centrifuged for 5 minutes at 15,000 rpm (4°C). The upper aqueous phase was applied to RNeasy midi column (Qiagen) and centrifuged for 5 minutes at 3,000 × g. The column was washed once with 4 ml RW1 buffer (Qiagen), and then with 2.5 ml and 5 ml of RPE buffer (Qiagen). Total RNA was eluted with RNase-free water by centrifuging for 3 minutes at 3,000 × g. The concentration and quality of total RNA was confirmed by Affymetrix facility staff at the University of Texas Southwestern Medical Center at Dallas.

**Analysis by gene arrays and comparison profiles**

Transcript analysis was performed and the five comparison profiles were generated by the Affymetrix facility staff. The five comparison profiles are the hug1Δ/WT (2% dextrose), the WT HU/WT (0.2% dextrose), the hug1Δ HU/ hug1Δ (0.2% dextrose), and the hug1Δ HU/WT HU (0.2% dextrose). The hug1Δ/WT (0.2% dextrose) comparison profile shows the transcript level differences between hug1Δ and WT, grown on 0.2% dextrose containing YPD plate. The hug1Δ/WT (2% dextrose) comparison profile shows the transcript level differences between hug1Δ and WT, grown on 2% dextrose containing YPD plates. The WT HU/WT (0.2% dextrose) comparison profile shows the transcript level differences between WT grown on 100 mM HU + 0.2% dextrose containing YPD plate and WT grown on 0.2% dextrose containing YPD plate. The hug1Δ HU/hug1Δ (0.2% dextrose) comparison profile shows the transcriptional level differences between hug1Δ grown on 100 mM HU + 0.2% dextrose containing YPD plate and hug1Δ grown on 0.2% dextrose containing YPD plate. The hug1Δ HU/WT HU (0.2% dextrose) comparison profile shows the transcript level differences between hug1Δ
grown on 100 mM HU + 0.2% dextrose containing YPD plate and WT grown on 100 mM HU + 0.2% dextrose containing YPD plate. The samples were all harvested after 12 hours at 30°C. The profiles are on http://sites.google.com/site/siedelab.

**Cluster analysis within transcription profiles through group assignment**

The transcription identities (ID)s were assigned to the gene symbols in www.affymetrix.com. In www.affymetrix.com, batch query was performed with Yeast2 information and the selected transcription IDs to obtain gene symbols for each transcription ID. We considered the level of transcript differences in comparison profiles as significant if the difference was above +2.83 (+log_2 1.5) or below -2.83 (-log_2 1.5).

Therefore, only transcription IDs whose transcript level difference was above +log_2 1.5 or below -log_2 1.5 were selected from each comparison profile. (The transcript level above +log_2 1.5 in hug1Δ/WT [0.2% dextrose] means that the mRNA level of a gene in hug1Δ grown on 0.2% dextrose containing YPD plate is 2.83 fold higher than that of the gene in WT grown on the same plate.) The gene symbols obtained from batch query were used for grouping (by process or function) in www.yeastgenome.org (GO Term Finder). Groups that were too broad or repetitive were deleted. Grouping results are in table 3 and http://sites.google.com/site/siedelab.

**Obtaining % of stress responding and filamentation related genes from comparison transcript profiles**

All stress responding genes were selected from genes whose transcript level changes were above +2.83 (+log_2 1.5) fold or below –2.83 (-log_2 1.5) fold in the
hug1Δ/WT (0.2% dextrose) comparison profile, and the percentage of stress responding genes was calculated (Figure 17). All the filamentation related genes were selected among genes whose transcript level changes are above +4 (+log₂) fold or below -4 (–log₂) fold in the WT HU/WT (0.2% dextrose), the hug1Δ HU/hug1Δ (0.2% dextrose), and the hug1Δ HU/WT HU (0.2% dextrose) comparison profiles, and the percentages of the filamentation related genes were calculated (Table 1).

The stress responding genes and filamentation related genes used in the calculations were selected as follows. The stress responding genes included the genes involved in amino acid import, monosaccharide import, hexose import, disaccharide import, oxidative stress, ethanol & glycerol & fatty acid utilization, heat, cold, salt, drug resistance, ion homeostasis, stationary phase, and aerobic respiration. However, mitochondria structure and pheromone related genes are excluded. The filamentation related genes included the genes participating in cell wall organization, cytoskeleton organization, pathogenicity, invasiveness, and budding.
III. RESULTS

1. Studies on the role of Hug1 in DNA damage and stress responses

The following parameters were tested to determine the function of Hug1: 1) the involvement of Hug1 in the recovery from DNA damage checkpoint arrest, 2) the involvement of Hug1 in controlling the protein levels of Rnr3 and Rnr4, 3) the sensitivity of differently aged cultures of hug1Δ to UV, 4-NQO, t-BH, and heat shock, 4) the cell cycle distribution of 1 day cultured hug1Δ, 5) filamentation and growth of hug1Δ under conditions of slowed DNA synthesis, 6) mRNA expression profile of hug1Δ with or without HU treatment, and 7) the comparison of phenotypes among hug1Δ, sml1Δ, and dif1Δ.

HUG1 deleted strain is not different from WT in the cell cycle recovery from DNA damage induced G1 and G1/G0 phase checkpoint arrest. HUG1 deletion does not influence the protein level of Rnr3 and Rnr4 after HU treatment and during the recovery from HU treatment. HUG1 deleted strain has more resistance to oxidative and heat shock stresses, forms less filamentation induced by slowed DNA synthesis stress, is arrested in G1/G0 phase earlier, and has higher transcript levels of many stress responding genes than WT. It was assumed in literature that Hug1 is a negative regulator of dNTP pool (8, 68, 120). Our results show that hug1Δ has the same phenotypes as sml1Δ and dif1Δ. We
think that the phenotypes of hug1Δ observed in this study are due to the increased dNTP pool and Hug1 influences Rnr2/Rnr4 distribution or activity (but not protein levels).

The expression of Hug1 after HU or 4-NQO treatment

*Hug1 is detectable after HU or 4-NQO treatment.* It is already known that DNA damaging and replication fork arresting agents induce the transcription of HUG1 to a detectable level (8). Additionally, the induction of the Hug1 protein is also observed after HU treatment (8). Because we had observed the hug1Δ sensitivity with 4-NQO (Figure 10B), we wanted to determine if Hug1 is expressed after 4-NQO treatment like it is after HU treatment (Figure 6). The Hug1 protein level was monitored by performing western blot using a tandem affinity purification (TAP) tagged HUG1 containing WT. WT, containing HUG1 without a TAP tag, was used as the control. Hug1 protein is detected after HU or 4-NQO treatment, but is undetectable when there was no treatment (Figure 6).

The influence of HUG1 deletion on the cell cycle progression and on the cell cycle recovery from DNA damage checkpoint arrest

*The hypothesis that Hug1 is required for the (transcriptional) recovery from checkpoint arrest was tested.* Because Hug1 is a very small peptide, it has been impossible to detect it as an ORF. Basrai MA, et al. (1999), recognized Hug1 as an ORF by the serial analysis of gene expression (SAGE) technique from nonannotated open reading frames (NORFs), and studied extensively on Hug1. They suggested that Hug1 might have a role in the transcriptional recovery from checkpoint arrest (8). This hypothesis is withdrawn by the facts that 1) sml1 or crt1 mutants also rescue mec1Δ.
Figure 6. Hug1 expression after HU (100 mM or 200 mM) or 4-NQO (0.1 ug/ml) treatment. Western blot was performed to see the expression of Hug1 using WT which contains TAP tagged HUG1. The anti-TAP primary antibody was used to detect the expression of Hug1-TAP. Even though the protein is unknown, the highest band in each lane is considered the loading control. WT (without any tag) is the negative control. Hug1 expression is observed only after HU (100 mM or 200 mM) or 4-NQO (0.1 ug/ml) treatment.
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![Image of gel electrophoresis results](image-url)
lethality like hug1Δ; Sm11 and Crt1 are the negative regulators of RNR which increases the dNTP pool through Mec1-Rad53-Dun1 signaling, 2) overexpression of RNR1 or RNR3 rescues mec1Δ lethality; overexpression of RNR1 or RNR3 increases the dNTP pool, and 3) transcriptional induction of HUG1 is later than that of RNR3; HUG1 and RNR3 are transcriptionally induced only after DNA damage or replication fork arrest (8). After DNA damage repair, the down regulation of dNTP pool is a part of the recovery mechanisms from DNA damage checkpoint arrest for further cell cycle progression to occur (25). Therefore, the hypothesis of Basrai MA, et al. (1999) might mean that Hug1 is a feedback inhibitor for the DNA damage reactions including decrease of dNTP pool for further cell cycle progression (for the recovery from DNA damage checkpoint arrest). However, they did not give any clue on their statement, ‘transcriptional recovery’. To test the hypothesis that Hug1 has a role in the (transcriptional) recovery from checkpoint arrest, cell cycle progression of G1 and G1/G0 phase arrested WT and hug1Δ was observed with or without treatment of DNA damaging agents.

There are no differences between WT and hug1Δ in cell cycle progression without any treatment of DNA damaging agents. The cell cycle progression of G1 or G1/G0 arrested WT and hug1Δ were observed without any treatment of DNA damaging agents (Figure 7A, 8A). These studies were conducted as a control study for the cell cycle recovery from G1 or G1/G0 phase checkpoint arrests by DNA damaging agents (4-NQO or UV) [Figure 7B, 8B]. G1 arrest was induced by α-factor and G1/G0 arrest was by 7 days of culture. FACS analysis was performed to analyze cell cycle progression. There are no differences in cell cycle progression between G1 arrested WT and hug1Δ without any treatment (controls) [Figure 7A]. Until WT and hug1Δ reach the G2 phase, the cell
Figure 7. The effect of HUG1 deletion on cell cycle progression of G₁ synchronized cells with/without DNA damage checkpoint arrest. G₁ arrest is induced by α-factor. Cell cycle progression of G₁ arrested WT and hug1Δ with/without 4-NQO treatment are compared. After treatments (α-factor only, or α-factor and 4-NQO), cells were washed and incubated in YPD. To detect cell cycle progression, cells were collected in the same intervals during incubation, and fixed with ethanol. FACS was performed with the fixed samples after cells were stained with propidium iodide. A. The cell cycle progression of G₁ arrested WT and hug1Δ were observed without 4-NQO treatment. B. The cell cycle progression of G₁ arrested WT and hug1Δ were monitored after 4-NQO treatment. Cell cycle progression after 4-NQO treatment represents the cell cycle recovery from G₁ phase DNA damage checkpoint arrest.
cycle progression of both strains are the same. After 25 min without α-factor, both types of cells reach G\textsubscript{2} phase at the same time. There are also no differences between G\textsubscript{1}/G\textsubscript{0} arrested WT and hug1Δ in the cell cycle progression when no treatment had been added (Figure 8A). After 1.5 hours of incubation in new YPD, both types of cells re-start the cell cycle progression from 7 day induced G\textsubscript{1}/G\textsubscript{0} arrest. After 2 hours of incubation, both types of cells showed normal cell cycle distribution. Therefore, it is concluded that Hug1 does not play a role in the cell cycle progression from G\textsubscript{1} as well as G\textsubscript{1}/G\textsubscript{0} arrest.

_Treatment of 4-NQO or UV triggers the checkpoint arrest in G\textsubscript{1} or G\textsubscript{1}/G\textsubscript{0} arrested WT and hug1Δ._ Checkpoint proteins sense cellular damage/improper cellular mechanisms, and trigger protection mechanisms (for example, cell cycle arrest, repair mechanisms, and cell death). It is known that 4-NQO or UV treatment damages DNA and triggers the activation of DNA damage checkpoints. DNA damage checkpoints arrest the cell cycle until the damage is repaired (122). In our data, delayed cell cycle progression is observed after 4-NQO or UV treatment, when cell cycle progression of treated cells (WT and hug1Δ) is compared with that of untreated cells (Figure 7, 8). When there is no treatment, G\textsubscript{1} arrested cells take 15 minutes to start cell cycle progression (Figure 7A). However, 4-NQO treatment delays cell cycle progression of G\textsubscript{1} arrested WT and hug1Δ for about 3 hours (Figure 7B). Without any treatment, G\textsubscript{1}/G\textsubscript{0} arrested cells take 1.5 hours to start cell cycle progression in new YPD (Figure 8A). However, 4-NQO treatment postpones cell cycle progression of G\textsubscript{1}/G\textsubscript{0} arrested WT and hug1Δ until 3 hours of incubation (Figure 8B). UV treatment also suspends cell cycle progression of G\textsubscript{1}/G\textsubscript{0} arrested WT and hug1Δ. UV treated G\textsubscript{1}/G\textsubscript{0} arrested WT and hug1Δ begin to progress in the cell cycle after 4 hours of incubation (Figure 8C).
Figure 8. The effect of HUG1 deletion on the cell cycle progression of G1/G0 phase synchronized cells with/without DNA damage checkpoint arrest. G1/G0 phase is induced by culturing cells (WT and hug1Δ) for 7 days. DNA damage checkpoint arrest is induced by 4-NQO or UV treatment. For the observation of cell cycle progression, cells were washed and incubated in YPD after 7 days culture and treatment with DNA damaging agents. During incubation, cells were collected and FACS was performed. A. The cell cycle progression of G1/G0 arrested WT and hug1Δ were observed without any treatment. B. The cell cycle progression of G1/G0 arrested WT and hug1Δ were monitored after 4-NQO treatment. C. The cell cycle progression of G1/G0 arrested WT and hug1Δ were monitored after UV irradiation. B. and C. represent cell cycle recovery from G1/G0 phase DNA damage checkpoint arrest.
C.

WT

hug1Δ

0 hr 1 hr 2 hr

Cell Number

3 hr 4 hr 5 hr

Cell Number

PI Fluorescence (DNA Content)
There are no differences in the cell cycle recovery from checkpoint arrest between

WT and hug1Δ. There are no differences in cell cycle progression between G1 arrested

WT and hug1Δ, as well as, between G1/G0 arrested WT and hug1Δ after 4-NQO or UV
treatment (Figure 7B, 8B, 8C). The cell cycle progression of 4-NQO treated G1 arrested

WT and hug1Δ are the same (Figure 7B). Both of them start to progress the cell cycle

after 3 hours of incubation in YPD (Figure 7B). The cell cycle progressions of 4-NQO

treated G1/G0 arrested WT and hug1Δ are the same (Figure 8B). Both cell types start to

progress the cell cycle after 3 hours of incubation in YPD (Figure 8B). The cell cycle

progression of UV treated G1/G0 arrested WT and hug1Δ are the same (Figure 8C). Both

of them begin the progression of cell cycle after 4 hours of incubation in YPD (Figure

8C). The resumption of cell cycle progression represents the recovery from DNA damage

checkpoint induced activities, including checkpoint triggered cell cycle arrest. Our data

show that HUG1 deleted strain does not differ from WT in the resumption of cell cycle

progression after DNA damage induced checkpoint arrest. Therefore, it is concluded that

Hug1 does not take part in the recovery from DNA damage checkpoint arrest in G1 or

G1/G0 phase.

The influence of HUG1 deletion on the protein levels of Rnr3 and Rnr4

The hypothesis that Hug1 is negatively regulating the level of RNR was tested by

measuring the protein levels of Rnr3 and Rnr4 in WT and in hug1Δ. This hypothesis is

part of the hypothesis that Hug1 is negatively regulating the dNTP pool (8). The

hypothesis that Hug1 is a negative regulator of the dNTP pool is suggested by Basrai MA,

et al (1999), and based on the several reasons. First, the transcriptional induction of
HUG1 and RNR subunits are through the same signal cascades (Mec1-Rad53-Dun1-Crt1). Second, deletion of HUG1 rescues mec1Δ lethality as high level of dNTP does. Third, HUG1 deletion represses the HU sensitivity of dun1Δ (HU reduces dNTP pool). Fourth, overexpression of HUG1 enhances HU sensitivity of mec1Δsml1-1 (8). We tested if Hug1 negatively regulates the level of RNR, since NTP is converted to dNTP only through RNR in *S. cerevisiae* (There are no salvage pathways in budding yeast) [92]. Among the RNR subunits, Rnr3 and Rnr4 were chosen because 1) Rnr3 expression occurs only after DNA damage or replication fork arrest like Hug1, 2) Rnr3 is one of large subunits of RNR and Rnr4 is one of small subunits of RNR (the localization of small subunits regulates the level of RNR holoenzyme and influences the level of dNTP pool) [26, 35, 68], as well as, 3) Rnr3 level is maintained by a stress regulating pathway, target of rapamycin (Tor) pathway (97).

*The protein levels of Rnr3 and Rnr4 are not negatively regulated by Hug1.* The protein levels of Rnr3 and Rnr4 in WT and hug1Δ were measured by monitoring the levels of green fluorescence of GFP tagged Rnr3 or GFP tagged Rnr4. FACS was used to detect green fluorescence.

There is no difference in the changes of Rnr3 levels between WT and hug1Δ during recovery from HU treatment (Figure 9A). Without HU treatment, Rnr3 is not detectable either in WT or hug1Δ; the peak positions of RNR3-GFP·WT and RNR3-GFP·hug1Δ are the same as that of the no GFP containing WT (Figure 9A). After 1 hour of recovery from 1 hour of HU treatment, the level of Rnr3 increases the most in both WT and hug1Δ; the peaks of WT and hug1Δ shift 31.6 % and 35.9 % to the left of the
Figure 9. The effect of HUG1 deletion on the protein levels of Rnr3 and Rnr4 during recovery from HU treatment. During recovery from HU treatment, RNR3-GFP (GFP tagged RNR3) or RNR4-GFP (GFP tagged RNR4) containing cells (WT and hug1Δ) were collected every hour and fixed with 100% ethanol. The GFP levels were monitored by FACS analysis. A. The changes in Rnr3 protein level in WT and hug1Δ are compared during recovery from HU treatment. B. The change in Rnr4 protein level in WT and hug1Δ were compared during recovery from HU treatment. % represents the percentage of shift of a peak to the right of control peak. The peak of WT without GFP tag represents background fluorescence (control).
control peak. For control, WT which does not contain any GFP tag is used. After 2 hours of recovery from 1 hour of HU treatment, the levels of Rnr3 begin to decrease in both WT and hug1Δ; the peaks of WT and hug1Δ shift 26 % and 25.8 % to the left of the control peak. Finally, after 3 hour of recovery from 1 hour of HU treatment, only a small amount of Rnr3 is present in both WT and hug1Δ; the peaks of WT and hug1Δ shift 7.6 % and 5.3 % to the left of the control peak. Therefore, it is concluded that Hug1 does not have a role in reducing the level of Rnr3 protein.

There are differences in the changes of Rnr4 protein level between WT and hug1Δ (Figure 9B). Unlike Rnr3, Rnr4 protein is present even without HU treatment; the peaks of RNR4-GFP·WT and RNR4-GFP·hug1Δ shift 50 % and 59.2 % to the left of the control peak even before HU treatment. After 0 hour of recovery from 1 hour of HU treatment, the levels of Rnr4 decrease in WT and hug1Δ; the peaks of WT and hug1Δ shift about 45.1 % and 26 % to the left of the control peak. After 1 hour of recovery from 1 hour of HU treatment, the Rnr4 levels of WT and hug1Δ reach maximum levels and persist until 2 hours of recovery (Figure 9B); the peaks of WT and hug1Δ shift 94.1 % and 97.3 % to the left of the control peak after 1 hour recovery, and 96.4 % and 94.2 % after 2 hours of recovery. The Rnr4 protein level is reduced after 3 hours of recovery from a 1 hour treatment of HU in both WT and hug1Δ; the peaks of WT and hug1Δ shift 68.6 % and 38.8 % to the left of the control peak. There are small differences in the Rnr4 level between WT and hug1Δ after 1 and 3 hours of recovery. However, this small differences display the opposite of what we expected. We expected a faster increase and slower decrease in RNR subunit levels in hug1Δ than WT, but there are slower increase
Figure 10. The effect of HUG1 deletion on the UV, 4-NQO, or t-BH sensitivities of differently aged cultures. These sensitivities of log phase, 1 day cultured, or 7 day cultured cells (WT and hug1Δ) were observed by measuring surviving fractions. Surviving fraction is the titer of colony forming cells with treatment divided by the titer of colony forming cells without treatment. A. The UV sensitivities of log phase, 1 day cultured, or 7 day cultured cells (WT and hug1Δ) were compared. B. The 4-NQO sensitivities of log phase, 1 day cultured, or 7 day cultured cells (WT and hug1Δ) were compared. C. The t-BH sensitivities of log phase, 1 day cultured, or 7 day cultured cells (WT and hug1Δ) were compared.
and faster decrease in Rnr4 level in hug1Δ. Therefore, it is concluded that Hug1 may not have a role in reducing the Rnr4 level. Like Rnr3, Rnr4 reaches the maximum level in WT and hug1Δ after 1 hour of recovery from HU treatment, and is reduced to about basal level after 3 hours of recovery from HU treatment (Figure 9).

**The sensitivity of hug1Δ to UV, 4-NQO, or t-BH in cultures of different age**

Sensitivity tests are often performed with a gene deletion strain to figure out the function of the protein. Therefore, we tested and compared the sensitivity of WT and hug1Δ to several DNA damaging agents. We tested the sensitivity of older WT and hug1Δ cultures, as well as that of log phase cultures, to DNA damaging agents (Figure 10, 11). Among treated agents, we find that hug1Δ has different sensitivity to 4-NQO than WT (Figure 10). 4-NQO is a UV mimicking and oxidative stress inducing agent (45, 91). Therefore, the UV and t-BH were also treated and the sensitivity of WT and hug1Δ were observed. The t-BH is an oxidative stress inducing reagent (34). 1 day and 7 day cultures were selected because 1 day represents diauxic shift and 7 day represents stationary phase (Figure 4) [116]. We performed the sensitivity test with 2 day cultures, but the sensitivity was not different from 1 day cultures (data not shown). Our FACS analysis confirmed that WT and hug1Δ reach at stationary phase before 7 days (data not shown).

*As cultures of WT and hug1Δ get older, they become more resistant to 4-NQO and t-BH but have constant sensitivity to UV.* The UV sensitivities of WT and hug1Δ remain the same throughout all the cultures (Figure 10A). However, the 4-NQO or t-BH sensitivities of WT and hug1Δ decrease as cultures get older (Figure 10B, 10C). Both of 4-NQO sensitivities of 7 day cultured WT and hug1Δ are decreasing when compared to
the 4-NQO sensitivities of log phase WT and hug1Δ (Figure 10B). The t-BH sensitivities of WT and hug1Δ are gradually decreasing as cultures get older (Figure 10C). 7 day cultured cells start to show sensitivity to t-BH at 50 mM, but log phase and 1 day cultured cells show sensitivity before 4 mM of t-BH treatment (Figure 10C). These mean that 7 day culture becomes tremendously resistant to t-BH. Therefore, it is concluded that older cultures have more resistance to oxidative stresses than younger cultures, but UV sensitivity remains the same regardless of culture age.

As cultures of WT and hug1Δ get older, the relative sensitivity of WT verses hug1Δ to 4-NQO or t-BH changes. However, the relative sensitivity of WT verses hug1Δ to UV stays constant throughout all the phases of cultures. The sensitivities of log phase WT and hug1Δ to 4-NQO or t-BH are the same (Figure 10B, 10C). However, 1 day/7 day WT and hug1Δ have different sensitivities to 4-NQO or t-BH. 1 day cultured WT has slightly less sensitivity to 4-NQO or t-BH than 1 day cultured hug1Δ (Figure 10B, 10C). The 4-NQO or t-BH sensitivity of 7 day cultured WT becomes more than that of 7 day cultured hug1Δ (Figure 10B, 10C). However, the relative UV sensitivity of WT is invariably less than that of hug1Δ in all cultures of different age (Figure 10A). Therefore, it is concluded that hug1Δ becomes more resistant to oxidative stress than WT in older cultures.

The sensitivity of hug1Δ to high temperature (heat shock) in cultures of different age.

The hypothesis that hug1Δ is related to stress responses was tested by measuring the heat shock (52 °C) sensitivity of hug1Δ. It was observed that hug1Δ is more resistant
to oxidative stress than WT in older cultures (Figure 10). Therefore, we wanted to test if hug1Δ is resistant to other stresses which relate to the stationary phase. Heat is a stress to which stationary phase cells show resistance (11, 116). Heat shock proteins (HSPs), the chaperone proteins, are highly expressed in the stationary phase (33). The synthesis of chaperone proteins is one of the three major yeast stress protection mechanisms, which also include trehalose accumulation and synthesis of antioxidative enzymes (111). The temperature of 52°C was selected since several studies have used 52°C to measure stress tolerance and adaptation (stress tolerance and adaptation; show more resistance after pre-stress treatment) [103, 111]. Additionally, it is reported that 50-55°C causes heat shock to yeast and the length of exposure to heat shock determines cell survival.

As cultures of WT and hug1Δ get older, they become more resistant to heat shock. Log phase WT and hug1Δ become sensitive to heat shock after 5 minutes of incubations at 52°C (Figure 11A). 1 day cultured WT and hug1Δ become sensitive to heat shock after 6 minutes of incubation at 52°C (Figure 11B). 7 day cultured WT and hug1Δ are much more resistant to heat shock than log phase and 1 day cultured cells. 7 day cultured WT and hug1Δ still do not show heat shock sensitivity even after 12 minutes of heat shock (Figure 11C). Therefore, this confirms that the cells become resistant to stresses as cultures age (Figure 10, 11) [11, 116].

The hug1Δ is more resistant to heat shock than WT from log phase. Log phase hug1Δ is more resistant to 52°C than log phase WT. Figure 11A shows that the survival of log phase hug1Δ is higher than that of WT after 5 or 6 minutes of heat shock (52°C). The higher heat shock resistance of hug1Δ compared to WT is also observed in 1 day culture (Figure 11B). 1 day cultured WT becomes dramatically sensitive to heat
Figure 11. The effect of HUG1 deletion on the heat shock (52°C) sensitivities of differently aged cultures. Heat shocked (52°C for 0-12 minutes) WT and hug1Δ (log phase, 1 day cultured, and 7 day cultured) were plated on YPD plates and incubated. Colonies were counted and surviving fractions were measured. A. Heat shock sensitivities of log phase WT and hug1Δ. B. Heat shock sensitivities of 1 day cultured WT and hug1Δ. C. Heat shock sensitivities of 7 day cultured WT and hug1Δ.
Heat Shock (52°C)

A. Early Log

B. 1 Day

C. 7 Day
shock after 8 minutes of incubation, but 1 day cultured hug1Δ does not (Figure 11B). That is, hug1Δ is more resistant to heat shock than WT. These experiments confirm that Hug1 may influence stress responses.

**The influence of HUG1 deletion on G₁/G₀ arrest.**

Cells adapt to the environment as cultures age. Cells change catabolic processes to utilize limited energy sources, increase stress resistance, and reduce growth rates when cultures get older (116). Additionally, to adapt to the environment of the culture, stationary phase cells arrest their cell cycle to G₁/G₀ phase (116). Therefore, we observed the cell cycle phases of WT and hug1Δ with differently aged cultures.

*The hug1Δ arrests in G₁/G₀ earlier than WT.* 1 day cultured hug1Δ is mostly composed of G₁/G₀ cells, but 1 day cultured WT contains cells in all the cell cycle phases (G₁, S, and G₂/M phase) [Figure 28]. That is, hug1Δ already arrests in G₁/G₀ after 1 day culture, but WT does not.

**The influence of HUG1 deletion on the responses to slowed DNA synthesis stress and stress from reduced dextrose content.**

*The hypothesis that Hug1 may influence cell responses to slowed DNA synthesis stress and stress from reduced dextrose content was tested by observing the formation of filamentation on HU and low dextrose containing YPD plates.* The hypothesis is based on the following reasons. First, our data show that Hug1 influences stress responses. Second, filamentation is one of the known stress responses (21, 72). Third, Hug1 and filamentation are commonly related to slowed DNA synthesis stress and Mec1 (a DNA
Figure 12. The cell cycle phases of 1 day cultured WT and hug1Δ. Cell cycle phases of 1 day cultured WT and hug1Δ were observed by performing FACS after cells were fixed with ethanol and stained with propidium iodide. A. The cell cycle phases of 1 day cultured WT. B. The cell cycle phase of 1 day cultured hug1Δ.
damage checkpoint) [8, 54, 122]. The overexpression of Hug1 enhances HU (a slowed DNA synthesis stress inducer) sensitivity of mec1Δsml1-1, and the deletion of HUG1 rescues the lethality of mec1Δ (8). Mec1/Rad53 signal cascade mediates slowed DNA synthesis stress (including HU treatment) induced filamentation (54, 122).

The hug1Δ forms less slowed DNA synthesis induced filamentation than WT. WT and hug1Δ were plated on YPD plates containing HU and dextrose (100 mM HU and 0.2% dextrose, or 100 mM HU and 0% dextrose), and then filamentous phenotype was observed (Figure 13B, 13C, 13E, 13F). Filamentation is defined as unipolar and branched chains of elongated cells (121). HU causes slowed DNA synthesis by lowering the level of dNTPs (54). A normal concentration of dextrose for YPD plates is 2%, but we used 0.2% for better observation. It is because the lowered dextrose concentration enhances the slowed DNA synthesis stress induced filamentation. No dextrose nor HU containing plates are used for control (Figure 13A, 13D). Figure 13B and 13C show that WT forms defined filamentation. However, figure 13D and 13E show that hug1Δ does not form filamentation. Both WT and hug1Δ show polarized chains of cells. However, hug1Δ cells are not as elongated as WT cells.

The hug1Δ grows slowly on 100 mM HU and 0% dextrose containing plates. After 6 days, it is observed that there is less growth of hug1Δ than WT on 100 mM HU and 0 - 0.2% dextrose containing plates (Figure 29 and data not shown). However, there is no growth difference between WT and hug1Δ on 100 mM HU and 2% dextrose containing plate (Figure 14). The normal dextrose content for YPD plates is 2%.

80
Figure 13. The effect of HUG1 deletion on HU (slowed DNA synthesis) induced filamentation. The filamentous phenotypes of WT and hug1Δ were observed by plating cells on different YPD plates that differ in concentrations of HU and dextrose. Photographs were taken after 12 hours at 30°C. A. WT plated on 0 mM HU and 0% dextrose containing YPD plate. B. hug1Δ plated on 0 mM HU and 0% dextrose containing YPD plate. C. WT plated on 100 mM HU and 0% dextrose containing YPD plate. D. hug1Δ plated on 100 mM HU and 0% dextrose containing YPD plate. E. WT plated on 100 mM HU and 0.2% dextrose containing YPD plate. F. hug1Δ plated on 100 mM HU and 0.2% dextrose containing YPD plate.
WT

A. No HU No Dextrose  
B. 100 mM HU No Dextrose

hug1Δ

C. No HU No Dextrose  
D. 100 mM HU No Dextrose

E. 100 mM HU 0.2 % Dextrose
F. 100 mM HU 0.2 % Dextrose
Figure 14. HUG1 deletion causes slow growth when cells are plated on 100 mM HU and 0% dextrose containing YPD plate. A. The growth of WT and hug1Δ plated on 100 mM HU and 0% dextrose containing YPD plate. B. The growth of WT and hug1Δ plated on 100 mM HU and 2% dextrose containing YPD plate: control. From left to right, serial dilutions of the same suspension are shown.
100 mM HU

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Figure 15. The effect of ECM7 deletion on HU (slowed DNA synthesis) induced filamentation. The filamentous phenotypes of WT and ecm7Δ were observed by plating cells on 100 mM HU and 0.2% dextrose containing YPD plates. A. WT plated on 100 mM HU and 0.2% dextrose containing YPD plate. B. ecm7Δ plated on 100 mM HU and 0.2% dextrose containing YPD plate. Photographs were taken after 12 hours.
100 mM HU
0.2 % Dextrose

A. WT

B. ecm7Δ
The influence of HUG1 deletion on transcription profiles without any treatment

The hypothesis that transcription profiles of hug1Δ will be significantly different from those of WT was tested by observing hug1Δ/WT (0.2% dextrose) and hug1Δ/WT (2% dextrose) comparison profiles. This hypothesis was constructed based on the following three facts. First, Basrai MA, et al. (1999), suggested that Hug1 has a role in transcriptional recovery from checkpoint arrest. This means that Hug1 may be involved in transcriptional regulation. Second, there is yeast two-hybrid data showing that Hug1 interacts with Tup1, a transcriptional co-repressor (www.yeastgenome.com). Third, blast hit with Hug1 shows several transcription regulators. Therefore, we assumed that Hug1 might be a transcriptional regulator and hug1Δ may have different transcriptional profile compared to WT. This hypothesis was tested by comparing transcriptional profiles between WT and hug1Δ. For the comparison, two comparison profiles (hug1Δ/WT [0.2% dextrose] and hug1Δ/WT [2% dextrose]) are used. The comparison profiles contain the ratios of each transcript level of WT and hug1Δ. The details on comparison profiles are described in the materials and methods section. The ratio of transcript level (increases and decreases) in comparison profiles are considered significant if the ratios are above +2.83 (+log₂1.5) or below -2.83 (-log₂1.5). Therefore, the considered genes have their transcriptional levels in the range of the criterion.

The HUG1 deletion causes more than 10% of the budding yeast genome to change their transcripts above +2.83 ( +log₂1.5) or below -2.83 ( -log₂1.5) fold in comparison to WT, when it is observed in hug1Δ/WT (2% dextrose) comparison profile (Figure 16). The normal dextrose concentration for YPD plates is 2%. Therefore, the hug1Δ/WT (2% dextrose) comparison profile may reveal the difference in transcripts between hug1Δ
Figure 16. The number of genes whose transcripts are modulated by HUG1 deletion and/or HU treatment. The number of genes whose transcript levels are above +2.83 (+log₂1.5) fold in each comparison profile are represented in gray with small upward arrows. The number of genes whose transcript levels are below -2.83 (-log₂1.5) fold in each comparison profile are represented in red with small downward arrows. The fold of the levels of each transcript in each comparison profile represent the influence of HUG1 deletion and/or HU treatment. Transcription profiles were obtained with WT and hug1Δ, which were grown on 2% dextrose (normal dextrose), 0.2% dextrose, or 100 mM + 0.2% dextrose containing YPD plates for 12 hours. Then, the five comparison profiles, hug1Δ/WT (0.2% dextrose), hug1Δ/WT (2% dextrose), WT HU/WT- (0.2% dextrose), hug1Δ HU/hug1Δ- (0.2% dextrose), and hug1Δ HU/WT HU (0.2% dextrose) were obtained. The five comparisons are represented as long black arrows in figure.
and WT in normal conditions. The hug1Δ/WT (2% dextrose) comparison profile shows that hug1Δ has 361 genes whose transcript levels are significantly above WT, and 398 genes whose transcript levels are significantly below WT (Figure 16) [http://sites.google.com/site/siedelab]. This means that the transcript level of 759 genes is significantly different between hug1Δ and WT. The 759 genes are more than 10% of total number of budding yeast genes (~6000).

In the literature, there is a transcriptional profile for the TUP1 deletion budding yeast strain. tup1Δ only has only 354 genes whose transcript levels are different from WT, and 334 are increased by TUP1 deletion (42). Most of transcript levels influenced by TUP1 deletion are increased. Tup1 is a transcriptional co-repressor, therefore, it is understandable that tup1Δ mostly derepresses transcriptions and causes the increase of transcripts. Compared to tup1Δ profile, hug1Δ/WT (2% dextrose) comparison profile contains similar numbers in genes whose transcripts are significantly increased and decreased. Therefore, it is assumed that Hug1 may influence transcriptional responses indirectly.

The transcript levels of many stress responding genes are significantly higher in hug1Δ than WT. The hug1Δ/WT (2% dextrose) comparison profile has increased transcripts of 71 (19.7%) stress responding genes out of 361, and decreased transcripts of 15 (3.7%) stress responding genes out of 398 (Figure 17, [http://sites.google.com/site/siedelab]). These results demonstrate that hug1Δ has more transcripts of stress responding genes than WT. The stress responding genes whose RNA levels are changed, are not only limited to oxidative stress or heat shock stress, but also include starvation, cold temperature, salt stress, etc. Additionally, the profile contains
Ras2 and Mns4 which are upstream kinase for stress signal and transcription factor for the induction of stress responding genes (Figure 17, http://sites.google.com/site/siedelab) [106, 117].

The transcript levels of two genes (SRL2 and CDC13) which are related to DNA damage checkpoint proteins (Mec1/Rad53/Tel1) are increased in hug1Δ. The transcript level of SRL2 and CDC13 is +3.48 (+log2 1.8) and +9.2 (+log2 3.2) fold increased in hug1Δ in comparison to WT when they are grown in normal YPD plates for 12 hours, and those levels are +3.73 (+log2 1.9) and +22.63 (+log2 4.5) fold increased in hug1Δ in comparison to WT when they are grown on reduced dextrose content (0.2%) containing YPD plates (http://sites.google.com/site/siedelab). However, their mRNA levels do not respond to HU. The comparison profiles of WT HU/WT (0.2% dextrose) and hug1Δ HU/hug1Δ (0.2% dextrose) do not show significant changes in transcript levels of these two genes (http://sites.google.com/site/siedelab). Both genes are related to DNA damage checkpoint proteins (www.yeastgenome.org). The overexpression of SRL3 rescues rad53Δ lethality, and Cdc13 is recruited to telomeres by Mec1 and Tel1 to stabilize telomeres.

The deletion of HUG1 changes the transcript level of many recombination related genes, but has that effect only on a few other DNA repair genes. The hug1Δ/WT (2% dextrose) comparison profile has only four repair genes (PMS1, RAD2, REV1, REV7) whose comparison transcript level change is more than ±2.83 (±log2 1.5) [http://sites.google.com/site/siedelab]. The PMS1 is a mismatch repair gene, the RAD2 is a NER gene, and REV1/REV7 are involved in translesion synthesis (TLS). Their comparison transcript levels range from -2.83 (−log2 1.5) to -3.48 (−log2 1.8). All of them
Figure 17. The potential functional significance of transcripts modulated by HUG1 deletion. Transcriptional modulation of hug1Δ/WT (2% dextrose) comparison profile and potential functional significance are diagramed. The hug1Δ/WT (2% dextrose) comparison profile shows that HUG1 deletion causes the increase of 361 gene transcripts (with small upward arrows), and the decrease of 398 gene transcripts by more than 2.83 (log$_2$1.5) fold (with small downward arrows). The number of genes whose transcripts are significantly changed in hug1Δ/WT (2% dextrose) comparison profile are circled with light green background. The number of genes whose transcript levels are modulated by HUG1 deletion are more than 10% of the yeast genome (boxed with light green background). Among those genes whose transcripts are increased by HUG1 deletion, 19.7% are stress responding genes. However, only 3.7% are stress responding genes whose transcript levels are decreased by HUG1 deletion. Additionally, several genes (HSP30, HSP26, RAS2, MSN4, SRL2, CDC13, and RAD2) which have potential significance are designated. HSP30, HSP26, MSN4, and RAS2 are stress responding proteins. SRL2 and CDC13 are DNA damage checkpoint protein related genes. RAD2 is a NER protein. Red dotted lines and letters are to explain the decreased transcripts in the comparison profile. Gray solid lines and letters are to explain the increased transcripts in the comparison profile.
decrease their transcript levels significantly by HUG1 deletion. The transcript levels of other DNA repair genes (excluding those four and recombination related genes) are not significantly different between WT and hug1Δ. The grouping results show that the transcript levels of many recombination related genes are different between hug1Δ and WT (Table 3) [http://sites.google.com/site/siedelab]. The grouping is performed by running a grouping program in www.yeastgenome.org with genes whose transcript levels are more than ±2.83 (±log21.5) in five comparison profiles. The detailed method for grouping is described in the materials and methods section.

*The grouping result of hug1Δ.* The grouping results of the genes whose comparison transcript level was more than + log21.5 or less than – log21.5 in hug1Δ/WT (0.2% dextrose) and hug1Δ/WT (2% dextrose) comparison profiles are summarized in the upper panel of table 3 [designated as hug1Δ/WT (0.2% and 2% dextrose)]. Table 3 and http://sites.google.com/site/siedelab show that compared to WT, hug1Δ has significantly increased transcripts whose proteins are participating in oxidoreductase activity, transcription regulator activity, response to external stimulus, telomere maintenance via recombination, catabolic process, and metabolic process. Whereas, the table displays that HUG1 deletion causes significantly decreased transcripts whose proteins are involved in protein binding, structural constituent of cytoskeleton, transcription regulator activity, ribonucleoprotein complex biogenesis and assembly, cell cycle, transcription, chromosome organization, regulation of nucleobase/nucleoside/nucleotide/nucleic acid metabolic process, chromosome segregation, regulation of metabolic process, negative regulation of biological process, DNA recombination, RNA splicing, and mitotic cell cycle (Table 3, http://sites.google.com/site/siedelab).
The influence of HU treatment on transcription profile

The following are the reasons why we wanted to get the transcription profiles of WT and hug1Δ grown on HU containing plates. First, WT forms clear filamentation but hug1Δ does not (Figure 13). Second, Hug1 can be a transcriptional regulator. There is yeast two-hybrid data showing the interaction between Hug1 and Tup1, a general transcriptional co-repressor (www.yeastgenome.org). Third, Basrai MA, et al. (1999), suggested that Hug1 influences transcriptional recovery from checkpoint arrest. Fourth, blast hit with Hug1 has several transcription regulators.

The hypothesis that HU treated hug1Δ has less transcript levels of filamentation inducing genes than WT was tested. This was tested by observing the percentage of the filamentation related genes whose transcripts are significantly changed (± log2) by HU treatment in WT and hug1Δ (Table 1). For the calculation of the percentage, the filamentation related genes are selected and divided by the total number of genes whose transcript level changes are above +4 (+log2) fold or below -4 (-log2) fold in each comparison profile (http://sites.google.com/site/siedelab). The comparison profiles are WT HU/WT (0.2% dextrose), hug1Δ HU/hug1Δ (0.2% dextrose) and hug1Δ HU/WT HU (0.2% dextrose) [Table 1]. The growth conditions for transcription profile were the same for photographs of filamentation in figure 13. Cells were incubated on 100 Mm HU and 0.2% dextrose containing YPD plates for 12 hours.

In the WT HU/WT (0.2% dextrose) comparison profile, the percentage of filamentation related genes is 2.4% of the genes whose transcript levels are increased more than +log2 fold. In the hug1Δ HU/hug1Δ (0.2% dextrose) comparison profile, among genes whose transcription is increased more than +log2 fold, 2.9% are
filamentation related genes (Table 1). Thus, there is no significant difference in the percentages of increased transcripts of filamentation related genes between the two comparison profiles. Furthermore, among genes whose transcript levels are decreased more than \(-\log_2{2}\) in the WT HU/WT (0.2% dextrose) comparison profile, 9.4% are filamentation related genes. However, in the hug1Δ HU/hug1Δ (0.2% dextrose) comparison profile, 0% are filamentation related genes (Table 1). This does not appear to correspond with filamentation results (Figure 13). That is, even though WT forms clearer filamentation than hug1Δ, WT has higher percentage of decreased transcript levels of filamentation related genes (9.4%) than hug1Δ (0%) after HU treatment. Additionally, HU treated hug1Δ has more up-regulated (7.5%) and down-regulated (5.9%) filamentation related genes than HU treated WT. This was observed in hug1Δ HU/WT HU (0.2% dextrose) comparison profile (Table 1). Therefore, it is concluded that percentage of filamentation related genes whose transcript levels are significantly changed by HU treatment does not explain the difference in HU (slowed DNA synthesis) induced filamentation phenotype between WT and hug1Δ. It is assumed that some specific filamentation related genes may influence the deficient filamentous phenotype of hug1Δ.

The transcript level of cell wall integrity related gene, ECM7, is significantly different between WT and hug1Δ after HU treatment. It has been observed that hug1Δ has polarity of budding, but less cell elongation in slowed DNA synthesis induced filamentaion than WT (Figure 13). This phenotype can possibly be explained by the difference in transcript levels of ECM7 in WT HU/WT (0.2% dextrose) and in hug1Δ HU/hug1Δ (0.2% dextrose) comparison profiles. The change in transcript level of ECM7
Table 1. The percentage of filamentation related genes among genes whose transcript levels are increased above +4 (+log₂2) or decreased below -4 (–log₂2) in each comparison profile. The filamentation related genes include cell wall organization, cytoskeleton organization, pathogenicity, invasiveness, and budding related genes.

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<th>hug1Δ HU/ hug1Δ- (0.2 % dextrose)</th>
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in the WT HU/WT (0.2% dextrose) comparison profile is +19.7 (+\log_2 4.3), and in the hug1Δ HU/hug1Δ (0.2% dextrose) comparison profile is -4.6 (-\log_2 2.2) [http://sites.google.com/site/siedelab]. The level of ECM7 transcript after HU treatment in WT is 24.3 fold higher than in hug1Δ. To confirm that ECM7 is participating in elongation during slowed DNA synthesis induced filamentation, the filamentation of ecm7Δ was observed under the same condition that was used for hug1Δ filamentation (Figure 13, 15). ecm7Δ has less cell elongation than WT (Figure 15). This proves that ECM7 plays a role in the elongation of cells during filamentation. However, the decreased transcript level of ECM7 in the hug1Δ HU/hug1Δ (0.2% dextrose) comparison profile does not appear to be the only reason for decreased elongation of hug1Δ in comparison to WT, because hug1Δ has even less elongation than ecm7Δ (Figure 13, 15).

*The grouping results of HU treatment.* The grouping results of HU treatment are summarized in the lower panel of table 3 [designated as WT HU/WT- (0.2% dextrose)]. The grouping was performed with genes whose transcript levels are more than +\log_2 1.5 or less than −\log_2 1.5 in the WT HU/WT (0.2% dextrose) comparison profile. The transcripts of sporulation, vitamin metabolic process, cell wall organization, meiotic cell cycle, developmental/differentiation process, cell division/reproduction, reciprocal meiotic recombination, monosaccharide transmembrane transporter activity, phosphopyruvate hydratase activity, and carbohydrate transport related genes are increased after HU treatment (Table 3, http://sites.google.com/site/siedelab). There are reductions in transcripts of the genes that are involved in snoRNA binding, RNA helicase activity, ribosome biogenesis, RNA metabolic process, and methionine biosynthetic process, after HU treatment (Table 3, http://sites.google.com/site/siedelab).
Table 2. The relative transcript levels of ECM7 in WT and hug1Δ with or without HU treatment, when the level of its transcript in WT without HU treatment is considered 1. HU treatment increases ECM7 transcript in WT tremendously, but the transcript level is decreased in hug1Δ. Without HU treatment, hug1Δ has more ECM7 transcript than WT. There is correlation between filamentous phenotype and ECM7 transcript level. The numbers in table represent numeric fold not logarithm fold.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>hug1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>HU -</td>
<td>1</td>
<td>6.5</td>
</tr>
<tr>
<td>HU +</td>
<td>19.7</td>
<td>1.25</td>
</tr>
</tbody>
</table>
Table 3. The grouping result of genes whose transcript levels changed more than $\pm \log_2 1.5$ ($\pm 2.83$) by HUG1 deletion or HU treatment. The genes whose transcript levels changed more than $\pm \log_2 1.5$ ($\pm 2.83$) in each comparison profiles were run on the yeast genome web site (www.yeastgenome.org [GO Term Finder]) in order to group them by function or process. The grouping results which had more than an 11.2% background frequency were rejected to avoid broad grouping. The results from grouping genes by their process and function are summarized in this table. The processes and functions of genes whose transcript levels are changed significantly by HUG1 deletion (hug1Δ/WT in this table) are from the grouping results of hug1Δ/WT (0.2 % dextrose) and hug1Δ/WT (2 % dextrose) comparison profiles. The processes and functions of genes whose transcript levels are changed by HU treatment (WT HU/WT- in this table) are from the grouping results of WT HU/WT- (0.2 % dextrose) comparison profile.
<table>
<thead>
<tr>
<th>Comparison Profile</th>
<th>Transcript Change</th>
<th>Processes and Functions of Transcripts</th>
</tr>
</thead>
<tbody>
<tr>
<td>hug1Δ/WT (0.2% and 2% dextrose)</td>
<td>Increase</td>
<td>oxidoreductase activity, transcription regulator activity, response to external stimulus, telomere maintenance via recombination, catabolic process, metabolic process</td>
</tr>
<tr>
<td></td>
<td>Decrease</td>
<td>protein binding, structural constituent of cytoskeleton, transcription regulator activity, ribonucleoprotein complex biogenesis and assembly, cell cycle, transcription, chromosome organization, regulation of nucleobase/nucleoside/nucleotide/nucleic acid metabolic process, chromosome segregation, regulation of metabolic process, negative regulation of biological process, DNA recombination, RNA splicing, mitotic cell cycle</td>
</tr>
<tr>
<td>WT HU/WT- (0.2% dextrose)</td>
<td>Increase</td>
<td>sporulation, vitamin metabolic process, cell wall organization, meiotic cell cycle, developmental/differentiation process, cell division/reproduction, reciprocal meiotic recombination, monosaccharide transmembrane transporter activity, phosphopyruvate hydratase activity, carbohydrate transport</td>
</tr>
<tr>
<td></td>
<td>Decrease</td>
<td>snoRNA binding, RNA helicase activity, ribosome biogenesis, RNA metabolic process, methionine biosynthetic process</td>
</tr>
</tbody>
</table>
The phenotypes of hug1Δ are compared with those of dif1Δ and sml1Δ

The hypothesis that hug1Δ may have high dNTP pool is tested by comparing the phenotypes of hug1Δ and those of dif1Δ and sml1Δ. There is high possibility that Hug1 down regulates dNTP pool. This hypothesis is from the following two facts. First, hug1Δ rescues mec1Δ lethality as other dif1Δ and sml1Δ (8, 24, 68, 120). Dif1 and Sml1 down regulate dNTP pool (24, 68, 120). Dif1 inhibits RNR by reducing the concentration of RNR holoenzyme through localizing Rrn2/Rnr4 complex (small subunits of RNR) to the nucleus (68, 120). While, Sml1 inhibits the activity of RNR by binding to it (24). Second, there is possibility that Hug1, Sml1, and Dif1 are evolved from the same ancestor protein (68). It is thought that duplication of the ancestor gene cause two copies of the ancestor gene, and the duplicated genes are evolved to three different protein coding genes (68, 120) [See discussion for more details]. The filamentation, the growth on low dextrose and HU containing YPD plate, and the resistance to heat shock are compared among dif1Δ, sml1Δ, and hug1Δ mutants. The results show that hug1Δ has similar phenotypes with dif1Δ and sml1Δ, which increase dNTP pools.

The defects in filamentous phenotypes are observed in dif1Δ and sml1Δ as in hug1Δ (Figure 18). The filamentous phenotypes of hug1Δ, dif1Δ and sml1Δ are observed after 12 hours of incubation in HU containing plates. WT forms HU induced filamentation, but mutants do not to the same extent. The dif1Δ and sml1Δ mutants have the polar growth pattern but similar defects in the elongation phenotypes as hug1Δ compared to WT. Among the three mutants, sml1Δ has more elongation phenotype than other two deletion mutants.
Figure 18. HU induced filamentation is affected by DIF1, SML1, and HUG1 deletion.

A, D, G, and J show the morphologies of WT, hug1Δ, dif1Δ, and sml1Δ, which were plated on 0.2% dextrose containing YPD plates. B, E, H, and K display the morphologies of WT, hug1Δ, dif1Δ, and sml1Δ, which were plated on 0% dextrose and 100mM HU containing YPD plates. C, F, I, and L display the morphologies of WT, hug1Δ, dif1Δ, and sml1Δ, which were plated on 0.2% dextrose and 100mM HU containing YPD plates. WT forms prominent filamentation (polar budding and elongation) on 0.2% dextrose and 100mM HU containing YPD plates. The filamentation is induced by HU, and low dextrose content enhances the phenotype. However, hug1Δ, dif1Δ, and sml1Δ do not induce filamentous morphology on HU containing plates. In all three mutants, there are polar budding pattern, but elongation at single cell level is defected.
Figure 19. hug1Δ, difΔ, and sml1Δ are sensitive to low dextrose and 100 mM HU.

Cell survival is observed, after the same numbers of cells (WT, hug1Δ, difΔ, and sml1Δ) are plated on the normal YPD plate, 0% dextrose and 100 mM HU containing YPD plate, and 0.2% dextrose and 100 mM HU containing YPD plate. All the mutants are sensitive compared to WT. A. Loading control: Cells were plated on 2% dextrose containing YPD plate (normal plate). B. Cells were plated on 0% dextrose and 100 mM HU containing YPD plate. C. Cells were plated on 0.2% dextrose and 100 mM HU containing YPD plate.
<table>
<thead>
<tr>
<th></th>
<th>No HU</th>
<th>100 mM HU</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% Dextrose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hug1Δ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dif1Δ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sml1Δ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0% Dextrose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2% Dextrose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 20. The surviving fractions of WT, hug1Δ, dif1Δ, and sml1Δ after heat shock (52°C). The surviving fractions of log phase cells were observed after they were treated with heat shock (52°C) for 5 and 6 minutes and plated on YPD plates. As hug1Δ, dif1Δ and sml1Δ, which have high dNTP pool, have higher resistance than WT.
Heat Shock (52°C)
The growth of dif1Δ and sml1Δ, as well as, that of hug1Δ on low dextrose and HU containing YPD plates are observed (Figure 19). WT grows better than dif1Δ, sml1Δ, and hug1Δ on the 0% dextrose/100 mM HU containing YPD plate and on the 0.2% dextrose/100 mM HU containing YPD plate. The three deletion mutants show the defects in growth on those plates compared to WT.

The heat shock resistance is observed in dif1Δ and sml1Δ as in hug1Δ (Figure 20). The surviving fractions of log phase WT, hug1Δ, dif1Δ, and sml1Δ are observed after heat shock treatment. WT has higher sensitivity to heat shock (52°C) than dif1Δ, sml1Δ, and hug1Δ.
2. Studies on the (suspected) role of Srl3 in DNA damage responses and spontaneous mutagenesis

In the literature, it is reported that overexpression of SRL3 rescues mec1Δ and rad53Δ lethality (31). Therefore, we became interested in the function of Srl3 when it showed spontaneously highly mutable phenotype during screening of spontaneous mutation frequency of a gene deletion BY4741 library in our lab. To determine the function of Srl3, the followings were tested using srl3Δ (=srl3Δuvs) strain: 1) comparison of spontaneous mutation rate and UV sensitivity between srl3Δ (=srl3Δuvs) and dNTP level regulating gene deleted mutants 2) cell cycle progression, DNA damage checkpoint arrest, and recovery from the arrest, and 3) sensitivity to several DNA damaging agents. During these experiments, we realized that the purchased srl3Δ vial contained a mixture of two strains, srl3Δ and srl3Δuvs. uvs is what we named the second mutation in srl3Δ, because the mutation seems to cause UV sensitivity. We decided to determine the function of UVS, and continued the study. Through epistasis study, we discovered that the protein encoded by the UVS gene functions in the same pathway as Rad5 and Rad50. The uvs is epistatic with rad5Δ and rad50Δ, but it is synergistic with rad52Δ. To identify the UVS gene, we made diploids by mating ‘uvs’ mutant with PRR pathway gene deleted strains (rad5Δ, mms2Δ, ubc13Δ, rad50Δ) or MRX complex gene deleted strains (rad50Δ, mre11Δ, xrs2Δ). Rad50 plays a role in both pathways. In complementation tests with the diploids, only mms2Δ/uvs diploid shows UV and MMS sensitivity. To verify that the UVS gene is MMS2, we sequenced the MMS2 gene in srl3Δuvs. The sequencing result demonstrates that the UVS gene is MMS2. There is a non-sense mutation in the MMS2
gene of srl3Δuvs. That is, it is proved that the reason for ‘uvs’ phenotypes (sensitivity to DNA damaging agents and spontaneously mutable) is the non-sense mutation occurred at the conserved trypotphan (W) encoding codon at the catalytic domain of MMS2.

**Spontaneous mutation rate and UV sensitivity of srl3Δ (=srl3Δuvs) and those of dNTP level regulating gene deleted mutants.**

*The hypothesis that srl3Δ will decrease dNTP levels was tested by comparing spontaneous mutation rates and UV sensitivity between srl3Δ (=srl3Δuvs) and dNTP level regulating gene deleted mutants.* High level of dNTPs rescues the lethality of mec1Δ or rad53Δ (22, 24, 31, 50). Therefore, it is possible that overexpression of Srl3 rescues mec1Δ lethality by increasing dNTP level. There are reports that overexpression of RNR large subunits or sml1 mutant (Sml1 is a RNR inhibitor) rescues the lethality of mec1Δ by increasing dNTP pool (24). Therefore, the spontaneous mutation rates and UV sensitivity between srl3Δ (=srl3Δuvs) and dNTP level regulating gene deleted mutants are compared (Table 4). In *S. cerevisiae*, RNR generates dNTPs by reducing abundantly presenting NTPs (92). Because there are no salvage pathways for dNTPs in budding yeast, the activity and level of RNR are critical for the level of dNTPs (92). RNR is composed of four subunits, two large subunits (Rnr1, Rnr3) and two small subunits (Rnr2, Rnr4) [36]. The activity and level of RNR is regulated by Mec1/Rad53/Dun1 DNA damage checkpoint activation. The activated Dun1 phosphorylates and inactivates Sml1, Crt1, and Dif1 to increase dNTP pool after DNA damage (23, 31, 50, 68, 120). Sml1 is the inhibitor of RNR activity. Crt1 is the transcriptional repressor of RNR subunits (RNR2, RNR3, RNR4), and Dif1 is the importer of Rnr2 and Rnr4 into the nucleus.
Table 4. The ratio of spontaneous mutation rate of srl3Δuvs to WT, and those of dNTP level regulating gene deleted mutants, srl3Δ, and ‘uvs’ to WT. The srl3Δuvs (BY4741) has a SRL3 deletion as well as uvs mutation. It is present in srl3Δ (BY4741) which was purchased from Open Biosystem, and used in this study. srl3Δ and ‘uvs’ (BY4744-3A) are haploid mutants isolated from srl3Δuvs/WT diploid. They contain only a single mutation, SRL3 deletion or uvs mutation.

<table>
<thead>
<tr>
<th>Deletion Mutant</th>
<th>The Ratio of Spontaneous Mutation Rate to WT</th>
</tr>
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<tbody>
<tr>
<td>srl3Δuvs</td>
<td>10.33 ± 1.3</td>
</tr>
<tr>
<td>rnr1Δ</td>
<td>2.99</td>
</tr>
<tr>
<td>rnr2Δ</td>
<td>lethal</td>
</tr>
<tr>
<td>rnr3Δ</td>
<td>2.24</td>
</tr>
<tr>
<td>rnr4Δ</td>
<td>0.89</td>
</tr>
<tr>
<td>dun1Δ</td>
<td>1.78</td>
</tr>
<tr>
<td>srl1Δ</td>
<td>0.89</td>
</tr>
<tr>
<td>srl3Δ</td>
<td>0.62</td>
</tr>
<tr>
<td>‘uvs’</td>
<td>8.61</td>
</tr>
</tbody>
</table>
srl3Δuvs and dNTP level regulating gene deleted mutants have different ratio of spontaneous mutation rate to WT. In Table 4, the ratio of spontaneous mutation rate of srl3Δ (=srl3Δuvs) to WT and those of dNTP level regulating gene deleted mutants to WT are compared. The ratio of spontaneous mutation rate of srl3Δ (=srl3Δuvs) to WT is 10.33 ± 1.3 (Table 4). The ratios of spontaneous mutation rate of rnr1Δ, rnr3Δ, and rnr4Δ to WT are 2.99, 2.24, and 0.89 (Table 4). The ratio of spontaneous mutation rate of sml1Δ to WT is 0.89 and that of dun1Δ to WT is 1.78 (Table 4). The verified srl3Δ (without uvs mutation) has 0.62 as the ratio of spontaneous mutation rate to WT (Table 4). That is, srl3Δ (=srl3Δuvs) has more than three to eight fold higher spontaneous mutation rate than dNTP level regulating gene deleted mutants. The ratio of spontaneous mutation rate of srl3Δ (=srl3Δuvs) to WT is significantly different from those of dNTP pool regulating gene deleted mutants to WT. When we obtained these results, we did not realize that we were performing experiments with the srl3Δuvs strain instead of the srl3Δ strain. Therefore, it was concluded that Srl3 may not positively regulate the increase of dNTP pool.

srl3Δuvs and dNTP level regulating gene deleted mutants have different UV sensitivity. The UV sensitivity of srl3Δ (=srl3Δuvs), rnr1Δ, rnr3Δ, and rnr4Δ are compared (Figure 21). There are differences between srl3Δ (=srl3Δuvs) and RNR subunit gene deleted mutants in UV sensitivity. RNR subunit gene deleted mutants have the same UV sensitivities as WT, while srl3Δ (=srl3Δuvs) has more UV sensitivity than WT (Figure 21). That is, the UV sensitivity of srl3Δ (=srl3Δuvs) is more than those of RNR subunit gene deleted mutants (Figure 21). Based on these phenotypic differences and the
Figure 21. The UV sensitivities of srl3Δuvs and RNR subunit deleted mutants. The UV sensitivities of srl3Δuvs, rnr1Δ, rnr3Δ, and rnr4Δ were compared with that of WT by measuring their surviving fraction after UV irradiation.
unawareness of the presence of the uvs mutation in the used srl3Δ strain, it was initially concluded that Srl3 is unlikely involved in the up-regulation of dNTP pools.

**Cell cycle progression, checkpoint arrest, and cell cycle recovery from DNA damage checkpoint arrest of srl3Δ (=srl3Δuvs)**

*The hypotheses that SRL3 may influence cell cycle progression, checkpoint arrest, and cell cycle recovery from DNA damage checkpoint arrest were tested.* The proper cell cycle progressions and the checkpoint activation induced cell cycle arrest are very important for maintaining genome integrity and survival (80). DNA damage checkpoints including Mec1 trigger cell cycle arrest when there is DNA damage or replication fork arrest (18). Even though it is understood that the lethality of mec1Δ is due to the instability of replication fork and the inability of replication fork to process (22), there are possibilities that the lethality of mec1Δ is caused by improper cell cycle regulation or by inappropriate checkpoint arrest. Additionally, we have observed that srl3Δ (=srl3Δuvs) has a high spontaneous mutation rate, which can be observed with checkpoint deficient mutants (80). Therefore, we tested the effect of SRL3 deletion on cell cycle progression, checkpoint arrest, and recovery from the checkpoint arrest. It was tested by observing cell cycle progression of srl3Δ (=srl3Δuvs) from G1, S, or G2/M phase arrest with/without treatment of UV, a DNA damaging agent.

*srl3Δuvs does not have defects in cell cycle progression from G1, S, or G2/M phase.* The cell cycle progression of WT or srl3Δuvs was observed using G1, S, or G2/M phase arrested cells. G1 phase arrest was induced by α-factor, S phase arrest by HU, and G2/M phase arrest by nocodazole. The cell cycle arrests were released by washing off the
arresting agents and incubating with YPD (liquid or plate). The cell cycle progression was monitored by measuring the percentage of budding or the percentage of microcolonies. For the measurement of the percentage of budding, cells were incubated in liquid YPD. For the measurement of the percentage of microcolonies, cells were streaked on YPD plate and incubated. Because a budding yeast starts forming its bud in early S phase and the bud is detached from mother cell after G₂/M phase, the percentage of budding tells how many yeast cells have passed G₁ phase. Microcolony is a small colony which contains more than two cell bodies. The percentage of microcolonies indicates how many cells have already progressed in the cell cycle from the arrest and produced their daughter cells. The cell cycle progression of G₁ arrested WT and srl3Δuvs are the same (Figure 22A). Both strains show the same changes in the percentage of budding cells after being released from G₁ arrest. G₁ arrested WT and srl3Δuvs start to bud within 15 minutes after incubation in YPD. They reach the maximum percentage of budding cells after 30 minutes of incubation; WT and srl3Δuvs have 64% and 53% of budding cells. After 60 minutes, most of WT and srl3Δuvs have passed one cell cycle, because their percentages of budding are reduced to less than 10% (WT: 4%, srl3Δuvs: 9%). S phase arrested WT and srl3Δuvs show the same cell cycle progression. There is no difference in the percentage of microcolonies between WT and srl3Δuvs after the cells are released from S phase arrest (Figure 22B). After 3 hours of incubation, S phase arrested cells (WT and srl3Δuvs) form approximately 80% of microcolonies. WT forms 90% of microcolonies and srl3Δuvs forms 99% at 5 hours after released from S phase arrest. G₂/M phase arrested WT and srl3Δuvs also progress their cell cycles at the same time (Figure 22C). The changes in the percentage of microcolonies are the same in G₂/M
phase arrested WT and srl3Δuvs. After 2.5 hours, the percentages of microcolonies of G₂/M phase arrested WT and srl3Δuvs reach 76% and 74% respectively. There are no differences between WT and srl3Δuvs in the progression of cell cycle from G₁, S, or G₂/M phase arrest. Therefore, it is concluded that Srl3 does not play a role in the cell cycle progression.

**srl3Δuvs does not have defects in checkpoint arrest triggered by UV.** G₁, S, or G₂/M phase arrested WT and srl3Δuvs were irradiated with UV after α-factor, HU, or nocodazole induced arrests. After UV irradiation, cells were incubated in liquid YPD or streaked on YPD plate. During incubation of the cells, the percentage of budding or the percentage of microcolonies was determined in appropriate intervals. There is UV induced checkpoint arrest in all G₁, S, and G₂/M phased cells (WT and srl3Δuvs), because there are prolonged cell cycle arrests after UV irradiation compared to the cell cycle arrests without UV treatment (Figure 22) UV irradiated G₁ phase arrested WT and srl3Δuvs reach their peak of percentage of budding later than UV un-irradiated G₁ phase arrested cells (Figure 22A) UV irradiated S or G₂/M phase cells form microcolonies slower than UV un-irradiated cells (Figure 22B, 22C).

**srl3Δuvs has more extended DNA damage checkpoint arrest triggered by UV than WT.** The recovery of WT from UV induced checkpoint arrest is faster than that of srl3Δuvs. In all cell cycle phases (G₁, S, and G₂/M phase), srl3Δuvs shows further elongated UV induced checkpoint arrest than WT (Figure 22). The cell cycle progression of UV irradiated G₁ phase srl3Δuvs is approximately 5 min slower than that of UV irradiated G₁ phase WT (Figure 22A). After 6 hours of incubation, UV irradiated S phase WT forms 80% of microcolonies but UV irradiated S phase srl3Δuvs forms only 43%
Figure 22. The cell cycle arrest and progression of WT and srl3Δuvs with or without UV irradiation. A. The cell cycle arrest and progression of α-factor induced G\textsubscript{1} phase arrested WT and srl3Δuvs are observed with/without UV irradiation. B. The cell cycle arrest and progression of HU induced S phase arrested WT and srl3Δuvs are observed with/without UV irradiation. C. The cell cycle arrest and progression of nocodazole induced G\textsubscript{2}/M phase arrested WT and srl3Δuvs are observed with/without UV irradiation.
(Figure 19B). After 5 hours of incubation, UV irradiated G<sub>2</sub>/M phase WT forms 80% of microcolonies but srl3Δuvs forms only 39% (Figure 22C). srl3Δuvs shows less recovery from S or G<sub>2</sub>/M phase arrest than WT during the time of observation (Figure 22). Therefore, it was initially concluded that Srl3 plays a role in the recovery from UV induced checkpoint arrest. However, additional studies showed that the phenotypes we were observing were from the uvs mutation.

The sensitivity of srl3Δuvs to UV in different cell cycle phases

The hypothesis that the slower recovery from checkpoint arrest of srl3Δuvs than that of WT is due to the higher UV sensitivity of srl3Δuvs than WT was tested. This was tested by observing the survival fraction of srl3Δuvs and WT, after UV irradiation. This hypothesis was suggested since defects in the repair of UV induced DNA damage can be the reason for the slower recovery of srl3Δuvs from UV induced checkpoint arrests in all cell cycle phases compared to that of WT.

srl3Δuvs has higher UV sensitivity in all the phases of the cell cycle compared to WT. G<sub>1</sub>, S, and G<sub>2</sub>/M phase arrested srl3Δuvs and WT were irradiated with several doses of UV, and surviving fractions were determined. G<sub>1</sub>, S, and G<sub>2</sub>/M phase arrested srl3Δuvs have more UV sensitivity than those of WT (Figure 23). Among G<sub>1</sub>, S, and G<sub>2</sub>/M phase arrested srl3Δuvs, G<sub>2</sub>/M phase arrested srl3Δuvs has least UV sensitivity (Figure 23). Therefore, it is concluded that the slower recovery from checkpoint arrest of srl3Δuvs compared to WT may reflect the higher UV sensitivity of srl3Δuvs than WT.
Figure 23. The UV sensitivities of G₁, S, and G₂/M phase arrested srl3Δuvs. The UV sensitivity of srl3Δuvs is compared with that of WT after being arrested in G₁, S, and G₂/M phases. G₁ phase arrest is induced by α-factor, S phase arrest is by HU, and G₂/M phase arrest is by nocodazole.
**Figure 24. The sensitivities of srl3Δuvs to UV, methyl-methane sulfonate (MMS), and streptonigrin.** Surviving fractions were determined as a function of dose (J/m² for UV, % for MMS, and µg/ml for streptonigrin). A. The sensitivity of srl3Δuvs to UV. UV induces cyclobutane-pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs), which are removed by base-excision repair (BER) and nucleotide-excision repair (NER). B. The sensitivity of srl3Δuvs to MMS. MMS alkylates bases and produces N7-methylguanine (N7-meG) and N3-methyladenine (N3-meA), which are repaired by BER, NER, and mismatch repair (MMR). C. The sensitivity of srl3Δuvs to streptonigrin. Streptonigrin is a γ-irradiation-mimetic agent which causes mainly double strand DNA breaks. Double strand DNA breaks are repaired by HR and NHEJ.
The sensitivity of srl3Δuvs to different DNA damaging agents

The hypothesis that srl3Δuvs has higher sensitivity to a specific DNA damaging agent than WT was tested by comparing the sensitivity of WT and srl3Δuvs to several specific DNA damaging agents. The sensitivity tests are usually performed to find out the function of a protein. Because different agents trigger different pathways, the sensitivity of a gene mutant to specific agents identifies in what pathway a protein has its function. Treatment with different DNA damaging agents will indicate more specific roles of Srl3 (=UVS). Thus, WT and srl3Δuvs were treated with UV, MMS, and streptonigrin, and the survival fractions were determined (Figure 24). UV forms photodimers, MMS (alkylating reagent) methylates bases, and streptonigrin produces double strand breaks in DNA.

srl3Δuvs has higher sensitivity to all the tested DNA damaging agents than WT. As a function of dose, srl3Δuvs has less surviving fraction than WT with all the three DNA damaging agents (Figure 24). srl3Δuvs is more sensitive to all agents than WT. Therefore, it is concluded that the sensitivity of srl3Δuvs is not specified to any specific DNA damaging agent among the three. It is also assumed that srl3Δuvs has defects in a DNA repair mechanism that handles different types of DNA damage.

Separation of uvs mutation from srl3Δ background mutation and the characterization of ‘uvs’ (uvs mutant) phenotype

The srl3Δ strain used for experiments is proved to be the srl3Δuvs strain. It is discovered that the purchased srl3Δ BY4741 vial contains two different strains, termed srl3Δ and srl3Δuvs. In the middle of the project to determine the function of Srl3, frozen srl3Δ stock was newly thawed and its UV sensitivity was determined, instead of srl3Δ
Figure 25. The comparison of UV sensitivities for WT, srl3Δuvs (BY4741), srl3Δ (BY4741), and srl3Δ (BY4742). The strains, srl3Δuvs (BY4741) and srl3Δ (BY4741), are different isolates from the same vial purchased from Open Biosystems. The srl3Δ (BY4742) strain was newly purchased from Open Biosystem to confirm the UV insensitivity of srl3Δ (BY4741). The genetic backgrounds, BY4741 (MATa) and BY4742 (MATα), represent opposite mating types.
(=srl3Δuvs) which was used for experiments for a while. It was observed that the UV sensitivity of the new srl3Δ isolate from stock is less than that of srl3Δ (= srl3Δuvs) which has been used for previous experiments (Figure 25).

To determine if the UV sensitivity is because of the deletion of SRL3 gene or the second mutation in the srl3Δ (=srl3Δuvs) strain, the UV sensitivities of newly thawed srl3Δ isolate (srl3Δ BY4741), newly purchased srl3Δ for this experiment (srl3Δ BY4742), and srl3Δ (= srl3Δuvs) were tested (Figure 25). srl3Δ BY4741 and srl3Δ BY4742 (the opposite mating type strain to the srl3Δ BY4741 strain) have the same UV sensitivity as WT, but srl3Δuvs has more UV sensitivity than WT (Figure 25). Therefore, it is concluded that the higher UV sensitivity of srl3Δuvs is due to the second mutation that occurred in the srl3Δ BY4741 strain. We named the second mutation as uvs which stands for “UV Sensitive”.

The ratio of spontaneous mutation rate of newly isolated srl3Δ BY4741 to WT was also measured. The ratio of spontaneous mutation rate of newly isolated srl3ΔBY4741 to WT is only 0.62 (Table 4). This is distinguished from the high ratio of spontaneous mutation rate of srl3Δuvs (10.33 ± 1.3) to WT [Table 4]. That is, srl3Δuvs has high UV sensitivity and spontaneously mutable phenotypes, but srl3Δ does not.

To confirm that the high UV sensitivity and high spontaneous mutation rate of srl3Δuvs are not due to the SRL3 deletion but completely due to the uvs mutation, SRL3 deletion and uvs mutation were separated from the srl3Δuvs strain. To separate two mutations (uvs mutation and SRL3 deletion), the following steps were performed. First, srl3Δuvs (BY4741) and WT (BY4742) were crossed and diploids (srl3Δuvs/WT) were
Figure 26. Separation of uvs mutation and SRL3 deletion from the srl3Δuvs strain.

A. A method for separating uvs mutation and SRL3 deletion. srl3Δuvs haploid (BY4741) and WT haploid (BY4742) were crossed to create a diploid. Sporulation was induced and the tetrad was dissected to haploids. B. and C. ‘uvs’ and srl3Δ were selected by their UV sensitivity and G418 resistance. The KanMX4 marker used to delete SRL3 confers G418 resistance to srl3Δ but not ‘uvs’.
A. $srl3\Delta uvs$\hspace{1cm} WT\hspace{1cm} srl3\Delta uvs/WT

\[\text{uvs} \quad \text{X} \quad \rightarrow \quad \text{uvs} \quad \downarrow \quad \text{srl3}\Delta\]

\[\text{uvs} \quad \neg \text{uvs}\quad \text{srl3}\Delta\]

B. 

C. 

$srl3\Delta$ : G418 resistant
\neg uvs : G418 sensitive

![UV Dose (J/m^2) vs Surviving Fraction Graph](image)

Legend:
- srl3\Delta
- uvs
created (Figure 26A). Second, sporulation was induced and tetrads were dissected to haploids (Figure 26A). Third, uvs mutant (‘uvs’) and srl3Δ were selected by UV and G418 sensitivity (srl3Δ carries kanMX4 which causes G418 resistance). srl3Δ strain was constructed by replacing the SLR3 gene with kanMX4 among the haploids (Figure 26B, 26C). The selected ‘uvs’ has UV sensitivity similar to srl3Δuvs and cannot survive on G418 (200 µg/ml) containing YPD plate (Figure 26B, 26C). However, the selected srl3Δ has UV sensitivity similar to that of WT and survives on G418 (200 µg/ml) containing YPD plate (Figure 26B, 26C). The meiotic recombination separates the uvs mutation and the SRL3 deletion, and allows for us to select ‘uvs’ and srl3Δ single mutant haploid spores.

The two phenotypes of srl3Δuvs are not from the SRL3 deletion but entirely from the uvs mutation. The UV sensitivity of ‘uvs’ was determined during selection procedure (Figure 26B). Figure 26 proves that the UV sensitivity of srl3Δuvs is entirely due to the uvs mutation. The ratio of spontaneous mutation rate of ‘uvs’ to WT is determined to 8.61 (Table 4). It is similar to the ratio of spontaneous mutation rate of srl3Δuvs (10.33 ± 1.3) to WT [Table 4]. The high spontaneous mutation rate of ‘uvs’ confirms that the spontaneously mutable phenotype of srl3Δuvs is caused by the uvs mutation.

Epistasis studies with ‘uvs’ (uvs mutant)

The hypothesis that UVS plays a role in a specific DNA repair pathway was tested by performing epistasis studies. We observed that srl3Δuvs has more prolonged checkpoint arrest and higher sensitivity to several DNA damaging agents than WT (Figure 22, 24). The extended checkpoint arrest and high sensitivity to DNA damaging
Figure 27. Definitions used in the epistasis study: epistatic, synergistic, and additive interactions. This method is used to determine the pathway a protein takes part in. To figure out the pathway, the phenotype of a gene mutant and the phenotype of double gene mutant are compared. The single gene mutant has a defect in a gene whose protein function is unknown. The double gene mutant contains one mutated gene whose protein function is unknown (the same gene as the single gene mutant) and another gene whose protein function is known. Epistatic: the two proteins have a role in the same pathway. The phenotype of a double gene mutant is the same as the phenotype of a single gene mutant. B. Synergistic: the two proteins have a role in different pathways, and the pathways are competitive. The phenotype of a double gene mutant is more than the addition of two single gene mutants. C. Additive: the two proteins have a role in different pathways, but the pathways are not competitive. The phenotype of a double gene mutant is the same as the addition of two single gene mutants. m1 and m2 represent single gene mutants. d represents a double gene mutant. Surviving fraction (sensitivity) is selected among phenotypes to explain these definitions.
A. 

![Graph showing surviving fraction vs. dose of DNA damaging agent with lines for WT, m1, and m2, d.]

B. 

![Graph showing surviving fraction vs. dose of DNA damaging agent with lines for WT, m1, m2, and d.]

C. 

![Graph showing surviving fraction vs. dose of DNA damaging agent with lines for WT, m1, m2, and d.]
agents are often observed with strains containing mutated DNA damage repairing genes. Defects in DNA repair will cause slower DNA repair. Slower DNA repair will result in a slower recovery from checkpoint arrest and higher sensitivity to DNA damaging agents. Because the slower DNA damage repair prolongs the presence of DNA damage, it continuously activates DNA damage checkpoint proteins and induces cell death. However, when checkpoint arrest prolongs so much, yeasts frequently progress cell cycle from checkpoint arrest even though it has un-repaired DNA damages (123). This is called adaptation and will cause mutations and sensitivity to DNA damaging agents. Epistasis studies were performed using UV or MMS sensitivity to determine the repair pathway UVS takes part in (Figure 27).

*Epistasis studies are used to determine the pathway a protein is participating in, by comparing a specific phenotype of two single gene mutants with that of a two gene mutant.* The two genes mutated in two single gene mutants and in a two gene mutant have to be the same. The pathway of one of the two genes must already be known, but the pathway of the other gene of interest is to be determined. In the epistasis study, there are three terms: Epistatic, Synergistic, and Additive. Epistatic means that the two proteins encoded by the deleted genes are participating in the same pathway. If the two proteins are epistatic, the phenotype of one of the single gene mutants is the same as that of the two gene mutant (Figure 27A). Synergistic means that the two proteins encoded by the deleted genes take part in different pathways and the different pathways are competitive. If two proteins interact synergistically, the phenotype of the two gene mutant is more than the added phenotypes of the single gene mutants (Figure 27B). Additive means that the two proteins encoded by the deleted genes have roles in different pathways, and the
Figure 28. Epistasis studies between ‘uvs’ and rad5Δ, as well as, ‘uvs’ and rad50Δ.

The UV and MMS sensitivities of rad5Δ, ‘uvs’ and rad5Δuvs were compared, as were rad50Δ, ‘uvs’, and rad50Δuvs. Double mutants, rad5Δuvs and rad50Δuvs were constructed by deleting RAD5 and RAD50 from ‘uvs’ respectively. A. The UV sensitivity was used for epistasis studies between ‘uvs’ and rad5Δ, and also ‘uvs’ and rad50Δ. B. The MMS sensitivity was used for epistasis studies between ‘uvs’ and rad5Δ, and also ‘uvs’ and rad50Δ.
A. 

B. 

Graph showing survival fraction against UV dose (J/m²) and MMS concentration (%) for different genotypes.
pathways cannot overlap. One of them participates in a pathway that responds to one part of the stimulation, and the other has a role in another pathway which reacts to the other part (the remnant) of the stimulation. If two proteins are additive, the phenotype of the two gene mutant is the addition of those of the single gene mutants (Figure 27C).

‘uvs’ is epistatic to rad5Δ in repairing UV or MMS induced DNA damage. The epistasis between rad5Δ and ‘uvs’ was studied. Rad5 is a ubiquitin ligase and plays a role in PRR (28). DDT pathways is composed of PRR and TLS. They allow the replication apparatus to bypass DNA damage during replications to prevent the collapse of the replication fork. (110). The collapse of the replication fork often results in cell death and gross chromosomal rearrangement (GCR) as well as DSB (78, 110). We chose rad5Δ to perform the epistasis study with ‘uvs’, because Rad5 plays a role in the PRR pathway which is related to cell death, and rad5 mutant has the UV and MMS sensitivity and spontaneously mutable phenotypes (101). Figure 28 displays the UV and MMS sensitivities of rad5Δ, ‘uvs’, and rad5Δuvs. The ‘uvs’ was obtained from tetrads (Figure 26) and rad5Δ is purchased from Open Biosystem. Double gene mutant, rad5Δuvs, was constructed by deleting RAD5 from the ‘uvs’ mutant as described in the materials and methods section. Figure 28A. shows that the UV sensitivity of rad5Δ and rad5Δuvs are the same; rad5Δ and ‘uvs’ are epistatic to UV stimuli. They have a role in the same pathway to repair UV induced DNA damage. The MMS sensitivity of rad5Δ and rad5Δuvs are also similar (Figure 28B) [The small difference in survival can be considered insignificant]. rad5Δ and ‘uvs’ are epistatic in response to MMS. Rad5 and Uvs participate in the same repair pathway to repair MMS induced DNA damage.
‘uvs’ is epistatic to rad50Δ at a low dose of UV and all doses of MMS. The epistasis between ‘uvs’ and rad50Δ was studied using UV and MMS. Rad50 is a component of the MRX complex which participates in DSB sensing and repair (5, 59, 67). It is also known that Rad50 is epistatic to Rad5 and both of them take part in the PRR bypass pathway (28). The UV and MMS sensitivity of rad50Δ, ‘uvs’, and rad50Δuvs were compared (Figure 29). The double mutant, rad50Δuvs was constructed by deleting RAD50 from ‘uvs’. The ‘uvs’ was obtained from tetrads (Figure 26). rad5Δ was purchased from Open Biosystems. The UV sensitivity of rad50Δuvs is the same as that of rad50Δ at lower doses, but is possibly additive to rad50 at higher doses (Figure 29A). The MMS sensitivity of rad50Δuvs and rad50Δ are the same (Figure 29B). That is, rad50Δ is epistatic to ‘uvs’ when MMS or lower doses of UV are used. In other words, Rad50 is involved in the same repair pathway as Uvs to repair MMS and lower UV dose induced DNA damage, but they may take part in different and not competitive repair pathways to repair high UV dose induced DNA damage.

‘uvs’ is synergistic to rad52Δ in repairing UV and MMS induced DNA damage. The epistasis study between ‘uvs’ and rad52Δ was performed. It is already known that rad52Δ and rad51Δ (HR genes) are synergistic to rad5Δ (PRR gene) [28]. HR pathway is inhibited during the activation of the PRR pathway (Figure 3). The sumoylation of PCNA recruits Srs2 helicase and interrupts the formation of Rad51-DNA filaments which is the early step of HR. This inhibition is to prevent improper activation of HR which causes GCR (78). HR may repair DSB caused by the collapse of the replication fork, and the PRR pathway occurs to prevent the collapse. The HR may operate after the collapse and the PRR pathway works before the collapse. They both deal with the same DNA
Figure 29. Epistasis studies between ‘uvs’ and rad52Δ. The UV and MMS sensitivities of rad52Δ, ‘uvs’, and rad52Δuvs are compared. The triple mutant, rad52Δsrl3Δuvs was constructed by deleting RAD52 from srl3Δuvs. srl3Δuvs was used instead of ‘uvs’ because srl3Δ does not have any sensitivity to UV or MMS. A. The UV sensitivity was used for the epistasis study between ‘uvs’ and rad52Δ. B. The MMS sensitivity was used for the epistasis study between ‘uvs’ and rad52Δ.
A. 

B.
structures which block replication at different times and in different ways. Therefore, rad51Δ and rad52Δ are synergistic to rad5Δ.

The UV or MMS sensitivities of rad52Δ, srl3Δuvs, and rad52Δsrl3Δuvs were compared (Figure 29). rad52Δsrl3Δuvs was constructed by deleting RAD52 from the srl3Δuvs mutant as described in the materials and methods section. srl3Δuvs was from the srl3Δ vial purchased from Open Biosystems. rad52Δ was also purchased from Open Biosystems. We consider srl3Δuvs to be equivalent to ‘uvs’, since the SRL3 deletion does not have any UV sensitivity and there is no difference in UV sensitivity between srl3Δuvs and ‘uvs’. Figure 29A. shows that the UV sensitivity of rad52Δsrl3Δuvs is more than the addition of the sensitivities of rad52Δ and srl3Δuvs. Figure 29B. displays that the MMS sensitivity of rad52Δsrl3Δuvs is more than the addition of the sensitivities of rad52Δ and srl3Δuvs. This means that Rad52 is synergistic to UVS. Rad52 and Uvs take part in different repair pathways which are competitive. In summary, our data show that ‘uvs’ is epistatic to rad5Δ and rad50Δ, but synergistic to rad52Δ (Figure 28, 29). It is known that Rad5 and Rad50 are the PRR pathway proteins, and Rad52 (HR protein) is inhibited by part of the PRR pathway (Figure 3). Therefore, it is concluded that UVS encodes a protein that plays a role in the PRR pathway.

**Studies on the allelism of UVS**

*UVS was identified as MMS2.* To identify the UVS gene, ‘uvs’ (BY4744-3A) was crossed with rad50Δ (BY4741), mre11Δ (BY4741), xrs2Δ (BY4741), rad5Δ (BY4741), ubc13Δ (BY4741), or mms2Δ (BY4741) on the mating plate and diploids (rad50Δ/uvs, mre11Δ/uvs, xrs2Δ/uvs, rad5Δ/uvs, ubc13Δ/uvs, and mms2Δ/uvs) were constructed.
(Figure 30A). The deletion mutants (BY4741 haploids) mated with ‘uvs’ were selected since UVS can be one of the genes (Figure 28, 29). Rad5, Ubc13, Mms2, and Rad50 are known PRR pathway proteins, and Rad50 is a component of the MRX (Mre11-Rad50-Xrs2) complex which senses and repairs DSB (28). The UV or MMS sensitivity of the diploids (rad50Δ/uvs, mre11Δ/uvs, xrs2Δ/uvs, rad5Δ/uvs, ubc13Δ/uvs, and mms2Δ/uvs) were tested by streak test. The mms2Δ/uvs diploid has higher UV and MMS sensitivity than other diploids, indicating the absence of complementation (Figure 30B). That is, mms2Δuvs diploid has deficient MMS2 loci at both sister-chromatids (Δ and uvs), but other diploids have only one flawed MMS2 loci (uvs) between both sister-chromatids (Figure 30C). The higher sensitivity of mms2Δ/uvs is because it can not produce any wild type Mms2 but other diploids can. Therefore, it is assumed that UVS is identical to MMS2.

**UVS was verified as MMS2 by sequencing. The sequencing results show that the non-sense mutation occurring at the conserved tryptophan (W) coding sequence of MMS2 is the reason for ‘uvs’ phenotypes.** To verify that UVS is MMS2 and to know the kind of mutation that occurred in the MMS2 loci of the srl3Δuvs haploid, the MMS2 gene of the srl3Δuvs haploid was sequenced after PCR amplification. The sequencing results show that srl3Δuvs has a point-mutation in MMS2 (Figure 31). G at +452 is transited to A. Therefore, the TGG codon is changed to the termination codon, TAG. The point mutation causes the conversion of tryptophan (W) to termination (Figure 31, 33). Our results demonstrate that the non-sense mutation causes the sensitivity to DNA damaging agents and high rates of spontaneous mutations.
Figure 30. Studies on the allelism of UVS. A. The ‘uvs’ (BY4744-3A) was crossed with MRE complex gene deleted mutants (rad50Δ, mre11Δ, and xrs2Δ [BY4741]) and with PRR bypass pathway gene deleted mutants (rad5Δ, ubc13Δ, and mms2Δ [BY4741]). ‘uvs’ and other one gene deletion mutants were mated by cross-replicaplating ‘uvs’ and other deletion mutants on mating plate. Because of the complementation between auxotrophic markers from BY4741 and BY4742 backgrounds, only diploids can grow on the mating plate. The diploids (rad50Δ/uvs, mre11Δ/uvs, xrs2Δ/uvs, rad5Δ/uvs, ubc13Δ/uvs, and mms2Δ/uvs) are shown as white square colonies. B. The complementation of UVS alleles of each diploid was tested through streak test. The diploids were streaked on a YPD plate and UV was irradiated, or they were streaked on a MMS containing YPD plate. mms2Δ/uvs has higher UV and MMS sensitivity compared to other diploids. This proves that UVS is potentially MMS2. C. The explanation for the sensitivity (non-complementation) of mms2Δ/uvs. The diploid which contains defects in both UVS (MMS2) alleles will display higher sensitivities than WT, due to the lack of a repair protein. However, the diploids which have flaws in only one of both UVS alleles (uvs only and deleted gene only) will show the same sensitivities to WT, because one normal allele produces the repair protein.
A. uvs

rad50Δ mre11Δ xrs2Δ rad5 Δ mms2Δ ubc13Δ

(rad50Δ/uvs mre11Δ/uvs xrs2Δ/uvs rad5 Δ/uvs mms2Δ/uvs ubc13Δ/uvs)

B. MMS

UV

C. uvs  Δ  Sensitive

WT  Δ  Insensitive
Figure 31. DNA sequence of MMS2 gene isolated from srl3Δuvs. Base sequence (black print) and amino acid sequence (blue print, single big letters) are shown. Intron is in gray print and underlined. ATG (start codon) is in front, and TGA (stop codon) is at the end of the sequence. The non-sense mutation that occurred in MMS2 is in bold red print which is boxed (TAG). A point mutation (A to G) changes the tryptophan (W) coding codon (TAG) to a termination codon (TGA) which caused termination improperly (TAG and TGA are boxed).
IV. DISCUSSION

1. Studies on the role of Hug1 in DNA damage and stress responses

The function of Hug1 is studied by characterizing the phenotypes of hug1Δ, by observing the transcript profile of hug1Δ, by testing the hypotheses suggested by a published study (i.e., Hug1 may have a role in the recovery from checkpoint arrest, and Hug1 can negatively regulate the level of RNR subunit proteins), and by comparing the phenotypes of dNTP pool increasing mutants (sml1Δ and dif1Δ) with those of hug1Δ.

We identified several phenotypes of hug1Δ. Compared to WT, hug1Δ is more resistant to oxidative stress and to heat shock, arrests earlier in G1/G0 phase during culture, grows slower on low dextrose and HU containingYPD plate, and displays defect in HU induced filamentation (possibly only in elongation) [Figure 10, 11, 12, 13, 14]. However, WT and hug1Δ do not have difference in the recovery from G1 or G1/G0 phase DNA damage induced checkpoint arrest, and in the protein levels of Rnr3 and Rnr4 during recovery from HU treatment (Figure 7, 8, 9).

The transcript profile of hug1Δ shows that more than 10% of yeast genes are significantly changed by HUG1 gene deletion (Figure 16, 17). This is a tremendous change demonstrating that Hug1 has an important role in regulating transcription profile directly or indirectly (Figure 17). Among those changes, we noticed that hug1Δ contains
more increased stress related transcripts than WT, and that some genes among whose transcript level is altered by HUG1 deletion can be connected to the phenotypes of hug1Δ. For example, the reduced mRNA level of ECM7 in hug1Δ is correlated with the hug1Δ’s defect in filamentation, the increased mRNA levels of SRL2 (and perhaps CDC13) in hug1Δ can explain the published hug1Δ phenotype that deletion of HUG1 rescues the lethality of mec1Δ, and the increased mRNA level of HSP30, HSP26, MSN4, and RAS2 in hug1Δ may be the reason for the resistance of hug1Δ to heat shock and t-BH (Figure 10, 11, 15, 17, http://sites.google.com/site/siedelab [www.yeastgenome.org, 8]).

The phenotypes of dif1Δ and sml1Δ are compared with those of hug1Δ, because literature suggests Hug1 has a role in down regulation of dNTP pool (Figure 18, 19, 20) [68, 120]. The phenotypes compared are HU induced filamentation, growth on low dextrose and HU containing YPD plate, and resistance to heat shock. The results show that all three mutants have similar phenotypes (Figure 18, 19, 20). These results support the hypothesis that Hug1 has a role in down-regulation of dNTP pool as Sml1 and Dif1 do. However, to confirm this hypothesis, it will be necessary to measure the dNTP pool of hug1Δ directly, or to compare transcripts of hug1Δ with those of sml1Δ and dif1Δ.

**A role of Hug1 in the recovery from checkpoint arrests in cell cycle phases other than G1 or G1/G0 phase?**

We have checked that if Hug1 has a role in the recovery from DNA damage checkpoint arrest by observing the difference between WT and hug1Δ in their cell cycle recovery from G1 or G1/G0 phase after DNA damage (Figure 7, 8). G1 or G1/G0 synchronized WT and hug1Δ do not display differences in cell cycle progression from 4-
NQO or UV induced checkpoint arrest. Therefore, we concluded that Hug1 does not play a role in the recovery from DNA damage induced G₁ or G₁/G₀ phase checkpoint arrest. Since Hug1 is expressed in all cell cycle phases (8), it can be assumed that Hug1 does not have a role in the recovery from the checkpoint arrest in other cell cycle phases (S and G₂/M phases). However, because HUG1 transcriptional induction is one of genes most highly induced by HU (http://sites.google.com/site/siedelab) [8], it will be of special interest to test if hug1Δ has different recovery from S phase checkpoint compared to WT (HU is an inhibitor of dNTP pool increase and used to arrest cell cycle in S phase).

**Why are there differences between GFP fluorescence peaks of Rnr3 and those of Rnr4 (Figure 9)?**

There are two differences between GFP fluorescence peaks of Rnr3 and those of Rnr4. First, there is no Rnr3-GFP fluorescence peak (no movement of Rnr3-GFP peak to the right from background fluorescence) in WT and hug1Δ at no treatment and 0 hour of recovery from HU treatment, but the peaks of Rnr4-GFP fluorescence present constantly. Second, Rnr3-GFP has only one peak, but Rnr4-GFP has two peaks.

The first difference can be explained by the difference in the regulation of Rnr3 and Rnr4 expression (35). The Rnr3 presents undetectable level in unchallenged cells, but its expression is enhanced by DNA damage or replication fork arrest to more than 100 fold to the level of unchallenged cells. Conversely, Rnr4 is constantly present in detectable level so that it can be detected even in unchallenged cells (35). Figure 9 re-confirms these facts: there is the absence of Rnr3-GFP fluorescence peak in 0 hour recovery from HU treatment, but the constant presence of Rnr4-GFP fluorescence peak.
The second difference can be reasoned by the three facts that 1) HU induces replication fork arrest only in S phase, 2) Rnr3 is expressed only after DNA damage or replication fork arrest, and 3) FACS methodology can detect different cell types. That is, one peak represents S phase cells (budding cells) expressing Rnr3-GFP by HU treatment. Two peaks illustrates existence of all the phases of cells (non-budding [single cell] and budding cells [two cells] with 1× and 2 × signal) containing Rnr4-GFP which is constantly present. In other word, FACS detects Rnr4-GFP which is normally expressed in all phases (non-budding and budding cells) even without HU treatment as well as is induced in S phase (budding cells) by HU treatment.

The resistance of hug1Δ to heat shock and to t-BH can be explained by the transcript profile of hug1Δ and stress tolerance mechanisms

From the sensitivity results, it is found that all the cultures of hug1Δ (log phase, 1 day, and 7 day cultures) have more resistance to heat shock than WT, and 7 day culture of hug1Δ has more resistance to t-BH than WT (Figure 10, 11) [data not shown].

It is thought that the transcript profile of hug1Δ can be the reason for the more resistance to oxidative stress and to heat shock (environment stresses). The transcript profile of elevated transcripts in hug1Δ (grown on normal YPD) has more transcripts of stress related genes than that of WT (Figure 16, 17, http://sites.google.com/site/siedelab). The stress responding genes are more than 19.7% of genes whose transcripts are increased in comparison profile of hug1Δ/WT (2% dextrose) [Figure 16, 17]. The grouping results also show that the part of transcription profile, whose transcripts are significantly elevated in hug1Δ compared to WT, is external stimuli (including nutrition)
responding genes (Table 3, http://sites.google.com/site/siedelab). That is, we think that the pre-existing stress responding gene transcripts may give hug1Δ more tolerance to t-BH and to heat shock than WT. There are reports that pre-stressed cells become more resistant to following stresses than non-pre-stressed cells (102).

Then, why does hug1Δ have higher resistance to heat shock in log phase but have higher resistance to t-BH only in 7 day culture (Figure 10, 11)? It may be that the altered transcript levels of hugΔ are sufficient for hug1Δ to have tolerance to heat shock, but not to t-BH. The tolerance of hug1Δ to t-BH may require the altered transcript profile of log phase and additional factors which are up- or down-regulated during 7 days of culture. The cultures start to experience oxidative stress after diauxic shift (respiration begins at this time to gain energy from the byproducts of fermentation) [116]. Therefore, yeast cells may activate the oxidative stress resistance pathways after diauxic shift. Before diauxic shift, because yeasts perform fermentation to get energy, yeasts may have no or little oxidative stress response. Yeasts respond to environment very flexibly by responding to environment stresses immediately and in long term to adjust to their environment (41, 116, 117). Therefore, we think that the transcription profile and conditions of hug1Δ alone are not sufficient to be tolerant to t-BH. This is indicated by the result that log phase and 1 day cultured hug1Δ do not have resistance to t-BH (Figure 10). In laboratory, diauxic shift occurs after 1 days of culture (116).

The hug1Δ/WT (2% dextrose) comparison profile does not match published environment stress response (ESR) profile, fermentation stress response profile, mec1Δ profile, tup1Δ profile, and pho80Δ profile
We wondered if hug1Δ profile has similarity with the profiles of some specific stresses, mec1Δ, tup1Δ, and pho80Δ. It is because 1) hug1Δ has increased transcripts of stress responding genes (including starvation) [Figure 17, Table 3, http://sites.google.com/site/siedelab], 2) hug1Δ has resistance to heat and t-BH (two kinds of stresses), 3) hug1Δ shows starvation related phenotypes (slow growth in low dextrose and HU containing YPD plate) [Figure 10, 11, 13, 14, 18, 19], 4) hug1Δ rescues mec1Δ lethality (8), 5) the interaction of Hug1 with Tup1 has been proved by yeast-two hybrid experiment (www.yeastgenome.org), and 6) proteomic study reports that Pho80/Pho85 phosphorylates Hug1 (83).

There are published profiles for environment stress response (ESR), fermentation conditions, and the MEC1, TUP1, or PHO80 deletion mutant (3, 39, 40, 42, 74, 83). The transcript profiles for ESR were established following exposure to specific stresses such as heat, starvation, osmotic stress, oxidative stress, and combination of them (40). We compared the genes whose transcripts are changed in our hug1Δ/WT (2% dextrose) comparison profile with these published profiles (http://sites.google.com/site/siedelab) [3, 39, 40, 42, 74, 83]. However, less than 10% genes with altered expression in each profile (published data only) can be matched with those of genes whose transcripts changed as the consequence of HUG1 deletion. It is understandable that hug1Δ/WT (2% dextrose) comparison transcript profile hardly matches ESR profiles, because our profile is a gene deletion profile. If the gene (HUG1) does not have a major role in sensing the applied specific stresses of ESR, transcript profiles will not be similar. Furthermore, the little similarity between hug1Δ and pho80Δ transcript profiles indicates that Hug1 is not involved, at least, in all the Pho80 pathways. Literature reports that Pho80 is an inhibitor.
of Pho4 (a transcription factor that targets genes increasing phosphate level) and of Rim15 (112, 123). However, we suggest that Hug1 may be a downstream target of Pho80 in Rim15 pathway. It is because 1) Pho80 is known to inhibit G\textsubscript{1}/G\textsubscript{0} arrest by inhibiting Rim15, 2) Pho80 phosphorylates Hug1, and 3) hug1Δ arrests earlier in G\textsubscript{1}/G\textsubscript{0} than WT (Figure 12) [88, 112]. However, Hug1 phosphorylation by Pho80 has so far only been demonstrated in a proteomics screen (88).

**Early arrest of hug1Δ in G\textsubscript{1}/G\textsubscript{0} may indicate hug1Δ has more activation of stress responses**

Our data show that hug1Δ arrests in G\textsubscript{1}/G\textsubscript{0} during an earlier stage of culture (Figure 12). The G\textsubscript{0} phase (quiescence) arrest occurs when cells experience stresses, especially starvation (126). Therefore, this data also suggests that hug1Δ has more active stress responses than WT.

An important protein for the G\textsubscript{0} arrest is Rim15 (86, 115, 126). Stress response signaling pathways such as Ras/PKA, Sch9, and Tor pathways, as well as, Pho80/Pho85 converge on Rim15 (83, 86, 105, 115, 126). They inhibit Rim15 by phosphorylation, when cells can obtain abundant nutrients to grow. However, the inhibition is reduced during starvation. The dephosphorylated and activated Rim15 localizes to nucleus by 14-3-3, and induces G\textsubscript{0} phase arrest (86, 115, 126). Rim15 causes G\textsubscript{0} arrest by activating the stress response transcription factors (Msn2/Msn4) and a post diauxic shift transcription factor (Gis1), by stimulating the interaction between Msn2/Msn4 and transcription factor IID (TFIID), and by activating enzymes such as Tps1, a component of the stationary-phase specific trehalose synthase (126).
Our hug1Δ/WT (2% dextrose) comparison profile indicates increased MSN4 (log$_2$2.1) transcript level and increased RAS2 (log$_2$5.5) [http://sites.google.com/site/siedelab]. The increased mRNA level of MSN4 and RAS2 in hug1Δ may represent two factors that connect transcript profile of hug1Δ with early G$_0$/G$_1$ arrest by Rim15. The mRNA level of Rim15 itself is not increased in hug1Δ, but this does not exclude Rim15 as a reason of the earlier G$_0$/G$_1$ arrest. It is because its activity not its level is important for G$_0$ arrest (86, 115, 126).

**How does Hug1 influence the transcript levels of 10% of genome?**

The yeast genome website (www.yeastgenome.org) lists yeast two hybrid data showing the interaction of Tup1 and Hug1, and BLAST search with Hug1 amino acid sequence presents many hits with transcription factors. Tup1 is a general transcription corepressor which does not bind directly to the promoter elements, but is recruited to promoters and represses transcriptions by interacting with other transcription regulating proteins (50, 126). These facts suggest the possibility that Hug1 works as a transcription factor.

However, Hug1 is unlikely be a direct effector of transcription, such as a transcription factor. We examined the transcript profiles of MSN4 (a transcription activator which binds to stress responding element and trigger expression of stress responding genes) overexpression and tup1Δ (Raw data from Dr. Gasch’s lab, 42). Overexpression of Msn4 causes the increase of 193 transcripts and the decrease of 42 transcripts. TUP1 deletion results in the increase of 334 transcripts and decrease of the 20 transcripts. So, both profiles have much higher number for increased than for decreased
transcripts, confirming the transcription activator function of Msn4 and transcription repressor function of Tup1. Compared to them, hug1Δ has similar numbers of both increased and decreased transcripts. Therefore, it is less likely that Hug1 regulates transcription directly. We assume that the changes in transcripts in hug1Δ are due to high dNTP pools.

It is likely that hug1Δ increases dNTP pool, and the increased dNTP pool changes transcript profile

There is a high possibility that Hug1 negatively regulates dNTP pool. Our results show that the phenotypes of sml1Δ and dif1Δ are similar to those of hug1Δ (Figure 18, 19, 20). Literature also predicts that Hug1 affects dNTP pool. Basrai MA, et al. (1999), reported that hug1Δ rescues the lethality of mec1Δ, and that hug1Δ suppresses the HU sensitivity of dun1Δ. Both phenotypes are observed when there are high levels of dNTP pools (24, 31, 50, 68). sml1 and dif1 mutants have similar phenotypes with hug1 mutant: their mutation rescues the lethality of mec1Δ, and overexpression of DIF1 enhances dun1Δ sensitivity to HU (24, 68, 120). Sml1 reduces dNTP pool by inhibiting the activity of RNR (24), Dif1 does by lowering the level of RNR (68, 120).

Very recently, Lee YD, et al. (2008), even suggested that Dif1, Sml1, and Hug1 have evolved from the same protein. They aligned the domains and sequences of Hug1 with other dNTP regulators, and showed sequence similarity between regions of Dif1 and Sml1 as well as between Dif1 and Hug1 (Figure 32B, 32C). This suggestion is convincing when the locations of SML1 and HUG1 genes are considered. The SML1 and HUG1 genes are localized contiguously on the same template strand of chromosome 13.
Figure 32. The domains and amino acid sequences of Hug1 and other dNTP regulating proteins are aligned. A. The HUG1, SML1, and DIF1 genes are evolved from the same ancestor gene of *S. cerevisiae*. The ancestor gene is duplicated to chromosome 12 and 13. Then, they are evolved to three genes (HUG1, SML1, and DIF1). DIF1 is evolved as a gene which encodes Rnr2-Rnr4 protein nuclear localization protein, but HUG1 and SML1 are evolved to two different genes. The localization of Rnr2-Rnr4 causes lower RNR level. Sml1 inhibits the activity of RNR. Dif1 and Sml1 are eventually reducing dNTP pool. B. The domains of Hug1 and other dNTP regulating proteins are aligned. Hug, Sml, and R1B represent Hug domain, Sml domain, and Rnr1-binding domain. The domains are named by Lee YP, et al. (2008). Hug1 has only Hug domain, Sml1 has Sml and R1B domains, and Dif1 has all of them. It is proved that R1B domain of Dif1 is not working. Dif1 and Sml1 both are phosphorylated and inhibited by Dun1 though Sml domain. C. The amino acid sequences of Hug1 and other dNTP regulating proteins are aligned. There is some similarity in hug domain sequences among Hug1 and other dNTP regulating proteins. Sml1 has least similarity. From Mol Cell. 2008 Oct 10;32(1):70-80.
Table 5. The grouping result of hug1Δ HU/WT HU (0.2% dextrose) comparison profile. The genes whose transcript levels changed more than $\pm \log_2 1.5$ ($\pm 2.83$) in hug1Δ HU/WT HU (0.2% dextrose) comparison profile were run on the yeast genome web site (www.yeastgenome.org [GO Term Finder]) in order to group them by process. P-vale shows the significant difference between Cluster frequency and Background frequency. A. shows the grouping result of genes whose transcript levels are increased in hug1Δ HU/WT HU (0.2% dextrose) comparison profile. B. shows the grouping result of genes whose transcript levels are decreased in hug1Δ HU/WT HU (0.2% dextrose) comparison profile.
A. hug1Δ HU / WT HU - (0.2% dextrose) Increase

<table>
<thead>
<tr>
<th>Gene Ontology term</th>
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<th>Background frequency</th>
<th>P-value</th>
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<td>telomere maintenance via recombination</td>
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<td>regulation of cellular process</td>
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</tbody>
</table>

B. hug1Δ HU / WT HU - (0.2% dextrose) Decrease

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<th>P-value</th>
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Dif1, is localized on chromosome 12 (Figure 32A) [68]. Lee YD, et al. (2008), assumed a common ancestor gene of DIF1 and SML1-HUG1 was duplicated onto two chromosomes, and the three genes evolved separately. In *S. cerevisiae*, duplicated genes compose 16% of total genome (63). Hug1 consists entirely of Hug domain. Sml1 has Sml and RIB domain. Dif1 has well conserved Hug1 and Sml domains, and a weekly conserved RIB domain (Figure 32B). Sml1 uses RIB domain to bind to the large subunit of RNR (Rrn1) and inhibits the activity (23). Sml domain is required to be phosphorylated and inhibited by Dun1 (68, 120). Sml domains of Dif1 and Sml1 are phosphorylated by Dun1, and the proteins are degraded as a consequence (68, 120).

Then, what can be the role of Hug domain? Dif1 inhibits the increase of dNTP pools by localizing the Rnr2-Rnr4 to the nucleus, (which is completely different from Sml1), and the Hug domain is necessary for the localization of Rnr2-Rnr4 to the nucleus (68, 120). Therefore, it will be interesting to observe if hug1Δ also influences the localization of Rnr2-Rnr4, which causes reduction in the cytoplasmic RNR holoenzyme level. However, it looks like Hug1 and Dif1 work in different time, since their expressions are different (8, 120). Dif1 presents in unchallenged cells and is degraded by Dun1, a downstream protein of DNA damage checkpoints (120). However, Hug1 barely presents in unchallenged cells, but highly induced by DNA damage (8). These expression differences support the hypothesis that Hug1 has a role in reducing dNTP pool for the recovery from DNA damage induced checkpoint arrest (8). This hypothesis was suggested by Basrai MA, et al (1999), mainly because the transcriptional induction of HUG1 is later than RNR subunits, even though the genes are regulated by the same DNA damage signaling pathway (Mec1-Rad53-Dun1-Crt1).
However, it should also be noted that BLAST searches did not demonstrate sequence similarity between Hug1 and Dif1. The similarity between Dif1 and Hug1 is only convincing when their sequences are aligned with other dNTP regulator genes from other species such as, Spd1 (*S. pombe*) and Aer122c (*A. gossypii*) [Figure 32C].

Our transcript profile also supports the possibility that Hug1 is involved in the regulation of dNTP pools. Table 5, which shows the grouping result of hug1∆ HU/WT HU comparison profile, has groups of genes related to nucleotide metabolism. This means that hug1∆ has more changes in nucleotide metabolism related transcripts than WT following HU treatment. We assume that these differences between WT and hug1∆ are probably due to the involvement of Hug1 in controlling the level of dNTP pool.

**Conclusions and future studies for Hug1**

We have presented phenotypic characterizations of hug1∆ that provides important clues to the function of Hug1. In literature, it was reported that HUG1 deletion rescues mec1∆ lethality, and enhances HU sensitivity of mec1∆sml1-1 and dun1∆. We newly discovered novel phenotypes of single gene mutant, hug1∆. Additionally, we observed the influence of HU treatment on transcript profile for the first time.

Although we could not define the exact function of Hug1, available data suggests that Hug1 negatively controls dNTP pool. This assumption is supported by the similar phenotypes of hug1∆, sml1∆, and dif1∆, as well as by the hypotheses and data in literature as discussed above (Figure 18, 19, 20, 32, Table 5) [8, 24, 31, 50, 68, 120]. We assume that the deletion of HUG1 increases dNTP pool, the increased dNTP pool
regulates transcriptions and other cellular mechanisms differently from normal condition, and this abnormal condition is reflected to hug1Δ phenotypes.

To confirm if Hug1 negatively regulates dNTP pool as Sml1 and Dif1 do, it is necessary to measure dNTP pool of hug1Δ directly. Additionally, similar transcript profiles in sml1Δ and dif1Δ could also confirm the potential function of Hug1 (a negative regulator of dNTP pool). Because the phenotypes of hug1Δ can be determined by the levels of specific transcripts, it will be easier to measure the levels of specific transcripts such as, ECM7, SRL2, MSN4, RAS2, HSP26, and HSP30 in dif1Δ and sml1Δ than to perform microarray with dif1Δ and sml1Δ to confirm the role of Hug1.

How may Hug1 negatively regulate dNTP pool? Since Hug domain is the potential Rnr2-Rnr4 binding site and is required for the localization of Rnr2-Rnr4 to the nucleus, it is required to study the interaction of Hug1 with Rnr2-Rnr4 and the localization of Rnr2-Rnr4 to nucleus in hug1Δ.
2. Studies on the (suspected) role of Srl3 in DNA damage responses and spontaneous mutagenesis

The second project was started to look for the function of Srl3, but was finished by identifying the second mutation, uvs in srl3Δuvs strain, as MMS2. The srl3Δ vial purchased from Open Biosystem contained two strains, srl3Δ and srl3Δuvs, and we used srl3Δuvs to determine the function of Srl3 unluckily. The UV sensitivity and highly mutable phenotype of srl3Δuvs was interesting because it was known that overexpression of Srl3 rescues the lethality of mec1Δ and rad53Δ, and because it was thought that srl3Δuvs is srl3Δ strain. However, in the middle of research, it was determined that the two phenotypes (UV sensitivity and highly spontaneously mutable phenotypes) were from uvs mutation but not from SRL3 deletion. To identify UVS gene, following three steps were performed. First, epistasis study was performed to figure out that Uvs is the PRR pathway protein (Figure 28, 29). Second, complementation tests were done with diploids which were created by mating ‘uvs’ and several deletion mutants, in which PRR pathway and assumed PRR pathway genes are deleted (Figure 30A). ‘uvs’/mms2Δ diploid showed more UV and MMS sensitivities than other diploids. Therefore, UVS was assumed MMS2 (Figure 30B). Third, to confirm UVS is MMS2, MMS2 gene from srl3Δuvs was sequenced (Figure 31). Sequencing results show that srl3Δuvs contains a point mutation in MMS2 gene. Therefore, it is concluded that UVS is MMS2 by sequencing as well as the phenotypes of ‘uvs’.
In the discussion, Mms2 families are overviewed, and the phenotypes (sensitivity to DNA damaging agents and spontaneously mutable phenotypes) of mms2Δ (results reported in the literature) and ‘uvs’ (mms2 mutant) [our results] are compared.

We assumed that Srl3 may not play a role in increasing dNTP pool. This assumption is due to the spontaneous mutation rate of srl3Δ and RNR large subunit deleted strains being different, even though overexpression of SRL3 rescues mec1Δ and rad53Δ lethality just as a high dNTP pool does (31). However, this assumption has to be confirmed by measuring srl3Δ’s dNTP pool directly.

**Overview of Mms2 family**

*The function and structure of Mms2.* MMS2 is composed of 499 bases including one intron (45 bases) [Figure 31], and encodes Mms2 protein which has 137 amino acids and is 15.5 kDa (www.yeastgenome.org). Mms2 is a ubiquitin-conjugating enzyme variant which has significant sequence homology with ubiquitin-conjugating proteins, but lacks a cysteine residue in the conjugating active site (13). Figure 31 shows the lack of a cysteine in the Mms2 catalytic domain, which is unlike other ubiquitin-conjugating enzymes (89). The cysteine residue is essential for ubiquitin-conjugating enzymes.

Ubiquitination occurs as follows: 1) a ubiquitin forms a thioester bond with the cysteine residue of a ubiquitin-activating enzyme (E1), 2) then is transferred to the cysteine residue of a ubiquitin-conjugating enzyme (E2), 3) finally, a ubiquitin ligase (E3) stimulates the isopeptide bond formation between lysine residue of a protein and the ubiquitin (89). Mms2 participates in the PRR pathway by forming a heterodimer with the true ubiquitin-conjugating enzyme, Ubc13 (48). They function together as a uniquitin-
conjugating enzyme and interact with a ubiquitin-ligase, Rad5, to poly-ubiquitinate PCNA (Figure 3) [110]. Even though Mms2 is not a true ubiquitin-conjugating enzyme, Mms2 is just as important as Ubc13. This is demonstrated by the fact that mms2Δ, ubc13Δ, and mms2Δubc13Δ have the same MMS sensitivities (16). Furthermore, there are reports that Mms2 inhibits TLS polymerase, Polζ, during PRR bypass (Figure 3) [7, 13]. Mms2 is conserved throughout eukaryotes. The human homologues of budding yeast Mms2 are hMms2 and CROC-1. hMms2 and CROC-1 share over a 90% consensus sequence with each other. hMms2 shares 50% but CROC-1 shares 75% of the consensus sequences with yeast Mms2 (124). It has been proved that hMms2 participates in the PRR pathway (15, 71).

The catalytic domain of Mms2 is compared to that of other ubiquitin-conjugating enzymes. Figure 33 displays aligned amino acid sequences from the catalytic domain of several ubiquitin-conjugating enzymes (89). There are differences in length and sequences among catalytic domains of ubiquitin-conjugating enzymes. Their different structures demonstrate their participation in diverse cellular mechanisms (89). Figure 34 represents the comparison of the insertions/extensions of E2s’ catalytic domain with that of standard UBC4 and UBC5 catalytic domain. The catalytic domain sequence of MMS2 is closest to that of RAD6 and UBC6 catalytic domains. The homologue tree diagram shows that there is some distance between MMS2 and UBC13, even though they are working together as a heterodimer for poly-ubiquitination of PCNA. Among the amino acid sequences, tryptophan (W) which is mutated in ’uvs’, is the common amino acid for all of the ubiquitin-conjugating enzymes displayed in figure 33.
The epistatic or synergistic relationship of Mms2 to Rad5, Rad50, and Rad52

*The epistatic relationships of Mms2 to Rad5 and Rad50 are confirmed.* Figure 28 (our data) shows that ‘uvs’ (mms2 mutant) is epistatic to rad5Δ when UV or MMS is treated. Figure 28 also displays that ‘uvs’ (mms2 mutant) is epistatic to rad50Δ in response to MMS and lower doses of UV. Epistatic means the two proteins play a role in the same pathway. All of the epistatic relationships we studied confirm what has already been published. The epistatic relationship between rad5Δ and mms2Δ for UV sensitivity is reported (106, 110). The epistatic relationships between rad5Δ and rad50Δ, as well as, rad50Δ and mms2Δ have been studied by Gangavarapu V, et al. (2006). Additionally, the fact that Ubc13 and Mms2 function in the same pathway has been proved by the same MMS sensitivity of ubc13Δ, mms2Δ, and ubc13Δmms2Δ (16). The proteins (Rad5, Mms2, Ubc13, and Rad50) are all PRR pathway proteins, and our data confirms that Rad5, Rad50, and Mms2 play a role in the same pathway.

*The synergistic relationship between Mms2 and Rad52 is confirmed.* Our data show the synergistic relationship between ‘uvs’ (mms2) and rad52Δ when UV or MMS is used (Figure 29). Synergistic means the two proteins play a role in different pathways for the same damage, and the two pathways are competitive to repair the damage. That is, Mms2 and Rad52 take part in different pathways to repair UV or MMS induced DNA damage. Rad52 is a HR pathway protein. Rad52 interacts with Rad51 nucleofilament, and mediates it with DNA replication machinery (98). Rad51 nucleofilament is necessary for the exchange of DNA strands in order to form heteroduplex DNA during HR (98). In general, HR is inhibited during PRR to prevent GCR which causes mutations and cell death (28, 78). The two pathways, HR and PRR, are competing for the DNA damage
located around the replication fork. The PRR bypasses the DNA damage during replication to prevent replication fork collapse, and HR may be involved in repairing double stranded DNA breaks after the collapse (78). During PRR, Ubc9 and Siz1 sumoylate PCNA and recruit Srs2 helicase (Figure 3). The helicase disrupts Rad51 nucleofilament to inhibit HR (87, 113). There are reports on the synergistic relationship between PRR and HR repair pathway proteins. Motegi A, et al. (2006), reported the synergistic MMS sensitivity of a double gene mutant (rad5rad51 or rad5rad52) to that of single gene mutants (rad5, rad51, or rad52). Hishida T, et al. (2002), showed the synergistic relationship between rad52Δ and rad18Δ. Rad18 and Rad6 mono-ubiquitinate PCNA, and mono-ubiquitinated PCNA is the pre-requisite for the PRR pathway (28).

However, Torres-Ramos CA, et al. (2002), reported that the UV sensitivity of rad52Δmms2Δ is additive to that of rad52Δ and mms2Δ. Additionally, Grangavarapu V, et al. (2006), suggested that Rad51, Rad52, and Rad54 operate in the PRR pathway of the lagging strand via the synthesis-dependent strand annealing (SSA) pathway, which is one of the HR pathways that uses homologous sequences to repair DDB.

**There is a possibility that Srl3 influences the recovery from checkpoint arrest.**

It is observed that srl3Δuvs have delayed recovery from DNA damage checkpoint arrest in all cell cycle phases (G₁, S, and G₂/M), compared to WT (Figure 22). We have assumed that these delays are due to defects in some of the repair mechanisms of srl3Δuvs. Several experiments (epistasis studies, complementation tests with diploids created by crossing ‘uvs’ with some PRR pathway gene deleted strains and Rad50 related gene deleted genes, and sequencing) were performed and ‘uvs’ was verified as mms2
mutant (Figure 28, 29, 30, 31). Mms2 is a PRR pathway protein (124). Therefore, it is thought that the prolonged DNA damage checkpoint arrest of srl3Δuvs is because of the impairment of the PRR pathway. However, it is still hard to say this conclusion, since the PRR pathway is related to DNA replication, which occurs in the S phase, but the delay of checkpoint arrest occurs in all cell cycle phases (Figure 22). One possibility is that SRL3 deletion causes the more prolonged DNA checkpoint arrest of srl3Δuvs compared to WT. To test this possibility, the DNA damage checkpoint arrest of srl3Δ or ‘uvs’ needs to be examined. The other possibility is that the PRR pathway plays a role in all cell cycle phases.

**DNA damage tolerance (DDT) pathway is required for bypassing UV, streptonigrin, and MMS induced DNA damage**

Our data show that ‘uvs’ (mms2 mutant) is more sensitive to UV, streptonigrin, and MMS than WT (Figure 24). The agents produce different types of DNA damage. It is also reported in the literature that DDT pathway mutants display higher sensitivities to different DNA damaging agents when compared to WT. The rad6 and rev3 mutants have more sensitivities to UV, ionizing radiation, and alkylating reagents than WT (7, 13). The rad5 mutant has higher sensitivity to UV and MMS than WT (124). The mms2Δ has higher UV and γ-irradiation sensitivity than WT (7).

Streptonigrin (bruneomycin) is an aminoquinone antitumor antibiotic, and considered a γ-irradiation-mimetic agent. It binds DNA irreversibly when there is a metal ion, and produces hydroxyl radicals and reactive oxygen species through its quinine moiety (10). It also inhibits topoisomerase II or forms covalent DNA adducts. Because of
these reactions, streptonigrin can induce single strand breaks, double strand breaks, and abnormal structures of DNA, which block replication fork progression (10). UV causes cyclobutane-pyrimidine dimmers (CPDs) and 6-4 photoproducts (6-4 PPs). These are mainly removed by BER and NER (44). Methyl methane sulfonate (MMS) is an alkylating agent. It alkylates bases and produces $N^7$-methylguanine ($N^7$-meG) and $N^3$-methyladenine ($N^3$-meA). These damaged sites are repaired by base-excision repair (BER), nucleotide-excision repair (NER), or mismatch repair (MMR) [51].

The high spontaneous mutation rate of ‘uvs’ may be due to Pol$\zeta$.

The mms2 mutants cause high spontaneous mutation rates. Our data show that ‘uvs’ (mms2 mutant) has an 8.61 higher spontaneous mutation rate than WT (Table 4). Others have reported that mms2Δ has a 22.1 and 31.0 higher spontaneous mutation rate than WT (7, 13). We determined the spontaneous mutation rate of ‘uvs’ (mms2 mutant) [BY4744-3A] by monitoring spontaneous mutations that occurred in arginine permease, while others examined the rate of mms2Δ (DBY747) by detecting the spontaneous reversion rate of trp1-289 amber mutation. It is still unclear whether it is reasonable to compare spontaneous mutation rates of deletion mutants and point mutants (we used ‘uvs’ strain which has a nonsense mutation in MMS2). However, regardless of the difference in strains and mutation systems, the common fact is that mms2 mutants show a high rate of spontaneous mutations.

It is reported that the high spontaneous mutation rate of mms2 mutant is because of active Pol$\zeta$ (13, 56, 76). Broomfield S, et al. (1998), observed for the first time that mms2Δ has a high spontaneous mutation rate. They discovered that the high spontaneous
mutation rate of mms2Δ is due to Polζ. They observed that mms2Δrev3Δ double mutant (Rev3 is the catalytic subunit of Polζ) eliminates the high spontaneous mutation rate of mms2Δ completely. mms2Δ has a 31 fold higher spontaneous mutation rate than WT, while the rates of rev3Δ and mms2Δrev3Δ are only 1.2 fold higher. Therefore, they concluded that Mms2 in an inhibitor of Polζ (Figure 3). However, the mechanism by which Mms2 inhibits Polζ is still unknown.

_A model of the DDT pathway for spontaneously induced DNA damage_ (76). In a model suggested in the literature, the DDT pathway for spontaneously induced DNA damage includes Rad6 dependent TLS pathway, Rad5 dependent error-free PRR pathway, and Rad5 dependent error-prone bypass pathway (76). Rad6 dependent TLS pathway and Rad5 dependent error-free PRR pathway for spontaneously induced DNA damage are the same for UV induced blockage. In contrast, Rad5 dependent error-prone bypass only participates in spontaneous DNA damage induced DDT pathways. In Rad5 dependent error-prone bypass, Rad5 homodimer and Polζ are in the same epistasis group. This model shows that Rad5 is involved in the stimulation of Polζ as well as in error-free PRR, and that Mms2 is the important protein that channels spontaneous damage away from Polζ (TLS polymerase) and to Rad5 dependent error-free PRR (76).

_A nonsense mutation occurred in the MMS2 gene of the ‘uvs’ strain_

_The nonsense mutation in the MMS2 gene of the ‘uvs’ strain, disrupts UV repair function of Mms2 as completely as thoroughly as that of the MMS2 deleted mutant. The nonsense in the MMS2 gene of ‘uvs’ (mms2 mutant) causes ‘uvs’ (our data) to have a similar UV sensitivity to that of mms2Δ (published by others). Gangavarapu V, et al.’s_
paper (2006), displays the same results as ours. At 20 J/m², the UV sensitivity of rad50Δ is the same as that of rad50Δmms2Δ, but at higher dose, the UV sensitivity of the double gene mutant increases to more than that of rad50Δ (Figure 28A). Additionally, Torres-Ramos CA, et al. (2002), published the UV sensitivities of mms2Δ, rad5Δ, rad5Δmms2Δ, rad52Δ, and rad52Δmms2Δ. The sensitivities are comparable to the UV sensitivities of our ‘uvs’, rad5Δ, rad5Δ’uvs’, srl3Δuvs, rad52Δ, and rad52Δsrl3Δuvs strains (Figure 28A, 29A). The UV sensitivity of their mms2Δ is same as that of our mms2 mutant, ‘uvs’. Therefore, it is concluded that the nonsense mutation in the MMS2 gene of the ‘uvs’ strain, disrupts the UV repair function of Mms2 completely.

The ‘uvs’ strain contains a point mutation that changes conserved W in the catalytic domain to a termination signal, and causes truncation of Mms2. Adenine (base) at +452 in MMS2 is changed to guanine in the ‘uvs’ strain. This transition changes W encoding codon to a termination encoding codon, resulting in a truncated translation product. This shorter version of Mms2 that originated from a nonsense mutation may cause the phenotypes of ‘uvs’. However, there is another possibility. The lack of W may be the reason for the malfunction of Mms2, because W is the amino acid conserved throughout the catalytic domain of ubiquitin-conjugation enzymes (Figure 33). Therefore, it would be interesting to observe and compare the phenotypes of point mutants which contain amino acids other than W with those from a truncated Mms2 containing strain (‘uvs’). This would verify the importance of W in the function of Mms2.
Figure 33. The amino acid sequence alignment of catalytic domains of ubiquitin-conjugating enzymes. a and b represent gaps between catalytic domains. The names of different ubiquitin-conjugating enzymes are shown in the left panel. MMS2 is circled in blue. MMS2 differs from others in that it lacks conserved C (cys) which is underlined in red. Activation of this cys site is important for ubiquitin-conjugation. Additionally, conserved W, which was mutated in our ‘uvs’ strain, is underlined in blue. Taken from Mol Cell Biol. 2001 19:6537-48.
Figure 34. Evolutionary relationships among ubiquitin-conjugating enzymes. The numbers of inserted (‘a’ and ‘b’ in here are same with ‘a’ and ‘b’ in figure 17) and extended amino acids of ubiquitin-conjugating enzymes are presented, and UBC4 and UBC5 are standard enzymes. Mol Cell Biol. 2001 19:6537-48.
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176
Is it still possible that Srl3 has a role in increasing dNTP pool?

Our data demonstrates that the deletion of SRL3 does not influence the phenotypes (UV sensitivity and highly spontaneous mutability) of ‘uvs’ (mms2 mutant). Therefore, Srl3 cannot be involved in the repair mechanism. However, there is still the question of whether Srl3 plays a role in increasing dNTP pool. This is due to the fact that overexpression of Srl3 and high dNTP pool rescue the lethality of mec1Δ and rad53Δ (24, 31). RNR controls the level of dNTPs, and overexpression of Rnr1 rescues mec1Δ and rad53Δ lethality (31). Therefore, it is reasonable to compare the phenotype of srl3Δ with that of rnr1Δ. If Srl3 rescues mec1Δ or rad53Δ lethality by increasing the dNTP pool, the phenotype of srl3Δ and rnr1Δ will be similar. Our data show that srl3Δ has the same UV sensitivity as rnr1Δ: they do not have more UV sensitivity than WT (Figure 21). However, the ratio of spontaneous mutation rate of rnr1Δ to WT (2.99) is somewhat different from that of srl3Δ to WT (0.62) [Table 4]. Thus, the difference in spontaneous mutation rate of rnr1Δ and srl3Δ to WT argues against the role of Srl3 in dNTP pool up-regulation. To verify the participants of Srl3 in dNTP pool upregulation, the level of dNTP pool must be measured after Srl3 deletion and overexpression.

Conclusions and future studies for Mms2 and Srl3

We used srl3Δuvs instead of srl3Δ to study the function of Srl3. The SRL3 gene deletion strain purchased from Open Biosystem was mixed with srl3Δ and srl3Δuvs, and then we used srl3Δuvs for experiments blindly. Later, we noticed that we were using srl3Δuvs instead of srl3Δ. UVS is named by our laboratory for the second gene whose mutation causes UV sensitivity in srl3Δuvs. We researched the ‘uvs’ mutants and could
identify UVS as MMS2. The sequencing results tell us that the phenotype of ‘uvs’ is due to the nonsense mutation in the MMS2 gene. A point mutation caused the conversion of a tryptophan (W) codon to a stop codon, and caused truncated Mms2. As mentioned earlier, it will be interesting to observe whether the W is important for the function of Mms2, by changing W to other amino acids. It is because the W is the conserved amino acid throughout all of the ubiquitin-conjugating enzymes presented in Figure 34.

For Srl3, we concluded that it does not positively regulate dNTP pool. This was determined by comparing only the UV sensitivity and spontaneous mutation rate of srl3Δ and rnr1Δ. The spontaneous mutation rate of srl3Δ and rnr1Δ are different. However, it will be more useful to measure dNTP pool directly, in order to conclude that Srl3 does not play a role in increasing dNTPs.
V. REFERENCE


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