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Genome-Wide Expression Changes in *Saccharomyces cerevisiae* in Response to High-LET Ionizing Radiation

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Abstract To understand the yeast response to high-linear energy transfer (LET) ionizing radiation (IR), we investigated global gene expression in yeast irradiated by three types of high-LET IR (fast neutrons, heavy ions, and thermal neutrons) and gamma rays using DNA microarray analysis. Stationary cells were irradiated by each IR and recultured in yeast–peptone–dextrose medium to allow repair for 40 min. RNA was then isolated from three independent samples of irradiated yeast. Genes involved in the Mec1p kinase pathway, which functions in DNA damage response, were induced by all forms of high-LET IR and by gamma rays. Some genes related to oxidative stress and the cell wall were induced by all forms of high-LET IRs. Gene expression patterns as a function of each type of high-LET IR were examined statistically by one-way analysis of variance. This analysis demonstrated the existence of irradiation-specific responses. For example, genes involved in ribosomal DNA synthesis were specifically induced by fast neutron irradiation, while the ubiquitin–proteasome system and heat shock response were specifically induced by thermal neutron irradiation. The study characterizes high-LET IR-induced gene expression and provides a molecular understanding of subsequent adaptation in yeast.

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Introduction

In general, ionizing radiation is known to cause DNA damage and various oxidative lesions. This damage elicits a wide variety of cellular responses, from DNA repair to cell cycle arrest, mutation, transformation, apoptosis, and cell death [1–3]. High-linear energy transfer (LET) ionizing radiation (IR) has been reported to have unique characteristics compared to low-LET IR. The relative biological effectiveness of high-LET IR is significantly more deleterious to cells than that of low-LET IR [4, 5]. High-LET IR produces unique types of complex DNA damage, which are relatively resistant to repair [6]. Two classes of complex DNA damage are induced by high-LET IR, double strand breaks (DSB), and non-DSB oxidatively induced clustered DNA lesions which cause a high mutagenetic or carcinogenic potential [7]. High-LET IR is more effective in killing cells [8–11] and enhancing apoptosis in lung cancer cells regardless of p53 status [12]. On the other hand, use of high-LET IR in cancer treatment is recognized as more effective than low-LET IR, thus current interest in better understanding the interactions between high-LET IR and cellular constituents.

Recent studies have reported on the effects of high-LET IR using DNA microarray analysis and have characterized a number of cellular processes linked to radiation-regulated genes and pathways. For example, based on DNA microarray analysis, it appears that L-selenomethionine and vitamin A acetate are protective toward the risk of irradiation-induced generation of Fe ions [13–15]. The cyclin-dependent kinase inhibitor *CDKN1A* has been shown to play an important role in several pathways that are functionally related to effects of high-LET IR by comparing the effects of Fe ions, protons, and X-rays on human lens cells [16]. Gene expression profiles in mouse and human cells after exposure to neutrons and α-particles have also been reported [17, 18]. However, global cellular responses to the effects of high-LET IR, particularly to fast neutrons and thermal neutrons, are not as well characterized as responses to low-LET IR [19–24].

In the present study, we compared and analyzed gene expression profiles of yeast cells exposed to three kinds of high-LET IR (fast neutrons, heavy ions, and thermal neutrons) and gamma rays. Here, the yeast cell, *Saccharomyces cerevisiae*, was used as a model eukaryote. Many disease genes and most of the genes associated with the repair of ionizing radiation-induced damage in mammalian cells were initially characterized in this model organism [25–27]. Because of its experimental tractability [28] and the fact that its complete genome was sequenced over 10 years ago, this organism has served as a useful in vitro test tube for analyzing human and mammalian gene function. We report here novel observations of shared and high-LET IR-specific gene expression patterns.

Materials and Methods

Strain and Culture Conditions

S. cerevisiae S288C (*Mat alpha SUC2 mal mel gal2 CUP1*) was used as the indicator strain for DNA microarray analysis. Yeast cells were grown aerobically in yeast–peptone–dextrose (YPD) medium (2% polypeptone, 1% yeast extract, and 2% glucose) at 25 °C to a cell density of 1.0 (optical density$_{660}$) [29].

Irradiation

Exponentially growing yeast cells were harvested, washed with saline, and resuspended in the same solution (5×10^8 cells per millimeter). During irradiation, cells were kept at 4–10°C. Cell suspensions were put into cuvettes and were irradiated with fast neutrons, thermal neutrons, or gamma rays. Cell suspensions were placed in tissue culture flasks for irradiation with heavy ions. After each irradiation, cells were incubated for 40 min in YPD at 25°C to allow repair. We previously reported the highly dynamic nature of gene expression changes in yeast during 40 min of repair following X-ray and gamma ray irradiations [21]. Three independent samples were irradiated for each condition. Control samples were placed under identical conditions for the same amount of time without irradiation. Three independent control samples were prepared for each condition. Cell viability of control and treated cells was determined by counting colonies on YPD plates containing 2% *w/v* agar. Irradiation conditions are shown in Table 1.

Fast Neutron Irradiation

Fast neutron irradiation was performed with a neutron-irradiating system, the Hiroshima University Radiobiological Research Accelerator (HIRRAC; Nisshin-High Voltage, Kyoto, Japan) in the Research Institute for Radiation Biology and Medicine of Hiroshima University (RIRBM). HIRRAC was operated at an average proton beam current of 73 μA. Fast neutrons were produced with the 7 Li (p, n) 7 Be reaction. Cells were exposed to total doses of 3.3, 9.7, and 21.4 Gy in 336 min, with dose rates of 10, 29, and 64 mGy/min, respectively. For each irradiation, the contribution of net fast neutrons was 3.3, 9.2, and 20.3 Gy.

Table 1 Irradiation conditions and cell survival.

Radiation	Radiation facility	Generator	Dose (Gy)	Viability (%)
Fast neutrons	RIRBM	HIRRAC (7Li (p, n) 7Be reaction)	3.3 (FN; 3.3+γ; 0.2)	118.7±9.3
			9.7 (FN; 9.2+γ; 0.5)	105.7±2.4
			21.4 (FN; 20.3+γ; 1.1)	83.2±2.4
Heavy ion	NIRS	HIMAC (carbon-12 ions)	6.0	80.5±4.4
			12.0	53.9±2.6
			25.0	47.5±6.3
Thermal neutrons	KUR	HWNIF (U fission)	4.0 (TN; 0.3+FN; 0.3+γ; 3.3)	120±26.5
			6.9 (TN; 0.51+FN; 0.5+γ; 5.8)	105±3.6
			20.8 (TN; 2.5+FN; 1.7+γ; 16.7)	92±7.2
Gamma rays	RIRBM	137 Cs γ ray irradiation device	3.3	99.2±5.0
			5.8	90±19.2
			17	72.5±9.3

RIRBM Research Institute for Radiation Biology and Medicine Hiroshima University, *HIRRAC* Hiroshima University Radiobiological Research Accelerator, *NIRS* National Institute of Radiological Science, *HIMAC* Heavy Ion Medical Accelerator, *KUR* Kyoto University Research Reactor, *HWNIF* Heavy Water Neutron Irradiation Facility, *FN* fast neutrons, *TN* thermal neutrons, γ gamma rays

Heavy Ion Irradiation

An experiment was carried out at a physics/general beam line at the heavy ion medical accelerator (HIMAC) in the International Space Radiation Laboratory (NIRS). Carbon-12 ions were accelerated by HIMAC up to a monoenergetic 290 MeV/u. Exposure was conducted using horizontal carbon beams with a dose rate of 3 Gy/min. Cells were exposed to total doses of 6, 12, and 25 Gy.

Thermal Neutron Irradiation

The Heavy Water Neutron Irradiation Facility of the Kyoto University Research Reactor was used for thermal neutron irradiation. The dose rate of global irradiation was 17 mGy/min, and the dose rate of thermal neutrons was 1.0–2.0 mGy/min. The net thermal neutrons and net fast neutrons were measured by gold foil activation analysis [30]. Net dose of gamma rays including net secondary gamma rays was measured with a thermoluminescence dosimeter [30]. Cells were exposed to total doses of 4.0, 6.9, and 20.8 Gy.

Gamma Ray Irradiation

A Cs-137 γ-ray source (Sangyo Kagaku, Tokyo, Japan) was used for gamma ray irradiation in RIRBM. The dose rate was 1.4 Gy/min. Cells were exposed to total doses of 3.3, 5.8, and 17 Gy.

Microarray Procedures

Microarray experiments were carried out as previously described [31]. Yeast cells were harvested after irradiation by centrifugation and stored at −80 °C until extraction of RNA. Total RNA was extracted by the hot phenol method. Poly(A) + RNA was purified from total RNA with an Oligotex-dT30 messenger RNA (mRNA) purification kit (TaKaRa, Kyoto, Japan). Fluorescein-labeled cDNA was synthesized in the presence of Cy3- or Cy5-labeled dUTP with the CyScribe First-Strand cDNA Labeling kit (Amersham Biosciences, Buckinghamshire, UK). In all microarray experiments, cDNA made from poly(A) + RNA obtained from control cells was labeled with Cy3, while that made from irradiated samples was labeled with Cy5. The reactions to generate fluorescein-labeled DNA from mRNA were performed at 42 °C for 90 min. The two-color labeled cDNA pools were mixed and hybridized with yeast DNA microarrays (DNA Chip Research, Kanagawa, Japan) containing 5,989 spots for 18–20 h at 65 °C. After hybridization, the labeled microarrays were washed and dried. Subsequently, labeled microarrays were scanned using a Scan Array 4000 laser scanner (GSI Lunomics, Billeria, MA, USA). Array images were analyzed with Gene Pix 4 (Axon Instruments, Union City, CA, USA). The DNA microarray data obtained in this report are available from Gene Expression Omnibus, accession no. GSE9464 (http://www.ncbi.nlm.nih.gov/projects/geo/).

Data Analysis

The microarray study design consisted of triplicate experiments for each of the irradiated samples. Details of the microarray validation and reliability assessments under our experimental conditions have been described [31]. We have found that reliable yeast DNA microarray data were obtained from three independent experiments under our

experimental conditions [31]. Signal intensities (the spot intensity minus the background intensity) exceeding the mean plus two standard deviations of background intensity were selected as high reliable spots and used to obtain Cy5/Cy3 ratios [31]. GeneSpring (Agilent technologies, Palo Alto, USA) was used for normalization and the following analysis. Detected signals for each ORF were normalized by intensity-dependent (locally weighted scatter plot smoothing) methods (http://www.silicongenetics.com/cgi/SiG.cgi/index.smf). Expression data were evaluated using the average of three ratios from independent scanning images. Initially, 3,637 genes were selected whose *t* test *p* value was less than 0.05 in at least one profile to remove the fault data and unchanged mRNA. Subsequently, 1,742 genes were selected by one-way analysis of variance (ANOVA) [32, 33] with multiple testing correlations (Benjamini and Hochberg false discovery rate), in order to identify genes whose induction or repression were characteristic of each type of irradiation and were statistically significant. Genes whose expression ratios were higher than those of the other three irradiation treatments were selected as specifically induced for the given type of irradiation, while genes whose ratios were lower than those of the other three irradiation treatments were selected as specifically repressed. To determine a shared set of genes whose expression ratios were changed similarly in response to all four types of irradiation, genes whose expression levels changed more than twofold or less than 0.5-fold in all experiments were selected as commonly induced or repressed from the list of 3,637 genes. To determine a shared set of genes whose expression was altered similarly by high-LET IR, genes whose expression level was greater than twofold or less than 0.5-fold in all three high-LET IR experiments were selected from the list of 3,637 genes. Selected genes were categorized by biological process using the *Saccharomyces* Genome Database Gene Ontology Slim Mapper (*SGD* GO Slim Mapper, http://db.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl) and Gene Ontology Term Finder tools (*SGD* GO Term Finder, http://db.yeastgenome.org/cgi-bin/GO/goTermFinder.pl). The oPOSSUM program (http://www.cisreg.ca/shosui/yeast_oPOSSUM/Yeast_results.php) was used to determine whether or not the selected genes regulated by common promoter elements [34, 35]. Regions upstream of the translation start site to the nearest stop codon (up to 500 bp) were analyzed. Putative transcription factor binding sites within promoter regions were identified using Z scores of >10 and Fisher *p* values of <0.01.

Results

Conditions for Irradiation Treatments

The purpose of this study was to characterize gene expression patterns characteristic of yeast response to high-LET IR. High-LET IR is generally available only at large facilities and is usually shared for use in medical and experimental treatments, and thus, is not as widely available as other types of irradiation. For DNA microarray analysis, appropriate experimental conditions must be selected. Without any biological or physiological characterization of the treatment, it is not possible to establish that the induction or repression of specific genes is due to the treatment. Lack of growth inhibition would merely show that the treatment did not cause cell stress and that the results obtained did not reflect stress. For example, IC50 and LD50 values can be used as indicators for toxicological analysis [36, 37]. To find appropriate conditions, we first estimated dose for the four kinds of IR by assessing cell survival. Table 1 shows that survival decreased in reverse proportion to dose under all IR conditions. Thus, radiation doses were chosen which had a maximum

effect on cell viability under the selected conditions, though an IC50 value for the neutron treatment could not be defined. Based on this dose–response analysis, 21.4 Gy of fast neutrons, 20.8 Gy of thermal neutrons, and 25 Gy of heavy ions were selected for DNA microarray analysis. The dose of gamma rays (17 Gy) was used to adjust the dose of gamma rays for the thermal neutron irradiation.

Shared Gene Expression Patterns in Response to High-LET IR

We performed DNA microarray experiments in order to compare gene expression responses to three types of high-LET IR (fast neutrons, heavy ions, and thermal neutrons) and to gamma rays, with reference to nonirradiated control cells. Table 2 lists genes whose expression patterns were shared upon irradiation treatment. Only five genes were induced in response to all three types of high-LET IR and gamma irradiation under these experimental conditions. The most highly induced gene was *HUG1*, followed by *YNL194C*, *RNR4*, *RNR2*, and *FMP16*. *HUG1*, *RNR4*, and *RNR2* are known to be involved in the Mec1p kinase pathway due to stalled DNA replication forks [38]. Only one gene, *SFG1*, was found to be repressed in response to all four treatments. *SFG1* encodes a nuclear protein and putative transcription factor required for growth of superficial pseudohyphae.

Table 2 Genes with shared expression patterns in response to high-LET IR and gamma rays.

Systematic name	Fold change				Common name	Description
	FN	HI	TN	G		
Induced by all irradiation types						
YML058W-A	7.38	8.87	2.96	3.92	HUG1	Protein involved in the Mec1p-mediated checkpoint pathway
YNL194C	2.15	5.47	3.92	6.83		Sequence similarity to SUR7 and FMP45
YGR180C	5.92	6.49	3.36	2.60	RNR4	Ribonucleotide-diphosphate reductase
YJL026W	4.95	5.42	2.46	2.53	RNR2	Ribonucleotide-diphosphate reductase
YDR070C	3.15	3.36	2.20	2.56	FMP16	Putative protein of unknown function
Repressed by all irradiation types						
YOR315W	0.33	0.28	0.38	0.40	SFG1	Transcription factor required for growth of superficial pseudohyphae
Induced by all three types of high-LET IR						
YFL014W	5.19	4.25	3.57	0.86	HSP12	12 kDa heat shock protein
YER150W	2.07	3.33	4.34	1.14	SPI1	GPI-anchored cell wall protein involved in weak acid resistance
YKR076W	2.10	2.11	4.04	1.86	ECM4	(Putative) involved in cell wall biogenesis
YLR109W	2.55	2.23	2.74	0.76	AHP1	Thiol-specific peroxiredoxin
Repressed by all three types of high-LET IR						
YDL227C	0.30	0.24	0.18	0.65	HO	Homothallic switching endonuclease
YGL028C	0.24	0.25	0.44	0.64	SCW11	Cell wall protein with similarity to glucanases
YHR143W	0.32	0.30	0.35	0.86	DSE2	Daughter cell-specific secreted protein
YBR158W	0.38	0.41	0.45	0.55	CST13	Protein required for daughter cell separation
YNL066W	0.45	0.46	0.42	0.59	SUN4	Cell wall protein related to glucanases

FN fast neutrons, *HI* heavy ions, *TN* thermal neutrons, *G* gamma rays

Similarly, we identified genes with similar expression patterns to the various forms of high-LET IR only, excluding the response to gamma ray irradiation. Four stress-related genes were found to be induced by high-LET IR (Table 2). The most highly induced was *HSP12*, followed by *SPI1, ECM4*, and *AHP1*. *HSP12* and *AHP1* are involved in response to oxidative stress while *ECM4* and *SPI1* are associated with cell wall-related processes and toxin response. Five genes involved in the process of cell division were found to be repressed by high-LET IR.

Characterization of High-LET IR-Specific Gene Expression

One-way ANOVA with multiple testing correlations was used to identify high-LET IR-specific gene expression patterns. This analytical method calculates a quintile function for each gene based on Fisher statistics, with a high score corresponding to a significant difference in expression between irradiated and nonirradiated samples [39]. One-way ANOVA is appropriate for the proposed analysis as it takes into account intraclass variance [39, 40]. A total of 1,742 statistically significant genes were selected by one-way ANOVA from the 3,637 total. Figure 1 shows hierarchical cluster analysis between the expression profiles for the four kinds of high-LET IR. The individual gene expression responses to fast neutrons, heavy ions, and thermal neutrons as forms of high-LET IR were more similar to one another than to the response to gamma rays (a low-LET IR). Among the 1,742 genes, those whose expression levels were higher for one specific form of high-LET IR than for the others were identified as significantly induced genes, whereas those whose expression levels were lower than for the others were recognized as significantly repressed.

Fast Neutron IR-Specific Gene Expression

Microarray analysis detected 31 genes that were uniquely induced by fast neutrons (Fig. 2a). Nine of these genes (*CSL4*, *IMP3*, *LIA1*, *NOP15*, *NUC1*, *RLP7*, *RRP1*, *SLX9*, and

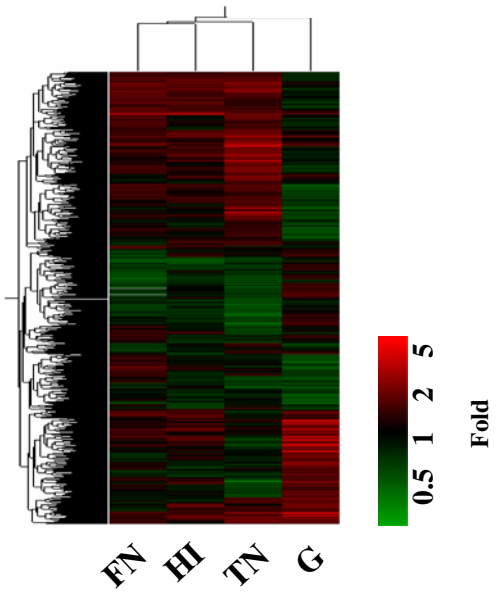

Fig. 1 Hierarchical clustering of high-LET IR. *Color* indicates deviation from the mean expression of each gene following treatment according to the scale below. *FN* fast neutrons, *HI* heavy ions, *TN* thermal neutrons, *G* gamma rays

(A)

FN	HI	TN	G	
1.29	0.73	0.79	0.85	BTS1
1.18	0.95	0.85	0.74	CSL4
1.22	0.68	0.90	0.87	CUP9
1.50	0.73	0.34	1.01	CYB5
1.88	1.16	1.18	1.28	FSH3
1.72	1.22	0.84	1.03	GIR2
1.31	0.71	0.66	0.85	ICS2
1.63	0.86	0.62	0.91	IMP3
1.20	0.84	0.40	0.82	INM1
1.38	0.67	0.94	0.81	INO2
1.27	0.87	0.89	0.76	LIA1
1.69	1.08	0.79	1.29	MSO1
1.32	0.73	0.40	0.85	NOP15
1.03	0.58	0.57	0.63	NUC1
1.26	0.78	0.76	0.75	PPM2
1.15	0.82	0.77	0.74	PSF2
1.30	0.75	0.93	0.87	RLP7
1.34	1.02	0.68	0.97	RPL28
1.28	0.98	0.99	0.93	RRP1
1.49	0.77	0.89	0.91	SEC20
1.03	0.49	0.40	0.80	SKS1
1.72	1.03	0.53	1.09	SLX9
2.08	1.05	0.72	0.99	SPO12
1.41	1.07	0.68	0.75	STS1
1.59	1.02	0.85	0.95	YAP5
1.30	0.96	0.67	0.93	YAL027W
1.60	0.84	0.73	0.69	YBL054W
1.24	0.69	0.98	0.80	YKL098W
1.28	0.92	0.60	0.90	YLR243W
1.14	0.58	0.58	0.81	YOR342C

(B)

FN	HI	TN	G	
0.27	0.45	1.09	0.51	CHA1
0.56	1.06	0.77	1.00	COS8
0.32	0.59	0.47	1.39	DIP5
0.49	0.77	0.74	0.88	EXG2
0.88	1.67	1.31	1.51	GAS5
0.44	0.70	0.64	0.64	GDH1
0.91	1.27	1.71	1.42	GFA1
0.62	0.87	0.82	0.81	GUS1
0.71	0.99	0.93	1.00	HOG1
0.84	1.13	1.15	1.21	KGD2
0.55	0.78	0.84	0.83	LEU1
0.59	1.47	1.49	1.28	MET8
0.67	0.97	1.14	1.00	MTO1
0.66	1.17	1.11	1.24	PDC1
0.99	1.50	1.59	1.50	PDC5
0.65	0.85	1.02	1.15	PGM1
0.93	2.71	1.58	2.88	PHM7
0.59	1.73	1.52	1.67	PNS1
0.78	1.04	1.36	1.41	PYC2
0.61	1.59	27.80	1.06	SSA4
0.57	0.88	0.80	1.03	STU2
0.53	1.09	0.85	1.18	TAF1
0.36	0.80	1.03	1.02	TPD3
0.84	1.12	1.31	1.31	TPS3
0.70	0.89	1.00	0.90	UBC9
0.95	1.18	1.42	1.12	UBR2
0.68	1.45	1.71	1.70	YBR139W
0.67	1.19	1.27	1.45	YDR029W
0.76	1.37	1.11	1.36	YEL074W
0.47	1.24	1.32	1.27	YFL032W
0.78	0.97	0.97	1.13	YJR114W

<0.5	0.5-0.75	0.75-1	1-1.5	1.5-2	2-2.5	2.5-3	3-3.5	3.5-4	4<

Fig. 2 Genes specifically over- and underexpressed in cells exposed to fast neutron irradiation. **a** List of 31 genes which were induced specifically by fast neutron irradiation. **b** List of 34 genes specifically repressed by fast neutron irradiation. *Color* indicates deviations from the mean expression of each gene following treatment according to the *scale below*. *FN* fast neutrons, *HI* heavy ions, *TN* thermal neutrons, *G* gamma rays

YBL054W) are involved in ribosome biogenesis (Fig. 2a), although their expression levels were not high. In contrast, 34 genes were more repressed by the fast neutron treatment than by any the other form of IR (Fig. 2b). Figure 2b shows that several genes involved in carbohydrate (*ATH1*, *GFA1*, *PGM1*, *PYC2*, and *TPS3*) and amino acid metabolism (*CHA1*, *GDH1*, *GUS1*, *LEU1*, *PDC1*, and *PDC5*) were repressed.

Heavy Ion IR-Specific Gene Expression

A total of 39 genes were selected by one-way ANOVA as responsive to heavy ion IR. Under the experimental conditions used, however, only two genes (*IPT1* and *RRM3*) were specifically induced by heavy ion relative to the three other types of IR (Fig. 3a). Twelve genes were detected as specifically repressed genes by heavy ion irradiation (Fig. 3b). However, induction and repression levels were not significant except for the repressed gene *CTS1,* which encodes chitinase that is required for cell separation during growth.

Thermal Neutron IR-Specific Gene Expression

A total of 254 genes were selected as responsive to thermal neutron IR from the list of 1,742. Among them, 162 genes were induced, and 88 genes were repressed. The induced genes were classified within accepted gene ontology process categories (Table 3; http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl). A large number of genes were induced within the "response to stress" category (25.9%). Within the categories of "protein

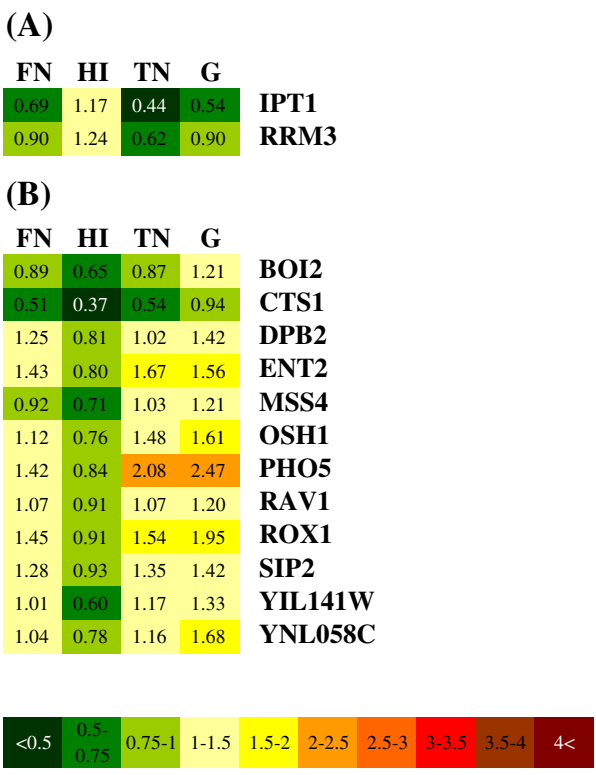

Fig. 3 Genes specifically over- and underexpressed in cells exposed to heavy ion irradiation. **a** List of two genes which were specifically induced by heavy ion irradiation. **b** List of 12 genes which were specifically repressed by heavy ion irradiation. *Color* indicates deviations from the mean expression of each gene following treatment according to the *scale below*. *FN* fast neutrons, *HI* heavy ions, *TN* thermal neutrons, *G* gamma rays

Table 3 Biological process classification of genes induced by thermal neutron irradiation.

GOID	GO term	Number of genes	Percentage
6950	Response to stress	42	25.9
30163	Protein catabolic process	31	19.1
6996	Organelle organization	27	16.7
6810	Transport	27	16.7
6464	Protein modification process	19	11.7
6457	Protein folding	18	11.1
8150	Biological process unknown	17	10.5
6519	Cellular amino acid and derivative metabolic process	14	8.6
42221	Response to chemical stimulus	13	8
7049	Cell cycle	10	6.2
16070	RNA metabolic process	10	6.2
16192	Vesicle-mediated transport	10	6.2
5975	Carbohydrate metabolic process	9	5.6
6259	DNA metabolic process	8	4.9
7165	Signal transduction	8	4.9
16044	Membrane organization	7	4.3
6350	Transcription	7	4.3
6091	Generation of precursor metabolites and energy	5	3.1
6629	Lipid metabolic process	5	3.1
6412	Translation	5	3.1
6725	Cellular aromatic compound metabolic process	4	2.5
51186	Cofactor metabolic process	4	2.5
7010	Cytoskeleton organization	4	2.5
30435	Sporulation resulting in formation of a cellular spore	4	2.5
7047	Cell wall organization	3	1.9
19725	Cellular homeostasis	3	1.9
46483	Heterocycle metabolic process	3	1.9
746	Conjugation	2	1.2
7124	Pseudohyphal growth	2	1.2
9653	Anatomical structure morphogenesis	1	0.6
45333	Cellular respiration	1	0.6
7126	Meiosis	1	0.6
6997	Nucleus organization	1	0.6
32196	Transposition	1	0.6
6766	Vitamin metabolic process	1	0.6

Classification of genes is process using *Saccharomyces* Genome Database Gene Ontology Slim Mapper

catabolic process" (19.1%), "protein modification" (11.7%), and "protein folding" (11.1%), a large fraction of genes were found to encode proteolytic functions. Highly significant p values were detected for the categories of "protein catabolic process" (7.94×10^{-16}), "protein folding" (8.18×10^{-12}), and "response to stress" (6.75×10^{-11}) using the *SGD* GO Term Finder tool (http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl). In the category of "response to stress," 13 genes function in heat shock stress in response to

protein denaturation (Fig. 4). For example, *HSP26*, *HSP42*, *HSP78*, *HSP104*, and *SSA4* were highly induced, more than sevenfold. Many genes encoding *HSP100* family (*HSP104*), *HSP70* family (*SSA3* and *SSA4*), *HSP90* family (*AHA1*, *CDC37*, *HCH1*, *HSC82*, and *HSP82*), and small HSP family (*HSP26* and *HSP42*) members involved in "protein folding" were also induced. Hsp proteins are known to be induced by

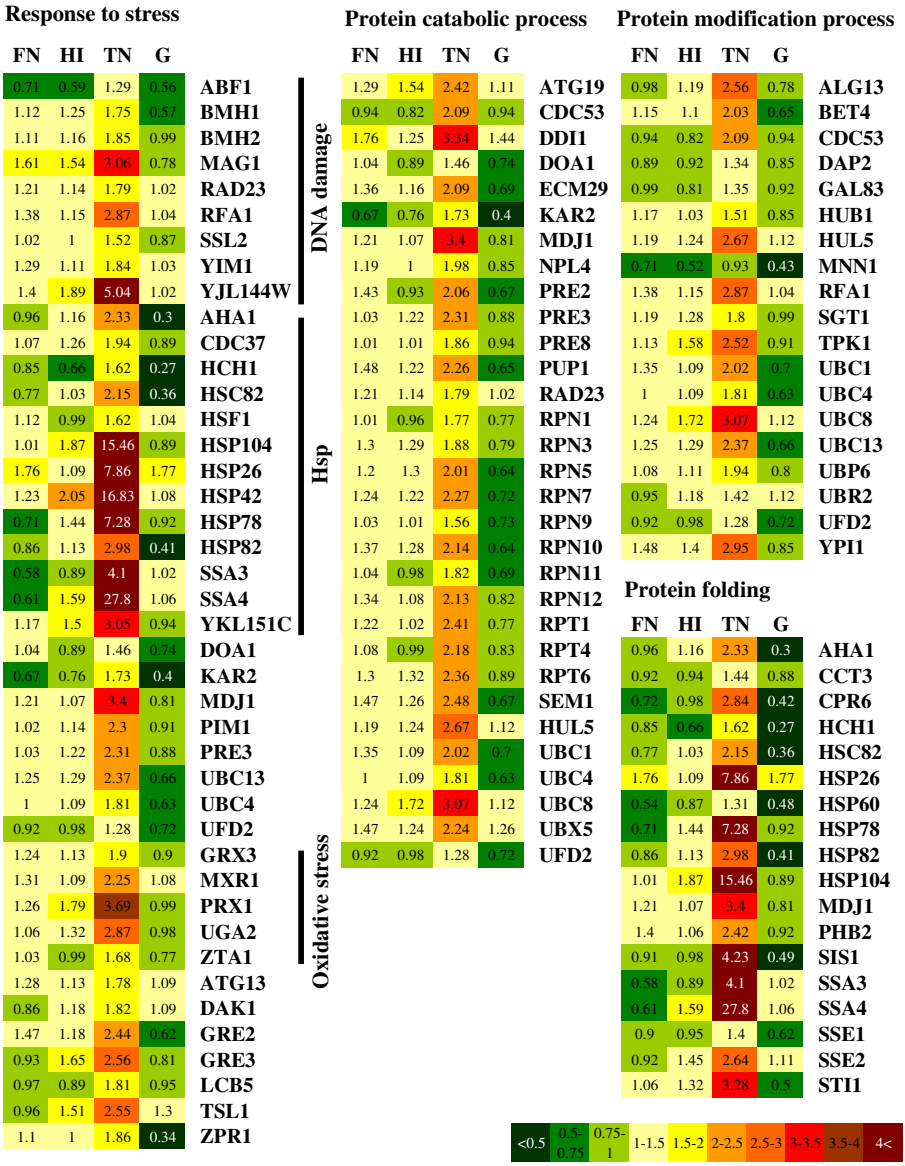

Fig. 4 Genes specifically induced by thermal neutron irradiation in the GO process categories of "response to stress," "protein catabolic process," "protein modification," and "protein folding". *Color* indicates deviations from the mean expression of each gene following treatment according to the *scale below*. *FN* fast neutrons, *HI* heavy ions, *TN* thermal neutrons, *G* gamma rays

environmental stress and function in protein metabolism as molecular chaperones or chaperone regulators [41–44]. Five genes involved in oxidative stress and nine genes involved in DNA damage and repair were also detected in the category of "response to stress."

As shown in Fig. 4, many genes involved in ubiquitin-dependent protein catabolism were detected in the categories of "protein catabolic process," "protein modification," and "protein folding" in addition to "response to stress." For example, *HUL5*, *UBC1*, *UBC4*, *UBC8*, *UBX5*, and *UFD2* are linked to ubiquitin function, and *UBC1*, *UBC4*, and *UBC8* encode ubiquitin-conjugating enzymes. *PRE2*, *PRE3*, and *PRE8* encode subunits of the 20S proteasome while *RPN1*, *RPN3*, *RPN5*, *RPN7*, *RPN9*, *RPN10*, *RPN11*, and *RPN12* encode non-ATPase regulatory subunits of the 26S proteasome. *RPT1*, *RPT4*, and *RPT6* contribute to ATPase function of the 19S regulatory particle of the 26S proteasome involved in the degradation of ubiquitinated substrates.

To determine whether the induced genes are regulated by common promoter elements, we searched for genes coregulated by the same transcription factor using the oPOSSUM system (Table 4). As shown, genes that were transcriptionally regulated by RPN4, HSE, and STRE (related to stress response) were detected.

Characterization of genes repressed by thermal neutron IR suggested significant association with "transport," "lipid metabolic process," and "organelle organization" (data not shown). Many genes involved in sugar (*HXT1*, *HXT2*, *HXT3*, *HXT4*, *HXT6*, *HXT7*, *HXT15*, and *HXT16*) and metal transport (*ALR2*, *NHA1*, and *ZRT1*) were also detected.

Discussion

This study aimed to characterize the biological effects of high-LET IR by DNA microarray analysis in *S. cerevisiae*. The responses of *HUG1*, *RNR2*, and *RNR4* were particularly interesting. These three genes were induced by fast neutrons, heavy ions, thermal neutrons, and gamma rays. *HUG1*, *RNR2*, and *RNR4* are involved in the Mec1p kinase pathway which is known to respond to DNA damage [38]. *MEC1*, which encodes a homolog of the human *ATM* gene product, is required for G1 arrest following radiation treatment, e.g.,

Table 4 Statistically over-represented TF binding sites within promoters of genes induced by thermal neutron IR.

TF1	TF class	Rank	Target gene hits	Z score	Fisher *p* value
RPN4	Unclassified	1	79	20.28	8.29×10^{-8}
UME6	C6_Zinc_finger	2	96	17.96	1.70×10^{-5}
REB1	Trp_cluster	3	59	9.32	4.09×10^{-2}
AFT1	Unclassified	4	55	8.84	3.62×10^{-2}
LEU3	C6_Zinc_finger	5	31	8.25	1.53×10^{-2}
CAR1_r	Unclassified	6	85	8.07	7.14×10^{-2}
ADR1P	Zinc_finger	7	118	7.91	1.65×10^{-1}
STRE	Zinc_finger	8	31	7.66	2.94×10^{-2}
PDS	Unclassified	9	83	6.36	2.38×10^{-2}
HSE	Unclassified	10	53	5.43	1.10×10^{-1}

Z scores >10 and Fisher *p* values <0.01 were considered statistically significant

TF transcription factor

ultraviolet (UV) light and gamma rays [45]. *RNR2* and *RNR4* encode small subunits of ribonucleotide reductase which catalyzes the rate-limiting step in dNTP synthesis [46]. An increase in dNTP levels is often associated with DNA damage and is considered necessary for DNA repair and resumption of normal transcription [46]. *HUG1* is transcribed in a kinase checkpoint-dependent manner and is thought to play a role in recovery from the transcriptional response to DNA damage and cell cycle arrest [38]. Benton et al. reported that these three genes (*HUG1*, *RNR2*, and *RNR4*) were induced by two DNA damaging agents, methyl methanesulfonate (MMS) and gamma rays [47]. Fry et al. reported that genes involved in the Mec1 pathway such as *RNR2* were induced in *S. cerevisiae* treated with gamma rays which cause single-strand breaks in DNA [48]. Our results suggest that ionizing irradiation causes DNA damage in yeast regardless of specific LET IR type. DNA damage caused by both high- and low-LET IR has also been reported in human cells [7, 49–51]. The *YNL194c* gene product when expressed as a GFP fusion protein has been shown to be induced in response to the DNA-damaging agent MMS [52]. Here, *YNL194c* was found to be induced by all four types of IR. However, no RAD genes involved in DNA damage-dependent checkpoints or nucleotide excision repair (NER) were detected under our experimental conditions.

The three types of high-LET IR were found to be highly inductive of genes involved in oxidative stress (*HSP12* and *AHP1*) and cell wall function (*ECM4* and *SPI1*). *HSP12* has been shown to be induced by oxidative stress in addition to heat shock and osmotic stress [53, 54]. *AHP1* encodes a thiol-specific peroxiredoxin which reduces hydroperoxides to protect against oxidative damage [55]. Two additional genes involved in oxidative stress (*SOD1* and *YCL068c*) were also induced more than 1.5-fold in cells treated with all forms of high-LET IR. The fold increases in *SOD1* expression were 2.21, 2.20, and 1.79 in response to fast neutrons, heavy ions, and thermal neutrons, respectively, while the values for *YCL003c* expression were 1.98, 1.95, and 2.52 in response to fast neutrons, heavy ions, and thermal neutrons, respectively. This may suggest that oxidative stress induced by high-LET IR may be greater than that following gamma ray treatment under our experimental condition, although a decrease has been reported for oxidatively induced clustered DNA lesions caused by high-LET IR relative to low-LET IR [7]. Two genes involved in cell wall function (*ECM4* and *SPI1*) have been associated with toxin resistance and acid stress. It has also been reported that neutron irradiation causes more damage to the cell wall and produces more early effects than X-ray treatment of the intestinal wall of mice [56].

Genes repressed by all forms of high-LET IR were found to be involved in cell division. In addition, many were found to be linked to growth-dependent functions, e.g., carbohydrate, amino acid, and lipid metabolism; transport; and organelle organization. Under stressful conditions, yeast tends to grow slowly while protein synthesis, including ribosomal protein synthesis, is repressed, complicating efforts to interpret data from the list of repressed genes.

Genes involved in ribosome synthesis were induced by fast neuron IR (Fig. 2). *NOP15*, *RLP7*, and *RRP1* are known to be associated with 66S preribosomal particles [57], and *SLX9* and *IMP3* are known to be associated with the 18S preribosome [58, 59]. Singh and Vadász reported that chain breaks in ribosomal RNA have been observed in inactive ribosomes of gamma irradiated-*Escherichia coli* [60]. Conconi et al. reported that UV irradiation caused ribosomal DNA damage and described repair-independent chromatin assembly onto active ribosomal genes after UV irradiation [61]. Under our experimental conditions, induction of these genes may reflect DNA damage of the yeast ribosome by fast neutron irradiation.

Following heavy ion radiation, there were few significant genes detected by ANOVA analysis, although 92 genes were overexpressed (>twofold), and 38 were underexpressed

(<0.5-fold; data not shown). These results are consistent with heavy ion irradiation causing damage similar to that caused by the other forms of irradiation used in this study.

Among the four types of radiation, thermal neutrons induced a unique pattern of gene expression. Genes that were induced significantly by thermal neutrons were repressed by gamma ray treatment and vice versa. In this study, thermal neutron irradiation (20.8 Gy) included thermal neutrons (2.5 Gy), gamma rays (16.7 Gy), and fast neutrons (1.7 Gy). With respect to their contribution to total energy, thermal neutrons can be considered similar to gamma rays. Thus, our results indicate that the effect of "thermal neutrons" was transferred from gamma rays to "thermal neutrons" by mixing the three types of radiation.

Many genes related to heat shock stress (*HSP100* family, *HSP70* family, *HSP90* family, and small HSP family) and the ubiquitin–proteasome system were induced by thermal neutron irradiation. Hsp proteins are known to be induced by environmental stress, and many function as molecular chaperones [41–44]. Ubiquitin is involved in targeting proteins for proteasomal degradation, while the proteasome is involved in the degradation of denatured proteins, caused in part, by environmental stress [62]. In addition, many genes induced by thermal neutrons were found to be regulated by common promoter elements (e.g., RPN4, HSE, and STRE). The RPN4 promoter contains an additional sequence that binds Yap1p, a bZIP-type transcription factor that plays an important role in the oxidative stress response and multidrug resistance. Rpn4p levels are in turn regulated by the 26S proteasome in a negative feedback control mechanism [63, 64]. The HSE element contains multiple inverted repeats of the nGAAn unit and regulates transcription under normal physiological conditions as well as in cells undergoing various stresses, including heat shock [65]. The stress response element (STRE) contains the AGGGG motif as a core component and is responsive to Msn2 and Msn4 transcription factors [53, 66, 67]. There have been some reports on the relationship between the ubiquitin–proteasome system and DNA repair. Maki et al. reported that thermal neutrons induced single- and double-stranded breaks in DNA in cultured mammalian cells [68]. In the present study, we found that nine genes involved in DNA damage response were among the genes induced by thermal neutron IR. The most highly induced genes, *MAG1* and *YJL144W*, are also known to be induced by alkylating and other DNA-damaging agents, respectively [52, 69]. *RFA1* is a subunit of the heterotrimeric replication factor A, which is a highly conserved single-stranded DNA binding protein involved in DNA replication, repair, and recombination [70, 71]. *RAD23* and *SSL2* are involved in NER which is required for resistance to DNA damage induced by such agents as UV light [72, 73]. These results may suggest that thermal neutrons cause DNA damage in yeast. Taken together with the present results, it suggests that the ubiquitin–proteasome system is important in the yeast response to thermal neutron IR. Some reports have shown that UV irradiation induces ubiquitination and degradation as highly conserved responses to DNA damage from yeast to humans [74, 75]. In general, thermal neutrons are known to generate a 14N (n, p) 14C reaction. If a 14N (n, p) 14C reaction is generated in yeast cells, 14N found in nucleic acid and protein might be converted into 14C, causing significant damage to both classes of polymers. Additionally, these reactions generate protons which may cause oxidative stress. Indeed, some genes involved in oxidative stress were overexpressed in cells exposed to thermal neutron IR.

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