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RAD2 and *PUF4* Regulate Nucleotide Metabolism Related Genes, *HPT1* and *URA3*

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Abstract

Yeast *RAD2*, a yeast homolog of human *XPG* gene, is an essential element of nucleotide excision repair (NER), and its deletion confers UV sensitivity and NER deficiency. 6-Azauracil (6AU) sensitivity of certain *rad2* mutants revealed that *RAD2* has transcription elongation function. However, the fundamental mechanism by which the *rad2* mutations confer 6AU sensitivity was not clearly elucidated yet. Using an insertional mutagenesis, *PUF4* gene encoding a yeast pumilio protein was identified as a deletion suppressor of *rad2*Δ 6AU sensitivity. Microarray analysis followed by confirmatory RT-qPCR disclosed that *RAD2* and *PUF4* regulated expression of *HPT1* and *URA3*. Overexpression of *HPT1* and *URA3* rescued the 6AU sensitivity of *rad2*Δ and *puf4*Δ mutants. These results indicate that 6AU sensitivity of *rad2* mutants is in part ascribed to impaired expression regulation of genes in the nucleotide metabolism. Based on the results, the possible connection between impaired transcription elongation function of *RAD2*/*XPG* and Cockayne syndrome via *PUF4* is discussed.

Keywords: *RAD2/XPG*, Cockayne syndrome, *PUF4*, Transcription

The human *XPG* gene is an essential element of nucleotide excision repair (NER). Consequently, its deletion confers UV sensitivity and NER deficiency. C-terminal deletions of *XPG* confer Cockayne syndrome (CS) that is characterized by retarded growth, impaired neurological development, mental retardation, and premature aging[1]. Except *XPG*, four other CS related genes were widely recognized to have functions in transcription[1]. Thus, the connection between CS and *XPG* had been a baffling problem.

6-Azauracil (6AU), a nucleotide analog that decreases GTP and UTP level in the cellular nucleotide pool[2], is a well known factor that interrupts transcription in the mutants with impaired transcription elongation factor. Thus, 6AU sensitivity is used as an indicator of transcription elongation function of a gene. Yeast *RAD2* gene is a yeast homolog of human *XPG*. Investigation of transcription elongation in *RAD2* mutations that mimic the XPG/CS mutation with 6AU revealed that *RAD2* had transcription elongation function[3]. Later, XPG was also found to be involved in transcription[4,5]. However, the mechanism by which the interrupted transcription due to mutations of CS related genes causes the symptoms of CS is not clearly understood yet.

In order to understand the detailed mechanism of obstructed transcription elongation by *rad2* mutations, the deletion suppressor of *rad2*Δ 6AU sensitivity was searched using a yeast transposon insertion library. As the result, *PUF4* gene, a yeast pumilio gene, was isolated. Deletion of *puf4* gene reverted 6AU sensitivity of *rad2*Δ mutant. In order to investigate the fundamental basis for restored 6AU resistance of *rad2*Δ mutant by *puf4* deletion, the gene expression pattern of *rad2*Δ*puf4*Δ mutant was compared to that of *rad2*Δ mutant using microarray analysis. Among many genes that were expressed differentially in wildtype, *rad2*Δ, *puf4*Δ, and *rad2*Δ*puf4*Δ strains, two genes involved in the nucleotide metabolism was analyzed further. Genetic analysis and induced gene expression of the two genes revealed that *RAD2* and *PUF4* regulate the expression of the genes.

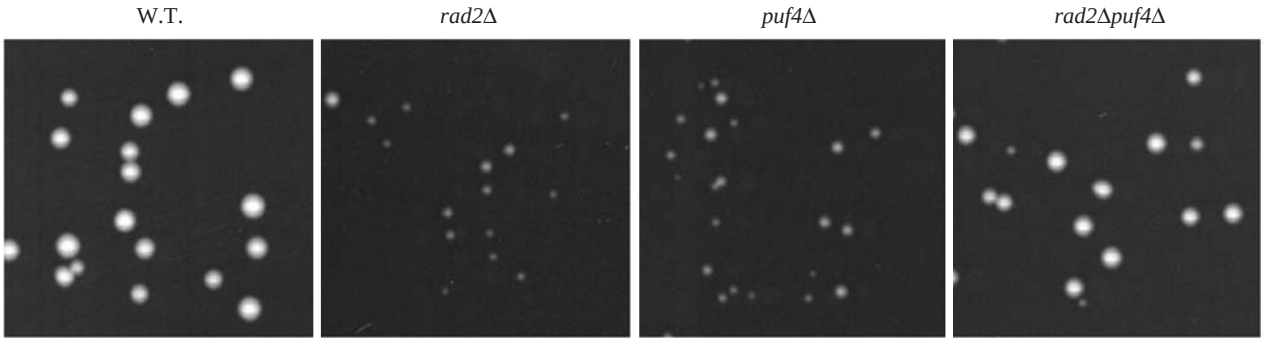

Figure 1. 6AU sensitivity of *rad2*Δ is reverted by *puf4* deletion. Yeast cells were plated on the uracil-depleted synthetic medium containing 75 μg/mL of 6AU. *rad2*Δ*puf 4*Δ mutants are resistant to 6AU while *rad2*Δ and *puf4*Δ mutants are very sensitive to 6AU.

Table 1. Relative expression of microarray-picked genes in microarray analysis and RT-qPCR.

Gene Name	*rad2*Δ*puf4*Δ*-1**	*rad2*Δ*puf4*Δ*-2**	*rad2*Δ/WT**	*rad2*Δ*puf4*Δ/WT**	*puf4*Δ/WT**
ORF:YBL086C	0.298	0.584	126.5	77.3	128.9
PHO3	2.820	1.726	90.6	170.8	78.8
ORF:YDL038C	2.890	2.174	95.6	143.0	123.3
PRM7	2.827	2.089	48.0	59.1	47.0
TPI1	3.587	1.638	80.1	103.6	66.4
HPT1	2.606	4.478	39.4	107.8	37.6
ORF:YDR539W	0.338	0.336	98.7	54.8	78.1
GLC3	0.598	0.387	150.7	244.5	329.5
URA3	3.831	2.501	21.0	57.9	26.8
MET6	2.764	1.850	77.7	290.1	74.8
GSY1	0.423	0.317	136.3	115.4	214.7
PUF4	0.203	0.218	57.7	0.4	0.3
ORF:YGL015C	11.050	7.882	121.8	594.0	642.8
ORF:YGR024C	0.387	0.623	108.3	78.3	103.3
SPT4	2.852	1.760	73.7	252.6	165.8
NSR1	1.956	2.589	62.7	197.4	111.1
OM45	0.462	0.358	170.1	127.6	171.8
MUC1	0.520	0.380	192.7	499.0	532.9
ORF:YLL044W	3.546	1.950	230.4	939.3	369.6
ILV5	3.803	2.729	78.2	263.1	123.2
PGM2	0.346	0.543	325.6	524.0	652.8
AQR1	3.675	1.773	68.9	115.0	86.7
ORF:YNL114C	6.396	1.541	156.3	275.7	112.1
RPL18B	3.900	1.796	111.0	420.7	82.7
RPL18A	2.251	1.540	40.8	63.8	14.4
STI1	0.309	0.623	99.5	62.0	26.1
GAC1	0.577	0.400	113.4	111.6	187.8
NOP58	2.606	1.608	72.9	114.3	81.0
NOG1	2.726	1.559	82.4	241.2	49.5
ORF:YPL251W	2.657	1.569	105.6	161.1	180.6

*Microarray analysis results. Results shown are expression ratio calculated by *rad*Δ*2puf4*Δ/*rad2*Δ.
**RT-qPCR results. Results shown are % gene expression level in each mutant against wildtype. Italicized data indicates discordance between microarray and RT-qPCR ones.

6AU Sensitivity of *rad2*Δ is Reverted by Deletion of a Pumilio Gene, *PUF4*

In order to find the genes that rescue 6AU sensitivity of *rad2*Δ, a yeast transposon insertion library was transformed into *rad2*Δ mutant, and 6AU resistant yeast colonies were isolated. Sequencing the flanking region of the library DNA insertion site revealed that the mutation of *PUF4* gene was the cause of reverted 6AU sensitivity. In order to verify the mutational effect of *PUF4* on *rad2*Δ 6AU sensitivity,

PUF4 was directly deleted. In accordance with the insertional mutation result, direct *puf4* deletion partially restored 6AU resistance of *rad2*Δ to the level of wildtype. Interestingly, *puf4* deletion itself caused 6AU sensitivity (Figure 1).

Many Genes are Differentially Expressed in *rad2*Δ and *rad2*Δ*puf4*Δ

The results that each *rad2* or *puf4* deletion confers 6AU sensitivity but *rad2*Δ*puf4*Δ recovers the sensitivity to near wildtype indicate that *RAD2* and *PUF4* may have conflicting effect on 6AU sensitivity or they modulate each other. In order to investigate the mechanism of 6AU resistance in *rad2*Δ*puf4*Δ, gene expression patterns between *rad2*Δ and *rad2*Δ*puf4*Δ after 2 hour 6AU treatment were compared by comparative hybridization of yeast genomic oligonucleotide microarray. Results from two independent experiments, one by direct comparison between *rad2*Δ and *rad2*Δ*puf4*Δ and the other by indirect comparison of *rad2*Δ/WT and *rad2*Δ*puf4*Δ/WT were analyzed. Twenty nine genes were selected for further analysis based on two-fold difference in either microarray analysis (Table 1).

Differential gene expression profile between *rad2*Δ and *rad2*Δ*puf4*Δ was confirmed by RT-qPCR of 2 hour 6AU treated yeasts cells (Table 1). RT-qPCR revealed that expression of 7 (24%) of the 29 microarray picked genes did not significantly change or the expression pattern was even reversed in opposite directions, which might be due to false positive of the microarray analysis. Among the 22 genes whose expression was changed in accordance both in microarray analysis and in RT-qPCR, expression of 5 genes were decreased and 17 genes were increased in *rad2*Δ*puf4*Δ cells in comparison with *rad2*Δ. Expression levels of 4 genes increased in *puf4*Δ cells were as high as in *rad2*Δ*puf4*Δ. Interestingly, *PUF4* expression in *rad2*Δ cells treated with 6AU was decreased by one half of the amount of wildtype cells implying that transcription elongation activity of *RAD2* was required for the expression of *PUF4*. This result implies that *RAD2* positively regulates *PUF4* expression.

Functional categorization of the 22 genes revealed that two involved in nucleotide metabolism, two in amino acid metabolism, three in protein biosynthesis and folding, two in rRNA processing and modification, one in transcription, one in transport and 11 in unknown function. Among them, one in protein folding and four in unknown function were down-regulated in 2 hour 6AU treated *rad2*Δ*puf4*Δ. 6AU reduces cellular GTP and UTP levels[2] and the defects in transcription elongation confer 6AU sensitivity. Consequently, one plain explanation of the restored 6AU resistance of *rad2*Δ by additional *puf4* deletion could be the altered regulation of nucleotide metabolism in the cell. Indeed, the microarray analysis demonstrated that *HPT1* and *URA3* that functions in the nucleotide metabolism were differentially expressed after 6AU treatment in *rad2*Δ and *rad2*Δ*puf4*Δ (Figure 2 and Table 1). For that reason, *HPT1* and *URA3* were selected for further analysis.

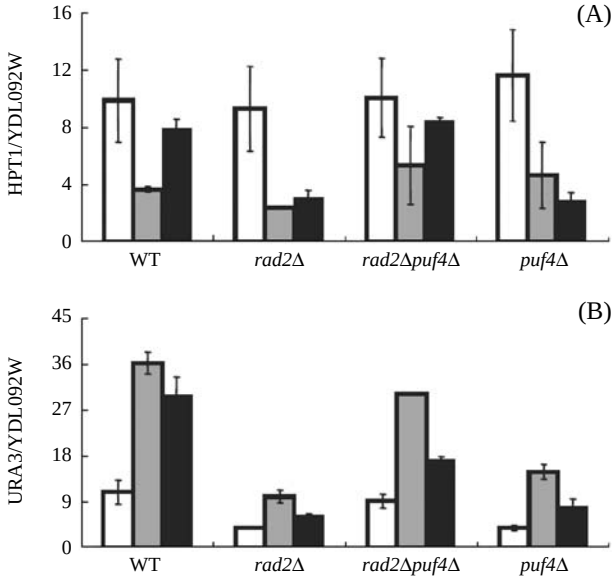

Figure 2. *HPT1* and *URA3* mRNA expression before and after 6AU treatment. Wildtype, *rad2*Δ, *puf4*Δ, and *rad2*Δ*puf4*Δ cells were treated with 6AU for indicated duration. Then, the altered mRNA expression pattern before and after 6AU treatment was analyzed using qRT-PCR. (A) *HPT1* mRNA expression. (B) *URA3* mRNA expression. open bar: without 6AU treatment, gray bar: 1 hour after 6AU treatment, black bar: 2 hours after 6AU treatment. Each experiment was repeated two times.

Purine and Pyrimidine Metabolism Related Genes are Differentially Expressed in *rad2* and *puf4* Deletion Mutants

The *HPT1* and *URA3* genes function in nucleotide salvage pathway and nucleotide *de novo* synthesis, respectively[6,7]. Also, mRNAs of *HPT1* and *URA7*/*URA8* that functions in the same pathway as *URA3* were identified as the targets of Puf4p binding[8] although the *ipso facto* effect of the binding was not identified. The microarray analysis disclosed that, in the presence of 6AU, both *HPT1* and *URA3* genes were down regulated in the *rad2*Δ and *puf4*Δ single mutants, while their expression levels in *rad2*Δ*puf4*Δ were higher than those of wildtype (Table 1). In order to examine the effect of 6AU treatment on the differ-

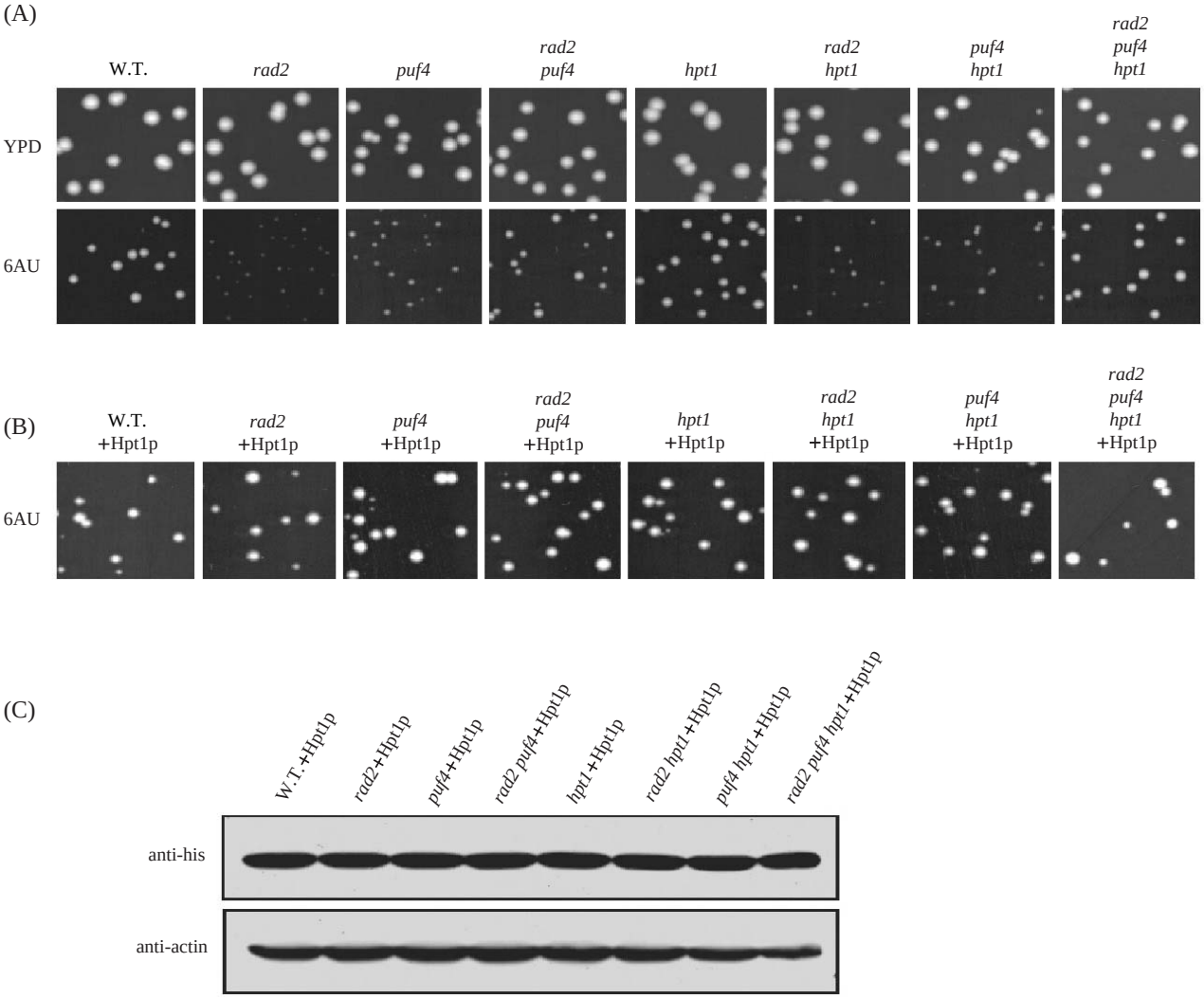

Figure 3. *HPT1* deletion and expression affect on cell growth of *rad2*Δ and *puf4*Δ. (A) Overnight cultured yeast cells were plated on YPD (top), and the uracil-depleted synthetic medium containing 75 μg/mL of 6AU (bottom). All the tested strains formed colonies well on YPD. *hpt1*Δ does not confer 6AU sensitivity. 6AU sensitivity of *rad2*Δ*hpt1*Δ and *rad2*Δ*puf4*Δ*hpt1*Δ are slightly restored. (B) All the strains used in A were transformed with *HPT1* expression plasmid. After induced expression of *HPT1*, cells were plated on the 75 μg/mL 6AU containing uracil depleted synthetic medium. *HPT1* induction restored 6AU sensitivity of all the tested mutants to the level of wildtype. (C) Expression of *HPT1* in the strains used in B was confirmed by Western analysis.

ential expression of *HPT1* and *URA3*, the expression levels of *HPT1* and *URA3* genes were analyzed in wildtype, *rad2*Δ, *puf4*Δ, and *rad2*Δ*puf4*Δ by RT-qPCR. In the absence of 6AU, *HPT1* expression level was similar in all four tested strains (wildtype, *rad2*Δ, *puf4*Δ and *rad2*Δ*puf4*Δ; Figure 2A). 6AU treatment initially decreased *HPT1* expression in all 4 strains. However, the decreased expression of *HPT1* in 2 hour 6AU treated wildtype and *rad2*Δ*puf4*Δ mutant was recovered to the level of untreated wildtype, while both *rad2*Δ and *puf4*Δ mutants were not able to restore the expression level (Figure 2A). On the other hand, *URA3* expression was lower in *rad2*Δ and *puf4*Δ mutants in the absence of 6AU treatment while its expression in *rad2*Δ*puf4*Δ mutant was similar to that of wildtype (Figure 2B). After 1 hour of 6AU treatment, *URA3* expression in all 4 strains was dramatically increased, but the 6AU induced expression levels were much lower in *rad2*Δ and *puf4*Δ compared to those of wildtype and *rad2*Δ*puf4*Δ. By 2 hours of 6AU treatment, *URA3* mRNA level was decreased in all four strains, but the level was maintained much higher than that of the untreated control (Figure 2B). These results indicate that *RAD2* and *PUF4* function

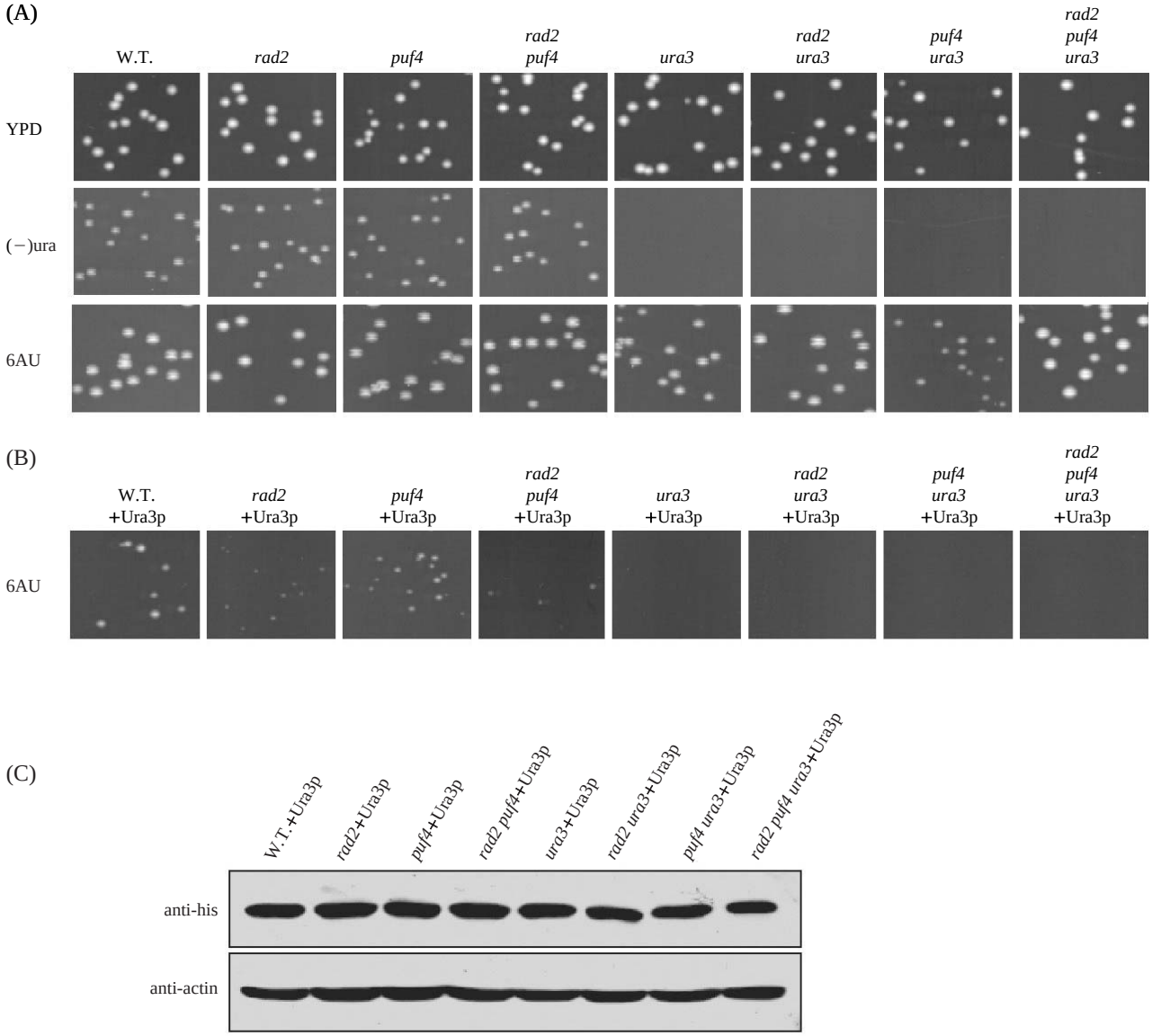

Figure 4. *URA3* deletion and expression affect on cell growth of *rad2*Δ and *puf4*Δ. (A) Overnight cultured yeast cells were plated on YPD medium (top) and the uracil-depleted synthetic medium (middle). All the tested strains formed colonies well on YPD. *ura3* deletion cells did not grow on the uracil-depleted medium. The same overnight cultured cells were plated on the synthetic complete medium containing 75 μg/mL of 6AU (bottom). Because the medium contains uracil, 6AU sensitivity of most strains was not easily recognizable. However, *puf4*Δ*ura3*Δ mutant was distinctively sensitive to 6AU. (B) All the strains used in A were transformed with *URA3* expression plasmid. After induced expression of *URA3*, cells were plated on the 6AU-containing tryptophan and uracil depleted medium. Effects of 6AU resistance by *URA3* induction varies in each mutant. (C) Expression of *URA3* in the strains used in B was confirmed by Western analysis.

together to increase *HPT1* and *URA3* expression in the presence of 6AU, and lack of either *RAD2* or *PUF4* causes negative influence on *HPT1/URA3* expression.

Effects of *HPT1* and *URA3* Deletion on 6AU Sensitivity of *rad2*Δ and *puf4*Δ

The expression profile of *HPT1* and *URA3* after 6AU treatment revealed that their mRNA levels after 6AU treatment were much lower in *rad2*Δ and *puf4*Δ than in wildtype, while those of *rad2*Δ*puf4*Δ were parallel to that of wildtype (Table 1). The decreased *HPT1* and *URA3* mRNA level in *rad2*Δ and *puf4*Δ is probably a cause of the decreased cell growth of *rad2*Δ and *puf4*Δ in the presence of 6AU (Figure 1). From these results, it was inferred that deletion of

either gene in the *rad2*Δ or *puf4*Δ mutants would not change 6AU sensitivity of the strains since *hpt1* or *ura3* deletion would act as the decreased mRNA level. On the other hand, *hpt1* or *ura3* deletion would increase the 6AU sensitivity of *rad2*Δ*puf4*Δ cells because *hpt1* or *ura3* deletion would obstruct their typically increased expression in *rad2*Δ*puf4*Δ after 6AU treatment. To test this, *HPT1* and *URA3* genes were deleted in wildtype, *rad2*Δ, *puf4*Δ, and *rad2*Δ*puf4*Δ cells, and then the effect of these deletions on 6AU sensitivity was examined.

As predicted, *hpt1* deletion did not confer 6AU sensitivity or have any influence on 6AU sensitivity of *puf4*Δ. Unexpectedly, however, *hpt1* deletion slightly restored 6AU sensitivity in the *rad2*Δ and *rad2*Δ*puf4*Δ mutants so that *rad2*Δ*puf4*Δ*hpt1*Δ conferred 6AU resistance to the level of wildtype (Figure 3A bottom panel). Since *ura3*Δ cells were unattainable to grow on uracil depleted medium (Figure 4A middle panel), 6AU sensitivity of *ura3*Δ were examined on a synthetic complete medium supplemented with 6AU. Unfortunately, however, 6AU sensitivity of the mutants with *ura3* deletion was not easily validated in most cells because of the presence of uracil on the test medium. Nevertheless, the result clearly revealed that *puf4*Δ*ura3*Δ cells were more sensitive to 6AU than either *puf4*Δ or *ura3*Δ cells (Figure 4A bottom panel). This result was unexpected since the *puf4*Δ*hpt1*Δ mutant displayed similar 6AU sensitivity to *puf4*Δ. This result indicates that functional *PUF4* is necessary for restoring 6AU resistance when *de novo* biosynthesis of ribonucleotide is impaired. Also, that result provides possible clues for the fundamental basis of 6AU sensitivity of *puf4*Δ. It is possible that the genes in the nucleotide salvage pathway are positively regulated by *PUF4*.

Effects of *HPT1* and *URA3* Overexpression on 6AU Sensitivity of *rad2*Δ and *puf4*Δ

The expression patterns of *HPT1* and *URA3* upon 6AU treatment analyzed by microarray analysis and RT-qPCR suggest that the 6AU sensitivity of *rad2*Δ and *puf4*Δ mutants are due to the decreased expression of *HPT1* and *URA3* and that *HPT1* and *URA3* expression are regulated by both *RAD2* and *PUF4*. In order to substantiate that, the *HPT1* and *URA3* expression plasmids were constructed and the genes were expressed in all the strains used in Figure 3A and Figure 4A, respectively. Expression of both *HPT1* and *URA3* was confirmed by Western analysis (Figures 3C, 4C). As predicted, the expression of *HPT1* restored 6AU sensitivity of all the tested mutants to the level of wildtype (Figure 3B). However, restored 6AU resistance of the mutants by *URA3* expression was varies (Figure 4B). *puf4*Δ recovered the 6AU resistance by *URA3* expression to similar level of wildtype. *URA3* expression slightly increased 6AU resistance of *rad2*Δ and *rad2*Δ*puf4*Δ but the level was much lower than that of wildtype. Interestingly, 6AU resistance of *puf4*Δ*ura3*Δ was barely restored by *URA3* expression. Moreover, *ura3*Δ and *rad2*Δ*ura3*Δ did not recover 6AU sensitivity at all. These results indicates that down-regulation of *HPT1* and *URA3* in 6AU treated *rad2*Δ and *puf4*Δ should elicit in 6AU sensitivity of them, but *URA3* involved nucleotide biosynthesis mechanism regulation has more complex effect on 6AU sensitivity.

Discussion

In order to understand the molecular mechanisms of 6AU sensitivity in *rad2*Δ, an insertion mutagenesis was applied. As the result, *PUF4* gene was identified as a deletion suppressor of *rad2*Δ 6AU sensitivity. Although a yeast aging study showed that *puf4* deletion decreased lifespan[9], the responsible biological mechanisms were not clearly understood. Recently, several studies elucidated the cellular functions of PUF proteins. PUF family proteins are found in most eukaryote, have characteristic RNA binding domains, and are known as translational regulators[10-13]. Yeast has five PUF genes that regulate mRNA stability by binding to the target mRNAs[14-17]. A microarray analysis using an affinity tag revealed that each PUF protein has a specific group of target mRNAs to binds[8]. Puf4p preferentially binds to mRNAs of nucleolar ribosomal RNA-processing factors[8]. The biological effect of Puf4p binding to mRNA was shown in the post-transcriptional regulation of *HO* gene. Puf4p represses translation and enhances removal of polyA of mRNA encoding HO endonuclease by binding to its 3′ untranslated region. Consequently, *puf4* deletion increased stability of HO mRNA[18]. Translational regulation of proteins by PUF proteins may allow cells to properly respond to the environmental changes. The target mRNA specificity of each PUF proteins indicates that different sets of proteins are controlled together by each PUF protein[8].

Even though understanding on post-transcriptional regulation by PUF proteins has been broadened, the regulation of PUF gene expression has not been studied that much. A recent study enlightened the expression regulation of *PUF5* gene, one of five PUF genes in yeast that is closely related to *PUF4*. *PUF5* expression is impaired when a transcription elongation factor activity of yeast *CEG1* (encoding a capping enzyme) is compromised[19], indicating that *CEG1* func-

tions as a positive regulator of *PUF5* expression. Our result showing decreased *PUF4* mRNA level in *rad2*Δ strongly indicates that *RAD2* should function as a positive regulator of *PUF4* expression. *RAD2* appears to function as a positive transcription elongation factor for many genes[3]. Thus, this result implies that transcription elongation function of *RAD2* possibly regulates *PUF4* expression. Meanwhile, our results showing variable changes in expression level of many genes in *puf4*Δ after 6AU treatment (Table 1) indicate that Puf4p might either up-regulate or down-regulate its target mRNAs even though Puf4p is known to destabilize some mRNA[18].

In consideration of known 6AU effect on cellular NTP pools and reported binding activity of Puf4p to mRNAs of nucleolar ribosomal RNA-processing factors, differential expression of genes of nucleotide metabolism and the ribosomal RNA-processing factors was anticipated. Indeed, two genes in nucleotide metabolism and three in nucleolar RNA processing were found up-regulated by microarray analysis and RT-qPCR (Table 1). Especially, *HPT1* and *URA3* were interested because they function in the nucleotide salvage pathway and the *de novo* biosynthesis of nucleotide, respectively[20,21]. Moreover, mRNAs of *HPT1* and two genes in *URA3* related pathway were identified as the targets of Puf4p binding[8] although the biological effect of the binding remains to be defined.

RT-qPCR results showing that either *RAD2* or *PUF4* deletion did not decrease *HPT1* expression, but did decrease *URA3* expression, indicated that the transcription elongation function of *RAD2* and the mRNA stabilization activity of *PUF4* were not necessary for the nucleotide biosynthesis pathway although both genes were required for the nucleotide salvage pathway in the absence of 6AU. However, *RAD2* and *PUF4* are necessary to increase the expression of genes in both the nucleotide biosynthesis and the salvage pathways when the nucleotide pool is limited by 6AU treatment. This result strongly implies that *RAD2* and *PUF4* genes function together for the regulation of *HPT1* and *URA3* when the nucleotide pool is limited by 6AU.

HPT1 and *URA3* expression was expected to complement 6AU sensitivity of all the mutants used, but 6AU sensitivity of some mutants was not recovered by *URA3* expression at all (Figure 4B). This is possibly due to the negative effect of overexpressed Ura3p. Ura3p converts orotidine-5′-phosphate to UMP that eventually converted to CTP by Ura7p and Ura8p[22-24]. In our experimental system, *URA3* is overexpressed by galactose induction in which *URA7/URA8* expression is not induced. Thus, discrepancy between *URA3* and *URA7/URA8* expression regulation can cause problems in the pyrimidine metabolism that can be implicated to regulation of cell proliferation.

RAD2 expression is changed in response to many environmental changes[25,26]. From this and the known function of Puf4p together with our result of the decreased *PUF4* expression in *rad2*Δ mutant after 6AU treatment, it can be deduced that *RAD2* positively regulates *PUF4* expression in the presence of 6AU, and then *PUF4* in turn regulates *HPT1* and *URA3* mRNA stability. In conclusion, 6AU sensitivity of *rad2*Δ and *puf4*Δ is the result of decreased expression of the genes in nucleotide metabolisms and *RAD2* and *PUF4* work together to regulate the genes. Thus, it is possible that increased or decreased *RAD2* expression influences on *PUF4* expression that modifies many gene expression in the posttranscriptional level in order to cope with the environmental changes. Taken together the variable expression of *RAD2* in accordance to the environment[25,26], target specificity of Puf4p[8], and our results showing the effects of *RAD2* and *PUF4* on the expression of nucleotide metabolism related genes, it can be reasoned that the miss-regulation of genes by mutations in *RAD2/XPG* may cause problems in purine and pyrimidine metabolism. Indeed, disordered nucleotide metabolism leads to many physiological consequences including mental retardation, neurological manifestations, and developmental delay[27-29]. Consequently, impaired function of Rad2p/XPG in transcription can cause imbalanced response to the environmental changes leading to the symptoms observed in CS patients.

Materials & Methods

Yeast Strains and Plasmids

EMY74.7 (MAT a *his3-Δ1 leu2-3 leu2-112 trp1*Δ *ura3*) and its isogenic strains were used in this study. *PUF4* gene was deleted using PCR based gene deletion method. *hpt1* and *ura3* were deleted by replacement most of the open reading frame (ORF) of each gene with marker genes contained in deletion generating plasmids, respectively. Deletion of each gene was confirmed by PCR using a pair of oligos flanking the ORF of target genes. *HPT1* and *URA3* genes were cloned into pYES2/NTB and pYES3/CT, respectively. Expression of *HPT1* and *URA3* gene was confirmed by Western analysis.

Isolation of Deletion Suppressors

*rad2*Δ cells were transformed with mTn3 (*LEU2 lacZ*) library that was generously provided by Dr. M. Snyder (Yale University), as described in Kumar *et*

Table 2. Sequences of primers used in RT-qPCR.

GENE	FORWARD PRIMER	REVERSE PRIMER
ORF:YBL086C	GTTGGCCTCCAAATCCAAGC	TCCGTGGGGTTATTGTCTTC
PHO3	GAATGGAACGTATTCGGCAG	CCAGGATGGACCAGGTTATG
ORF:YDL038C	CAGACCCAACGACTCTAGCC	TAGTGTCTTTGGGACCCGAG
PRM7	TGACTTCTCCTGCATCAACG	AGTAGTGGTGGTCGTGAAGG
TPI1	TGGAAGAAAAGAAGGCCGGT	CCAACTTGGAAGCCAAGAAC
HPT1	CGGGATTTTTGTTCTACACG	TATTGCTTGTGTTCCTGCTC
ORF:YDR539W	AGCATTGAAGACAACGCCTG	GGGAGCCAAAGGAAAAGAAG
GLC3	TGTTTACCCCGCTCCATAGG	CATAGCGGCATCCATCAACC
URA3	TGCAAGGGCTCCCTAGCTAC	GACCACATCATCCACGGTTC
MET6	TCCCAAGAGACGATGTCGAC	GCAGCCCAGGTGTAGTAAGC
GSY1	CCTTCACTGTGGAGGCACTG	GGTCTTCTCAGCGCCAATAC
PUF4	GCCAATTCATCTTGCACCAG	GAGGCGGAACCATTGCGTTG
ORF:YGL015C	ACGTCCATTAAATTACGCAG	CAGGGCGTCAGGTTGAAAGC
ORF:YGR024C	GGCTTAACTCCCCAGGAATC	TCATCTATCTGTGCGATGAC
SPT4	ATGTCTAGTGAAAGAGCCTG	GCCACTTTGCTACCCACGAC
NSR1	CGCTAAACACGGTGAAGTTG	ATCGTTGTTTGGTCTTGGAG
OM45	ACGGCACAGGAGTTTGGTAG	TCACCCCATCCTTCTAAACC
MUC1	GGCGAAAACACTGCACCTTC	TGTAGCGACTGCAGAACCAG
ORF:YLL044W	CTCCATTCGGTGCTAAGTCC	CAGCGATAGCGGCAGCTTCC
ILV5	AGAAGAGGTGCTTTGGACTG	CTTCCTTACCAACCTTCCAG
PGM2	TTCCAATGGTGCAAGATTCG	GTACGAACCGTTGGTTCTTC
AQR1	CCAACGAAATCATCTACAGC	TGGCTTTGACTGCCAAGGTG
ORF:YNL114C	CGCTACAGAGGTAACACCAC	GCATTTCCAAGCGTGTGATC
RPL18B	AAAGATCCGGTCATAGAACC	TCCTGGCATCGTCAGTAACG
RPL18A	CTCCATTCAACAAGGTTGTC	CTCTGACAGCTAATTGATCC
STI1	CCAACCTGGTACCAGTAACG	AGTCCGGATGATACCAGCAG
GAC1	TCGGGAATTTCCCAAGCTTC	GGATGGGTGGAGATGAACTG
NOP58	CGATTCAGGTGACATTGGTC	TCTTCATCATCAGAATCGGA
NOG1	ACGATGATTGCTGAAGCAAG	ACACCATCCAACAGTCTATC
ORF:YPL251W	CATGGTTTAAGAACGAAGAC	TCATACGAAATAGGCGCTTC
ORF:YDL092W	CTACCAACCATCCTGACTATG	GTCTTACTGATGGTACCGTTC

al.[30] and plated on the leucine-depleted medium to select the transformed cells. Once the colonies were formed, the 6AU resistant colonies were isolated by replica plating on the 6AU containing medium and incubation at 30˚C for 4 days. Twenty-six individual colonies were isolated and their 6AU sensitivity was compared with wildtype and *rad2*Δ strains. To identify the insertion site of library DNA, genomic DNA was prepared and the flanking region DNA sequence of the library DNA insertion site was determined by vectorette PCR and sequencing[30].

Cell Growth

To examine the effect of deletion of the genes on growth, the cells grown overnight in the uracil-depleted synthetic medium containing 2% dextrose were plated on the same medium and YPD (1% yeast extract, 2% Peptone, 2% Dextrose) medium. For 6AU sensitivity verification, the overnight cultured cells were diluted and plated on the uracil-depleted synthetic medium containing 75 μg/mL of 6AU. The plates were incubated at 30˚C for 2-3 days (YPD and -ura plate) or 4-5 days (6AU plate).

Galactose Induction

For galactose-induced gene expression, overnight cultures of cells containing the expression plasmid were diluted to the OD_{600} of 0.5 in the uracil-depeleted synthetic medium containing 2% galactose. After 7 hours of galactose induction, cells were plated on the control plates˚C (YPD and uracil depleting plates) and 6 AU containing plates. The rest of galactose-induced cells were harvested and the whole cell extracts were prepared with lysis buffer (50 mM Tris pH 7.5, 10% sucrose, 1 mM DTT) supplied with protein inhibitors. Thirty μg of whole cell extracts were separated on 10% SDS-PAGE and blotted onto a nylon membrane (Millipore). Hpt1p and Ura3p were detected with anti-his (R & D system) antibody (ABcam).

Microarray Analysis

Microarray analysis was carried out using an Agilent's Yeast Oligo Microarray (11K). cRNA probes were synthesized with Agilent's Low RNA Input Linear Amplification kit (Agilent Technology, USA) according to the manufacturer's protocol. Transcription template cDNA was prepared by reverse trans-

cription of 1 μg total RNA and subjected to transcription with T7 RNA polymerase in the presence of Cy3 or Cy5 CTP to generate fluorescent labeled cRNA probes. The cRNA probe was purified with cRNA Clean-up Module (Agilent Technology, USA), quantified with ND-1000 spectrophotometer, and fragmented. Mixture of cRNA probes of a control and a test was directly pipetted on an Agilent's Yeast Oligo Microarray (11K) (Agilent Technology, USA) and hybridized by incubation at 65˚C for 17 hours using Agilent Hybridization oven (Agilent Technology, USA). Hybridized chips were scanned with Agilent's DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technology). Data was normalized and functional annotation of genes was performed according to Gene OntologyTM Consortium (http://www.geneontology.org/index.shtml) by GeneSpringGX 7.3 (Agilent Technology).

RT-qPCR Analysis

Total RNA was prepared with WelPrep™ Total RNA Isolation Reagent (JBI, Seoul, Korea). Total RNA (5 μg) was reverse transcribed with oligo dT primer using Superscript RT II (Invitrogen) as described in the manufacturer's protocol. Real time PCR with an aliquot of reverse transcription reaction was performed by incubation at 95˚C for 10 min followed by 45 cycles of 95˚C for 10 s, 56˚C for 5 s and 72˚C for 20 s with Light Cycler 2.0 (Roche) using QuantiFast™ SYBR Green PCR kit (Qiagen). Relative mRNA amount was calculated by division of $(1/2)^{CT}$ of a specific gene $(1/2)^{CT}$ of YDL092W, an input control. Specificity of PCR products was determined by melting curve analysis. Sequences of primers used in RT-qPCR are listed in Table 2.

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