Antifolate Response in Replication Arrest Mutants of *Saccharomyces cerevisiae*

KEN DORNFELD

University of Minnesota Medical School, Duluth Campus, and Essentia Health Radiation Oncology, Duluth, MN, U.S.A.

Abstract. *Aim: Thymidine deprivation is a common cancer treatment. This study examines the role of replication arrest and uracil DNA repair in response to thymidine deprivation. Materials and Methods: Strains of S. cerevisiae deficient in various replication and DNA repair functions were tested for sensitivity to thymidine deprivation induced by the antifolate aminopterin. Cell survival and DNA content were assayed following drug treatment. Results: Most arrest mutants were more sensitive to aminopterin than was the parental strain. Inactivation of uracil glycosylase in arrest mutants led to a partial reduction in toxicity for some double-mutants. DNA content during exposure to aminopterin was similar in parental and single mutants. However, cells deficient in both arrest and uracil glycosylase functions exhibited continued DNA synthesis, suggesting that uracil glycosylase activity contributes to replication arrest during thymidine deprivation. Conclusion: Replication arrest and uracil DNA repair are important and overlapping determinants of cellular response to thymidine deprivation.*

Nucleotide pool limitations incite a number of responses in *Saccharomyces cerevisiae*. Depletion of deoxyribonucleotides (dNTPs), either by treatment with hydroxyurea or ribonucleotide reductase inactivation, disrupts replication resulting in S-phase arrest (1). DNA is structurally sensitive during replication arrest. It is unwound, partially single-stranded and significantly more vulnerable to damaging agents during S-phase arrest (2, 3).

A number of proteins are thought to be active in sensing and responding to replication stress caused by nucleotide pool depletion. These proteins serve to recognize replication difficulties or DNA damage, transmit appropriate signals and stabilize DNA. The roles of several proteins have been described during nucleotide pool depletion caused by decreased ribonucleotide reductase activity, which reduces the abundance of all four dNTPs. However, the role of these proteins in responding to selective depletion of thymidine has not been fully-elucidated.

Thymidine deprivation poses unique challenges. Thymidine monophosphate (dTMP) is produced by thymidylate synthase in a reaction that transfers a one-carbon group from reduced folate to dUMP to produce dTMP. Anti-folates block this reaction, leading to elevated levels of the precursor dUMP and subsequently of dUTP (4, 5). dUTP and dTTP are chemically similar and most DNA polymerases have poor discrimination between dUTP and dTTP. Therefore, when thymidylate synthase is inhibited, the dUTP level rises, the dTTP level drops and these conditions favor incorporation of dUTP into DNA (5, 6). Once incorporated into DNA, uracil becomes a target for uracil glycosylase DNA base repair, which involves base excision and strand incision. These activities could be harmful if performed on DNA already in vulnerable conformations due to stalled replication.

How do cells balance these potentially conflicting processes of cell-cycle arrest and uracil base excision repair? Understanding how arrest and repair activities are coordinated during thymidine deprivation may provide with unique insights into how these pathways interact. This study examines the role of replication arrest during thymidine deprivation. Interactions between arrest and uracil base excision repair are also examined.

Materials and Methods

Strains. Commercially available single-gene deletion strains of *S. cerevisiae* were used for this study. The parental strain BY4741 and deletion derivatives were obtained from Open Biosystems (Huntsville, AL, USA). Uracil DNA glycosylase mutant (*ung1*) strains were constructed by replacing the entire reading frame for *UNG1* with the selectable histidine biosynthetic gene *HIS3*.

Media. Cells were grown in minimal media supplemented with charcoal-adsorbed casamino acids, uracil and tryptophan and

Correspondence to: Ken Dornfeld, Essentia East Radiation Oncology, 400 E. Third Street, Duluth, MN, 55805, U.S.A. E-mail: Kenneth.dornfeld@essentiahealth.org

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adenine, as described previously (7). Thymidine deprivation was induced by adding sulfanilamide at 6 mg/ml to the media and resterilizing. Aminopterin was added at a final concentration of 200 μg per ml (7).

Thymidine deprivation. Cells were grown in supplemented minimal media overnight, diluted 1:100 and grown for 4-6 h. These log-phase cells were then diluted to 10^5 cells per ml in media containing sulfanilamide and aminopterin. Viable cell density (colony-forming units) was determined just after cells were added to the aminopterin culture and again after 24 hours. Cultures were diluted in water prior to plating to solid YPD media.

Survival calculations. The percentage survival was calculated by dividing the number of colony-forming units (viable cells) per ml at 24 hours by the colony-forming units per ml at time zero. Percentages shown in the tables are averages of a minimum of three independent cultures. Standard deviations were determined and Student's *t*-test performed to compare survival data.

Flow cytology analysis. Cultures were prepared for thymidine deprivation as described above with the exception of the use of a cell density of 10^6 cells per ml. Cultures were harvested at time 0 and 12 hours. Cells were lysed, stained for DNA content using propidium iodide and analyzed as described previously (7).

Table I. *Aminopterin sensitivity of S. cerevisiae mutants deficient in replication arrest. Mean survival of parental BY4741 and various replication checkpoint mutants following treatment with aminopterin. Survival was calculated as described in the Materials and Methods, SD is standard deviation. Mean survival rates for parental and single-mutants were compared and p-values are shown in the final column.*

Strain	Survival (%)	SD	Mutant *vs.* parent *p*-Value
BY4741	27	10	
tel1	43	10	0.002
mre11	12	0.4	0.02
rad50	10	5	0.009
xrs2	23	9	0.6
ddc1	7.4	6	0.00009
mec3	38	11	0.2
rad17	7.0	4.3	0.0002
rad24	7.8	3.2	0.0003
chk1	61	20	0.000021
rad9	39	29	0.14
mrc1	4.6	2.8	0.000013
tof1	0.63	0.31	9.1E-07
rad6	8.7	4.8	0.00019
dun1	4.6	5.8	6.4E-07
mgs1	31	6.1	0.58

Results

The role of S-phase and replication arrest in mediating the toxicity of thymidine deprivation was assessed by examining aminopterin sensitivity in mutants deficient in these processes. Mutants chosen were viable as a haploid deletion strain and had a defined role in S-phase or replication arrest (8). Parental and mutant strains were treated with the antifolate aminopterin, as described in Materials and Methods. Multiple cultures were tested for each strain and average survival values are presented in Table I. Most mutants display a significant sensitivity to aminopterin. Mutants lacking functions important for replication fork maintenance during replication stalling, including *tof1* and *mrc1*, are particularly sensitive to aminopterin. Mutants lacking *tel1* and *rad9* are exceptions and apparently not sensitive to aminopterin.

The *rad6* mutant was significantly more sensitive than the parental strain in this screening and by Game *et al*. in a screening for mutants sensitive to thymidine deprivation produced by the antifolate trimethoprim (9). Here, the aminopterin sensitivity of strains harboring mutations in various activities within the *RAD6* repair and ubiquitination pathways was determined. As shown in Table II, some of these mutant strains were sensitive to aminopterin. *UBI4*, coding for a poly-ubiquitin transcript, appears to play a critical role in responding to thymidine deprivation since ubi4 mutants are exquisitely sensitive to aminopterin. Indeed, *ubi4* and *doa1* mutants were more sensitive than strains deficient in other *RAD6* repair pathway activities. This implies a role for ubiquitin outside the *RAD6* pathway. Def1 functions to ubiquitinate RNA polymerase. *def1* mutants are very sensitive to aminopterin, suggesting multiple roles for ubiquitin in response to thymidine deprivation.

Uracil base excision repair plays a role in response to thymidine deprivation (5, 7, 10). Strains deficient for both arrest and uracil glycosylase functions were treated with aminopterin. Survival data for the double mutants in comparison to the uracil glycosylase proficient arrest single mutant are shown in Table III. The increase in aminopterin sensitivity of *rad17, rad24* and *tof1* single-mutants was partially relieved by the co-deletion of *ung1*. *rad50 ung1* double-mutant was marginally more sensitive to aminopterin than either single-mutant. *ung1* co-deletion did not uniformly reduce sensitivity to aminopterin, suggesting some arrest functions have a more direct interaction with uracil repair than others.

DNA content in various strains was examined using propidium iodine staining and flow cytometry during aminopterin treatment. The parental strain arrested in S-phase after 12 h of drug treatment. The patterns for *rad6* and *ung1* single-mutants both resembled the parental pattern. Interestingly, the *rad6 ung1* double-mutant appears to be able to continue DNA synthesis more than either single-mutant, as seen by an increase in the number of cells with greater than mid-S phase content of DNA (Figure 1). This suggests that the presence or activity of uracil glycosylase contributes to S-phase arrest during thymidine deprivation. Uracil

Table II. *Aminopterin sensitivity of S. cerevisiae mutants deficient in RAD6 pathway and ubiquitination activities. Mean survival of parental BY4741 and various ubiquitination mutants following treatment with aminopterin. Survival was calculated as described in Materials and Methods, SD is standard deviation. Mean survival rates for parental and single-mutants were compared and the p-value is shown in the final column.*

Strain	Survival (%)	SD	Mutant *vs*. parent *p*-Value
BY4741	27	10	
rad6	8.7	4.8	0.0002
rad18	22	1.8	0.5
bre1	25	2.6	0.06
doa1	1.1	0.06	0.0002
rad5	5.2	2.2	0.0003
lge1	28	8.3	0.9
ubc13	30	8.6	0.7
ubp8	39	13	0.05
mms2	33	8.3	0.4
rev1	52	36	0.01
rev3	74	9.7	1.1E-07
ubi4	1.2	0.7	0.0002
srs2	5.9	0.5	0.008
def1	12	10	2.7E-08

Table III. *Aminopterin sensitivity of paired mutants with and without uracil glycosylase. Mean survival values for wild-type UNG1 and mutant ung1 S. cerevisiae strains harboring additional mutations in various replication checkpoint functions. p-Values from comparisons for single-mutants compared to ung1 double-mutants are shown in the final column.*

Strain	Survival (%)	SD	Single-*vs*. double mutant *p*-Value
BY4741	27	10	
ung1-	36	6.7	0.097
tel1	43	10	
tel1 ung1	40	6.5	0.74
mre11	12	0.38	
mre11 ung1	15	2.9	0.18
rad50	10	4.9	
rad50 ung1	2.2	0.32	0.05
ddc1	7.4	5.8	
ddc1 ung1	4.5	1.9	0.38
rad17	7	4.4	
rad17 ung1	22	5.9	0.003
rad24	7.8	3.2	
rad24 ung1	20	6	0.016
mrc1	4.6	2.8	
mrc1 ung1	3.3	0.45	0.37
tof1	0.63	0.31	
tof1 ung1	5.8	1.1	3.2E-06
rad6	8.7	4.9	
rad6 ung1	2.9	0.42	0.16
dun1	4.6	5.8	
dun1 ung1	1.9	1.7	0.34

glycosylase may contribute to S-phase arrest in *rad6* mutants, and Rad6 may participate in arrest in uracil glycosylase mutants, but the double mutant lacks a normal arrest function. Similar findings were found for *rad17*, *mrc1* and *tof1* mutations in combination with *ung1* mutations (data not shown).

Discussion

Nucleotide pool imbalances pose a substantial threat to growing and dividing cells. dNTP depletion leads to stalled replication forks and activation of several S-phase checkpoint proteins (8, 11). Replication stress induced by hydroxyurea, which depletes all four dNTPs, has been a model system for studying the consequences of nucleotide pool depletion (2). Proteins participating in replication checkpoints, including those for DNA damage recognition, signaling, DNA repair and replication fork stabilization, are all important in managing the consequences of hydroxyurea treatment (12).

Proteins that play an important role in responding to hydroxyurea may also play an important role in response to selective dTTP depletion. Aminopterin is an anti-folate that inhibits the conversion of dUMP to dTMP leading to thymidine depletion. Many of the replication checkpoint mutants tested were found to be sensitive to aminopterin. *rad50* and *mre11* mutants, deficient in early stages of DNA damage recognition are sensitive to aminopterin. Mutants lacking various functions in replication fork stabilization (*tof1, mrc1, rad17, ddc1* and *rad24*) are also sensitive to aminopterin. *chk1* mutants are not sensitive to aminopterin despite Chk1 having a role in replication fork protection (GO annotation, SGD database, http://www.yeastgenome.org/). *chk1* mutants are not sensitive to hydroxyurea either (12), suggesting Chk1 is not essential in responding to the type of DNA damage caused by these drugs. Simon *et al*. also found *rad50* and *rad17* to be sensitive to a methotrexate analog (13).

Thymidylate synthase inhibition by raltitrexed in human cancer cell lines induced DNA damage foci containing RAD51, RPA, pChk1, pNBS1, and phosphorylated histone H2AX (14). Reducing the levels of ATM and ATR in colon cancer cell lines through siRNA leads to increased sensitivity to thymidine deprivation (15). These data in human cancer cell lines, and the data presented here confirm the importance of the replication checkpoint function in mediating the response to thymidine deprivation.

Inhibiting the conversion of dUMP to dTMP may increase levels of dUMP and therefore dUTP. Rising dUTP levels lead to the possibility of dUTP incorporation into DNA (5, 6). Uracil is removed from DNA by activities such as strand incision, which may create additional DNA damage and toxicity. Uracil glycosylase has been identified at the site of

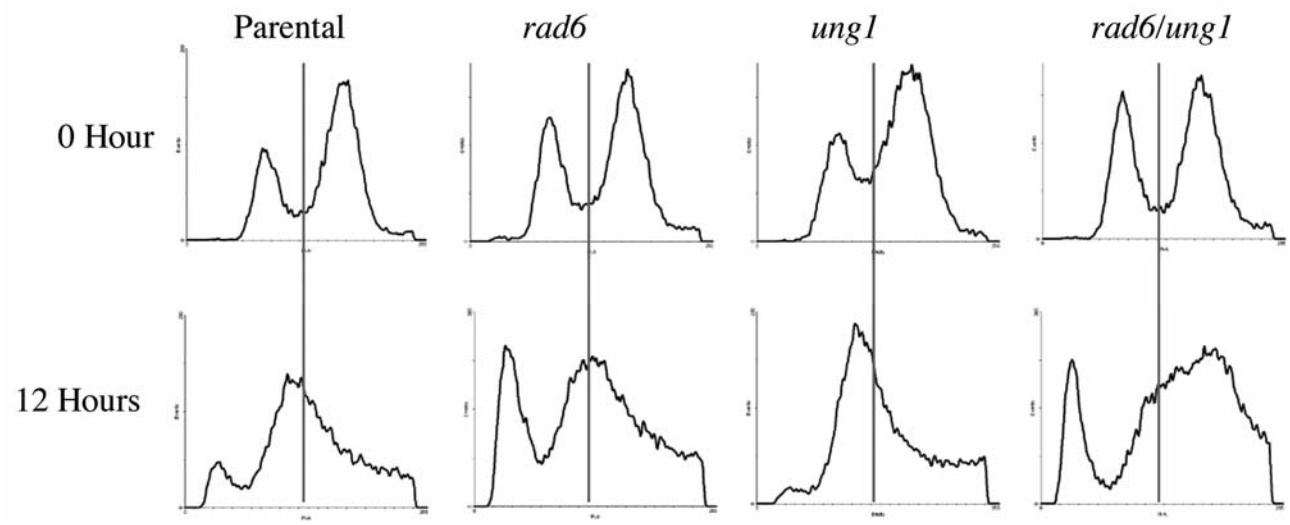

Figure 1. *Cultures of either BY4741, or rad6, ung1 or rad6/ung1 mutant S. cerevisiae were treated with aminopterin, harvested and analyzed by propidium iodide staining and flow cytometry as described in the Materials and Methods. Results are shown by strain (column) and time (rows).*

active replication forks (16). Uracil glycosylase activity contributes to aminopterin toxicity in *rad17*, *rad24*, and *tof1* mutants because simultaneous deletion of *ung1* from these replication checkpoint mutants alleviates some of their aminopterin sensitivity. While *ung1* mutants have close to parental sensitivity to aminopterin (5, 17), mutants lacking the second step in uracil base excision repair, *apn1*, are exquisitely sensitive to thymidine deprivation (7, 10). Collura *et al*. have also described functional interactions between Rad17 and uracil glycosylase (18). Their data show cells lacking *rad17* and *apn1* to have high mutation rates that are dependent on a functional uracil glycosylase. High spontaneous mutation rates or increased aminopterin sensitivity both suggest that uracil glycosylase activity in *rad17* mutants is potentially toxic, likely through difficulty in managing apyrimidinic sites.

Flow cytometric data suggest that uracil glycosylase contributes to cell-cycle arrest, at least in the absence of a fully functional replication checkpoint. DNA synthesis is halted in early S-phase following aminopterin treatment in parental, *rad6* and *ung1* cells, but progresses to late S or G_2 content of DNA per cell in the *rad6 ung1* double-mutant. This finding implies *rad6* and uracil glycosylase may have redundant abilities to halt progression of DNA synthesis. Overexpression of uracil glycosylase in *S. pombe* also leads to cell-cycle arrest (19). Uracil glycosylase binds tightly to apurinic and apyrimidinic sites in DNA until displaced by Apn1 (20). Uracil glycosylase bound to DNA may serve as a replication block. Alternatively, apurinic and apyrmidinic sites generated by uracil glycosylase could block replication (21). Additional studies are required to further define how uracil glycosylase contributes to replication arrest.

An important role for the *RAD6* pathway in mediating thymineless stress was identified over 35 years ago (9). The *RAD6* pathway includes multiple activities involved with error prone and error-free post-replication DNA repair. Members of this group have helicase activity (*SRS2* and *RAD5*), DNA polymerase activity (*REV1*, *REV3*) and ubiquitin ligase activities (*MMS2, UBC13, RAD5, RAD18*). The sensitivity of *rad5* mutant suggests the key defect responsible for sensitivity for members of the *RAD6* pathway lies in the ability of *Rad5* to resolve stalled replication forks (22). Srs2 is also a helicase and mutants show aminopterin sensitivity, supporting the importance of helicase activity in coping with thymidine deprivation.

Mutants with lower ubiquitin-producing capacity (*doa1* and *ubi4*) are more sensitive than mutants deficient in *RAD6* pathway functions. The relative insensitivity of mutant *RAD6* pathway members (*e.g. bre1*, *mms2*) suggests that inability to ubiquitinate targets such as PCNA or histone H2b is not a major impediment to survival during aminopterin treatment. Other targets of ubiquitination, such as RNA polymerase, may be even more important. Def1 is responsible for RNA polymerase ubiquitination in the presence of DNA damage and loss of *def1* renders cells sensitive to aminopterin. *ubi4* mutants are sensitive to oxidative stress (23) and thymidine deprivation may create oxidative stress due to preferential mitochondrial damage (24). Over 400 proteins are modified by ubiquitin (25), leaving many potential candidate proteins to explain the sensitivity of *ubi4* and *doa1*.

Thymidineless death continues to be an intriguing puzzle. Continued efforts to understand thymidineless death will hopefully lead to a better understanding of how repair, arrest and recovery interact during replication stress. Activities beyond DNA replication and repair including transcription, mitochondrial function, amino-acid metabolism and others, may also play a significant role in response to thymidine deprivation (12, 25, 26, 27). Understanding thymidineless death will also hopefully lead to improved outcome for thousands of patients with cancer whose treatment is based on thymidine deprivation.

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References

1 Diffley JF, Bousset K, Labib K, Noton EA, Santocanale C and Tercero JA: Coping with and recovering from hydroxyurea-induced replication fork arrest in budding yeast. Cold Spring Harb Symp Quant Biol *65*: 333-342, 2000.
2 Grallert B and Boye E: The multiple facets of the intra-S checkpoint. Cell Cycle *7(15)*: 2315-2320, 2008.
3 Shechter D, Costanzo V and Gautier J: Regulation of DNA replication by ATR: Signaling in response to DNA intermediates. DNA Repair *3(8-9)*: 901-908, 2004.
4 Berger SH, Pittman DL and Wyatt MD: Uracil in DNA: consequences for carcinogenesis and chemotherapy. Biochem Pharmacol *76(6)*: 697-706, 2008.
5 Tinkelenberg BA, Hansbury MJ and Ladner RD: dUTPase and uracil-DNA glycosylase are central modulators of antifolate toxicity in *Saccharomyces cerevisiae*. Cancer Res *62(17)*: 4909-4915, 2002.
6 Richardson CC, Schildkraut CL and Kornberg A: Studies on the replication of DNA by DNA polymerases. *In*: Synthesis and Structure of Macromolecules, 1st edn. Vol. XXVIII Frisch L (ed) Cold Spring Harbor Symposia on Quantitative Biology Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press p. 629, 1963.
7 Dornfeld K and Johnson M: AP endonuclease deficiency results in extreme sensitivity to thymidine deprivation. Nucleic Acids Res *33(20)*: 6644-6653, 2005.
8 Putnam CD, Jaehnig EJ and Kolodner RD: Perspectives on the DNA damage and replication checkpoint responses in *Saccharomyces cerevisiae*. DNA Repair *8(9)*: 974-982, 2009.
9 Game JC, Little JG and Haynes RH: Yeast mutants sensitive to trimethoprim. Mutat Res *28(2)*: 175-182, 1975.
10 Seiple L, Jaruga P, Dizdaroglu M and Stivers JT: Linking uracil base excision repair and 5-fluorouracil toxicity in yeast. Nucleic Acids Res *34(1)*: 140-151, 2006.
11 Lisby M and Rothstein R: Localization of checkpoint and repair proteins in eukaryotes. Biochimie *87(7)*: 579-589, 2005.
12 Hartman JL and Tippery NP: Systematic quantification of gene interactions by phenotypic array analysis. Genome Biol *5(7)*: R49, 2004.
13 Simon JA, Szankasi P, Nguyen DK, Ludlow C, Dunstan HM, Roberts CJ, Jensen EL, Hartwell LH and Friend SH: Differential toxicities of anticancer agents among DNA repair and checkpoint mutants of *Saccharomyces cerevisiae*. Cancer Res *60(2)*: 328-333, 2000.
14 Yang Z, Waldman AS and Wyatt MD: DNA damage and homologous recombination signaling induced by thymidylate deprivation. Biochem Pharmacol *76(8)*: 987-996, 2008.
15 Geng L, Huehls AM, Wagner JM, Huntoon CJ and Karnitz LM: Checkpoint signaling, base excision repair, and PARP promote survival of colon cancer cells treated with 5-fluorodeoxyuridine but not 5-fluorouracil. PLoS One *6(12)*: e28862, 2011.
16 Otterlei M, Warbrick E, Nagelhus TA, Haug T, Slupphaug G, Akbari M, Aas PA, Steinsbekk K, Bakke O and Krokan HE: Post-replicative base excision repair in replication foci. EMBO J *18(13)*: 3834-3844, 1999.
17 Luo Y, Walla M and Wyatt MD: Uracil incorporation into genomic DNA does not predict toxicity caused by chemotherapeutic inhibition of thymidylate synthase. DNA Repair *7(2)*: 162-169, 2008.
18 Collura A, Auffret Van Der Kemp P and Boiteux S: Abasic sites linked to dUTP incorporation in DNA are a major cause of spontaneous mutations in absence of base excision repair and Rad17-Mec3-Ddc1 (9-1-1) DNA damage checkpoint clamp in *Saccharomyces cerevisiae*. DNA Repair *11(3)*: 294-303, 2012.
19 Elder RT, Zhu X, Priet S, Chen M, Yu M, Navarro JM, Sire J and Zhao Y: A fission yeast homologue of the human uracil-DNA-glycosylase and their roles in causing DNA damage after overexpression. Biochem Biophys Res Commun *306(3)*: 693-700,2003.
20 Parikh SS, Mol CD, Slupphaug G, Bharati S, Krokan HE and Tainer JA: Base excision repair initiation revealed by crystal structures and binding kinetics of human uracil-DNA glycosylase with DNA. EMBO J *17(17)*: 5214-5226, 1998.
21 Boiteux S and Guillet M: Abasic sites in DNA: repair and biological consequences in *Saccharomyces cerevisiae*. DNA Repair *3(1)*: 1-12, 2004.
22 Minca EC and Kowalski D: Multiple Rad5 activities mediate sister chromatid recombination to bypass DNA damage at stalled replication forks. Mol Cell *38(5)*: 649-661, 2010.
23 Cheng L, Watt R and Piper PW: Polyubiquitin gene expression contributes to oxidative stress resistance in respiratory yeast (*Saccharomyces cerevisiae*). Mol Gen Genet *243(3)*: 358-362, 1994.
24 Barclay BJ, Kunz BA, Little JG and Haynes RH: Genetic and biochemical consequences of thymidylate stress. Can J Biochem. *60(3)*: 172-184, 1982.
25 Starita LM, Lo RS, Eng JK, von Haller PD and Fields S: Sites of ubiquitin attachment in *Saccharomyces cerevisiae*. Proteomics *12(2)*: 236-240, 2012.
26 Gresham D, Boer VM, Caudy A, Ziv N, Brandt NJ, Storey JD and Botstein D: System-level analysis of genes and functions affecting survival during nutrient starvation in *Saccharomyces cerevisiae*. Genetics *187(1)*: 299-317, 2011.
27 Fischer JA, Muller-Weeks S and Caradonna S: Proteolytic degradation of the nuclear isoform of uracil-DNA glycosylase occurs during the S phase of the cell cycle. DNA Repair *3(5)*: 505-513, 2004.

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