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Research Article

Global protein expression profiling of budding yeast in response to DNA damage

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Abstract

Exposure to DNA-damaging agents can activate cell cycle checkpoint and DNA repair processes to ensure genetic integrity. Such exposures also can affect the transcription of many genes required for these processes. In the budding yeast *Saccharomyces cerevisiae*, changes of global gene expression as a result of a DNA-damaging agent were previously identified by using DNA chip technology. DNA microarray analysis is a powerful tool for identifying genes whose expressions are changed in response to environmental changes. Transcriptional levels, however, do not necessarily reflect cellular protein levels. Green fluorescent protein (GFP) has been widely used as a reporter of gene expression and subcellular protein localization. We have used 4156 yeast strains expressing full-length, chromosome-tagged GFP fusion proteins to monitor changes of protein levels in response to the DNA-damaging agent, methyl methanesulphonate (MMS). Through flow cytometry, we identified 157 proteins whose levels were increased at least three-fold following treatment with MMS. Of 157 responsible genes, transcriptions of 57 were previously not known to be induced by MMS. Immunoblot experiments with tandem affinity-tagged yeast strains under the same experimental conditions confirmed these newly found proteins as inducible. These results suggest, therefore, that the 57 protein expressions are regulated by different mechanisms, such as post-translational modifications, and not by transcriptional regulation. Copyright © 2007 John Wiley & Sons, Ltd.

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Introduction

Genetic integrity is critical to the survival and propagation of all cellular organisms. DNA damage is continually at risk and can result from normal cellular metabolism as well as environmental stresses. Free oxygen radicals, generated either by metabolic processes or by exposure to ionizing radiation, can break phosphodiester bonds in the backbone of the DNA helix. Alkylating agents can modify the bases of DNA or cause intra- or inter-strand cross-links. Inhibitors of DNA topoisomerases can lead to enhanced single- or double-strand breaks, depending on which topoisomerase is inhibited and on the phase of the cell cycle. To survive, cells have evolved complex surveillance mechanisms that

continually monitor genomic integrity. Exposure to DNA-damaging agents can activate DNA repair mechanisms and cell cycle checkpoints or initiate the process of apoptosis. Such molecular insults can also activate some transcription processes to induce proteins that are required for these events. This core DNA damage response, however, is but one component of a global response to DNA damage. Cellular macromolecules other than DNA are also subject to modification from damaging agents and these damaged macromolecules need to be repaired or removed (Begley *et al*., 2002; Jelinsky *et al*., 2000; Jelinsky and Samson, 1999). Thus, global responses to DNA damage are coordinated precisely by many regulatory mechanisms, including transcriptional regulation, RNA and protein turnover, interactions between proteins, RNA and DNA, and post-translational modifications.

Transcriptional profiling studies (Birrell *et al*., 2002; Gasch *et al*., 2000, 2001; Hughes *et al*., 2000; Jelinsky *et al*., 2000; Jelinsky and Samson, 1999; Natarajan *et al*., 2001) and high-throughput phenotype analyses (Bennett *et al*., 2001; Birrell *et al*., 2002; Chang *et al*., 2002; Ross-Macdonald *et al*., 1999) have been used in yeast to identify genes and proteins important in the responses to DNA damage. The DNA alkylating agent, methyl methanesulphonate (MMS) is a known carcinogen and primarily modifies DNA at N7-methylguanine and N3-deoxyadenine. Although the N7-methylguanine adduct may be non-toxic and non-mutagenic, the N3-methyladenine is a lethal lesion that inhibits DNA synthesis and needs to be actively repaired. DNA damage caused by alkylating agents is repaired predominantly by base excision repair pathways and DNA alkyltransferases. Transcriptional responses to MMS have been studied by several research groups (Gasch *et al*., 2000, 2001; Hughes *et al*., 2000; Jelinsky *et al*., 2000; Jelinsky and Samson, 1999; Natarajan *et al*., 2001). In addition to DNA repair proteins, many genes not involved in DNA repair were identified. These genes function to degrade and synthesize proteins and control RNA metabolism, signal transduction and transcription. Despite the power of global genomic analyses using DNA chips, a significant shortcoming to this approach is that mRNA levels do not necessarily or completely reflect cellular protein levels, since post-transcriptional regulation also plays a role.

Recently a yeast GFP library, whose open reading frames were tagged with green fluorescent protein (GFP), was constructed and used for global localization studies (Huh *et al*., 2003). As all gene products can be detected using the GFP signal, we have been able to measure the amount of each tagged protein using flow cytometry. We tested 4156 proteins and determined that 157 proteins were induced by MMS treatment (Table 2). Among the 157 proteins, 100 were previously reported to be induced at a transcription level by MMS so that the 57 proteins were newly identified by this study. These 57 proteins were reported not to be induced on a transcriptional level by MMS, suggesting that post-transcriptional regulation may be involved in the induction of these proteins. Thus, we have demonstrated that protein expression profiling, using a GFP library and subsequent flow cytometric analysis, can identify valid new DNA damage-inducible proteins without evidence of change of the mRNA levels. Follow-up studies for these proteins will identify new regulation mechanisms of induction of these proteins and provide valuable insights into the understanding of the functions of these proteins in DNA damage response pathways or other stress response pathways.

Materials and methods

Strains, media and growth conditions

In this study, we used 4156 yeast strains expressing full-length, chromosome-tagged GFP fusion proteins to monitor changes of protein levels in response to the DNA-damaging agent, methyl methanesulphonate (MMS). The haploid parent yeast strain (ATCC201388: *MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) was used as a control. Yeast cells were grown in 1% yeast extract/2% peptone/2% glucose media at 30 °C.

Exponentially-grown cells were divided equally and MMS was directly added as a liquid (at 0.02%) to one of the two cell populations. Cells were then cultured with or without MMS for 4 h.

FACS analysis

Cellular fluorescence from GFP was determined quantitatively with a FACSCalibur flow cytometer (Becton Dickinson, CA) equipped with a 15 mW,

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488 nm argon ion laser. Voltage and gain setting, respectively, were set at 582 and 1.00 in log mode for FL1 readings and E00 and 1.00 in linear mode for forward scatter (FSC) readings. It was customary to analyse 30 000 cells/sample. Data acquisition and subsequent analysis were performed using CELLQuest software (BD).

The induction fold was calculated as follows:

$$\begin{aligned}\text{Induction fold} = &[(\text{MV}_{\text{sample, mms+}}) \\ &- (\text{MV}_{\text{control, mms+}})]/ \\ &[(\text{MV}_{\text{sample, mms-}}) \\ &- (\text{MV}_{\text{control, mms-}})]\end{aligned}$$

where MV is control, mms^+ and mms^- are mean median values, control cell lacking GFP, MMS treated and MMS not treated, respectively.

Western blotting

TAP-tagged yeast cells (Ghaemmaghami *et al*., 2003) were grown to $\text{OD}_{600} \approx 0.7$ at 30 °C and divided into two culture populations. MMS (0.02%) was added directly to one culture, and both cultures were incubated at 30 °C for 4 h. After centrifugation, a lysis buffer (1% SDS, 8 M urea, 10 mM MOPS, pH 6.8, 10 mM EDTA, 0.01% bromophenol blue) and phenyl methylsulphonyl fluoride (PMSF) were added to the cell pellets. The cell suspensions were vortexed with glass beads and boiled for 5 min. Lysed cells were centrifuged and 25 µl aliquots of the supernatant extract were loaded onto SDS–PAGE gels. The gels were run at 200 V for 70 min, transferred onto nitrocellulose membranes, and a constant current of 250 mA was applied to each gel for 120 min. The blots were probed using rabbit IgG (Sigma) at a 1 : 1000 dilution, and subsequently the blots were probed with a horseradish peroxidase (HRP)-conjugated goat secondary antibody (Pierce) (1 : 10 000) against rabbit IgG. The TAP-conjugated proteins were detected by using an ECL kit (Amersham). The transfer efficiencies and protein loads were monitored by fast green staining.

Results and discussion

The DNA chip method has been used to monitor global gene expression changes under certain conditions in yeast. Many genes were reported to be induced or repressed in response to the DNA-damaging agent MMS. These genome-wide studies yielded three unexpected and important findings about cellular responses to DNA-damaging agents. First, DNA-damaging agents cause damage to other macromolecules, including proteins and RNA. Second, the agents activate a proteasome-dependent protein degradation pathway that leads to the degradation of damaged proteins. Third, gene expression responses to specific damaging agents results in distinct expression profiles and a general stress response pathway.

Despite the power of global genomic analyses, one significant deficiency to this approach stems from the fact that changes in mRNA levels do not always correlate with similar changes in protein expression and that post-transcriptional regulation can also affect protein levels. Thus, complementary proteomic analysis may provide a more complete assessment of the distinct molecular profile under certain defined experimental conditions. Due to the diverse nature of proteins, global protein expression profiling studies are not well established. Recently, several yeast strain collections have tagged each of their annotated open reading frames (ORFs) with specific epitope tags, such as GFP or TAP. To study the global protein expression profiling of yeast in response to a DNA-damaging agent, we used 4156 yeast strains that expressed full-length, chromosome-tagged GFP fusion proteins, which were then used for the study of global protein localization in yeast (Huh *et al*., 2003). The parent yeast strain not harbouring GFP was used as a study control. Flow cytometric analysis was then applied to directly quantify GFP fusion proteins. We examined cellular green fluorescence quantitatively for four well-known DNA damage-inducible genes (RNR3, GTT2, FLR1 and HUG1). Induction fold was calculated by the ratio of normalized median values. Increases of protein levels due to these genes was easily detected by flow cytometry (Figure 1). We used this assay to monitor global protein expression with the 4156 yeast strains. Of 4156 ORFs, 568 (13.7%) showed a more than two-fold increase in protein level as a result of MMS treatment. Of these, 157 (3.8%) showed a more than three-fold increase, 65 (1.6%) a more than four-fold increase and 33 (0.8%) a more than five-fold increase. A three-fold change was arbitrarily chosen as the cut-off level for further studies (Table 1). Only 28 (18%)

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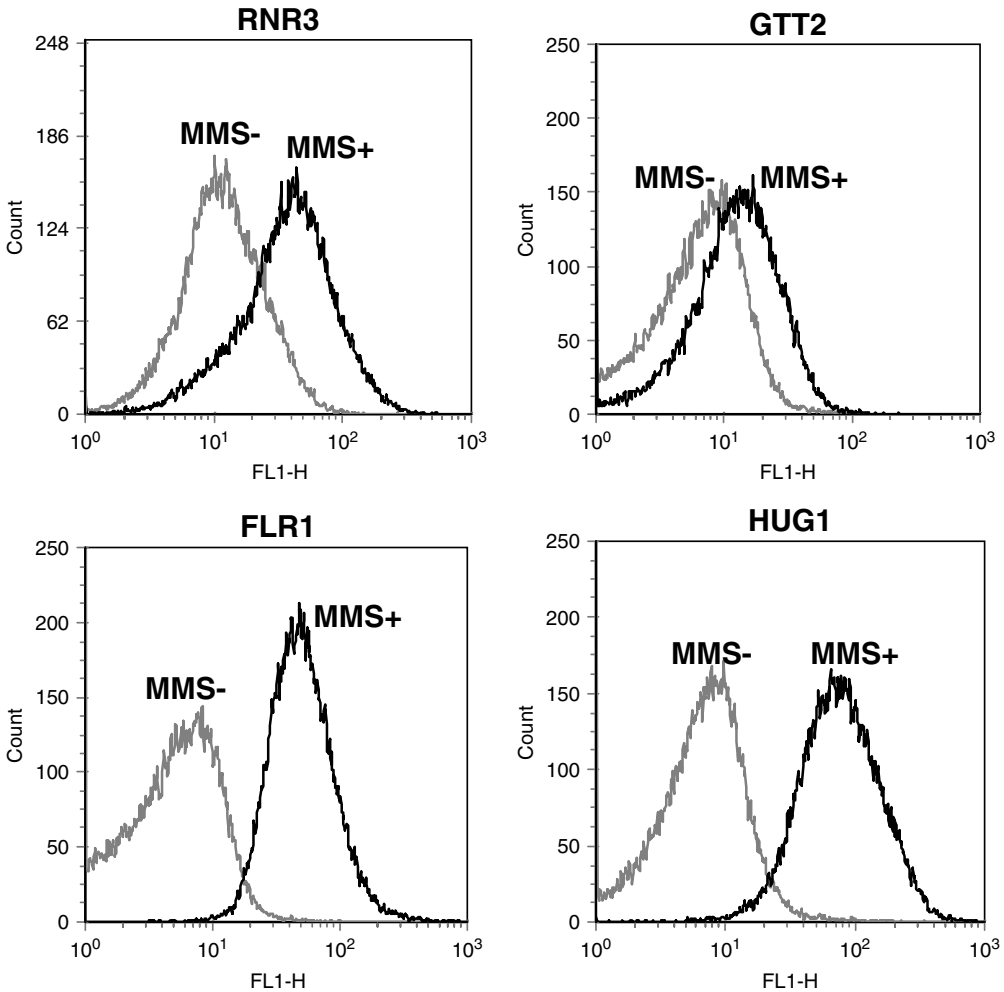

Figure 1. FACS analysis of yeast strains expressing GFP fusion genes whose transcriptions were previously reported to be increased by DNA damage treatments. Green fluorescence (FL1) data is presented on a logarithmic scale. RNR3, subunit of ribonucleotide-diphosphate reductase; GTT2, glutathione S-transferase; FLR1, plasma membrane multidrug transporter; HUG1, hydroxyurea and UV and γ-radiation-induced gene

were known to play roles in DNA repair and the cell cycle checkpoint. In addition to DNA repair and cell cycle checkpoint proteins, many proteins involved in general stress response/detoxification and protein modification/degradation were induced by MMS treatment. As MMS alkylates proteins as well as DNA, the protein degradation pathway has been proposed to remove damaged proteins. With the exception of some genes with known functions in DNA damage response pathway, it remains to be determined how other inducible proteins plays protective roles against MMS treatment and how these proteins are induced without transcription induction.

Four proteins, Hug1 (DNA damage checkpoint protein), Cbp4 (ubiquinol–cytochrome C reductase assembly factor), Aqy2 (water channel) and Prx1 (mitochondrial isoforms of thioredoxin peroxidase) were increased greater than 10-fold. We compared these results to DNA chip results previously reported by Jelinsky and Samson (1999). Of the 157 inducible proteins, 100 (64%) were previously reported to be induced on a transcriptional level by DNA-damaging agents; however, it is important to note that in the remaining 57 proteins it was previously reported that their transcription was not changed or even repressed by MMS. It is conceivable that post-transcriptional regulation

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Table 1. ORFs whose protein expression are induced more than three-fold by MMS ($n = 157$)

ORF	Gene	Fold	Function
Cell cycle and DNA repair			
YML058W-A	HUG1	46.2	Hydroxyurea, UV and γ-irradiation-induced
YAL009W	SPO7	6.6	Meiotic protein
YBR088C	POL30	6.4	Proliferating cell nuclear antigen (PCNA)
YIL066C	RNR3	6.1	Subunit of ribonucleotide-diphosphate reductase
YDL076C*	RXT3	5.7	Putative histone acetylase
YFL014W*	HSP12	5.6	Heat shock protein
YHR167W*	THP2	4.3	Subunit of the THO complex
YEL019C*	MMS21	4.2	SUMO ligase
YDR182W	CDC1	4.2	Cell division control protein
YPL241C	CIN2	4.2	Involved in chromosome segregation
YNL312W	RFA2	4.0	DNA replication factor A, 36 kDa subunit
YGR180C	RNR4	4.0	Ribonucleotide reductase, small subunit
YGR209C*	TRX2	4.0	Thioredoxin
YGL075C	MPS2	4.0	Protein of the nuclear envelope/endoplasmic reticulum
YLR086W	SMC4	3.7	Subunit of the condensin complex
YBL063W	KIP1	3.6	Kinesin-related motor
YOR265W	RBL2	3.6	β-Tubulin binding protein
YOL148C*	SPT20	3.5	Member of the TBP class of SPT proteins
YJR140C*	HIR3	3.4	Involved in cell cycle regulation of histone transcription
YLR135W	SLX4	3.3	Subunit of Slx1p/Ybr228p–Slx4p complex
YNR010W*	CSE2	3.3	Subunit of RNA polymerase II mediator complex
YLR310C	CDC25	3.3	GDP/GTP exchange factor for Ras1p and Ras2p
YKL052C	ASK1	3.2	Outer kinetochore protein (part of Dam1 complex)
YNL246W*	VPS75	3.2	Protein involved in vacuolar protein sorting
YNL088W	TOP2	3.2	DNA topoisomerase II (ATP-hydrolysing)
YHR090C	YNG2	3.2	Component of NuA4 histone acetyltransferase complex
YKR031C*	SPO14	3.2	Phospholipase D
YBR160W*	CDC28	3.1	Cyclin-dependent protein kinase
Stress response/detoxification			
YBL064C	PRX1	10.2	Mitochondrial isoform of thioredoxin peroxidase
YDR453C	TSA2	6.1	Thioredoxin-peroxidase
YBR173C*	UMP1	6.0	Proteasome maturation factor
YKL086W	SRX1	5.8	Sulphiredoxin
YBR244W	GPX2	5.6	Glutathione peroxidase
YFL014W*	HSP12	5.6	Heat shock protein
YLL060C	GTT2	4.9	Glutathione S-transferase
YKL150W*	MCR1	4.7	Mitochondrial NADH-cytochrome *b*5 reductase
YPL163C	SVS1	4.7	Vanadate sensitive suppressor
YDR533C	HSP31	4.3	Member of the DJ-1/ThiJ/PfpI superfamily
YJL179W	PFD1	4.3	Subunit of prefoldin
YGR209C*	TRX2	4.0	Thioredoxin
YML116W*	ATR1	3.9	Putative substrate-H^+ antiporter
YML028W	TSA1	3.8	Thioredoxin-peroxidase (TPx)
YMR022W*	QRI8	3.7	E2 ubiquitin-conjugation enzyme
YML014W*	TRM9	3.7	tRNA-methyltransferase
YKL007W	CAP1	3.6	F-actin capping protein α-subunit
YIL113W*	SDP1	3.5	Stress-inducible dual specificity phosphatase
YBR072W	HSP26	3.3	Small heat shock protein
YJL115W*	ASF1	3.2	Nucleosome assembly factor
YGL016W*	KAP122	3.2	Member of the karyopherin-β family, nuclear import
Carbohydrate metabolism			
YJR096W		6.0	Xylose and arabinose reductase
YGL156W	AMS1	4.5	Alpha mannosidase
YGL047W	ALG13	3.3	Essential protein
YBL001C	ECM15	3.1	Involved in cell wall biogenesis and architec
YPL050C	MNN9	3.1	Subunit of Golgi mannosyltransferase complex
Amino acid metabolism			
YJR137C*	ECM17	9.5	Involved in cell wall biogenesis and architecture
YLR146C	SPE4	4.2	Spermine synthase
YHL036W*	MUP3	3.8	Low affinity methionine permease
YCR059C	YIH1	3.7	Piecemeal microautophagy of the nucleus (PMN)
YBL036C		3.4	Putative unspecific racemase
Transcription			
YPR107C	YTH1	5.5	Pre-mRNA 3′-end processing and polyadenylation protein
YMR039C	SUB1	5.2	Transcriptional coactivator
YOL093W	TRM10	4.6	tRNA methyltransferase

Table 1. Continued

ORF	Gene	Fold	Function
YKL058W	TOA2	4.6	Transcription factor IIA subunit
YHR167W*	THP2	4.3	Subunit of the THO complex
YML112W*	CTK3	4.1	Subunit of C-terminal domain kinase I (CTDK-I)
YML014W*	TRM9	3.7	tRNA-methyltransferase
YKL095W	YJU2	3.6	Putative spliceosomal component
YOL148C*	SPT20	3.5	Member of the TBP class of SPT proteins
YJR140C*	HIR3	3.4	Involved in cell cycle regulation of histone transcription
YNR010W*	CSE2	3.3	Subunit of RNA polymerase II mediator complex
YKR052C*	MRS4	3.2	Protein of the mitochondrial carrier family (MCF)
YJL115W*	ASF1	3.2	Nucleosome assembly factor
YGL070C	RPB9	3.2	DNA-directed RNA polymerase II, 14.2 kDa subunit
YKR086W	PRP16	3.1	RNA helicase
YHR058C	MED6	3.1	RNA polymerase II transcriptional regulation mediator
Protein modification/degradation			
YBR173C*	UMP1	6.0	Proteasome maturation factor
YDL076C*	RXT3	5.7	Putative histone acetylase
YEL012W*	UBC8	4.9	E2 ubiquitin-conjugating enzyme
YEL019C*	MMS21	4.2	SUMO ligase
YMR089C*	YTA12	4.2	Protease of the SEC18/CDC48/PAS1 family
YML112W*	CTK3	4.1	Subunit of C-terminal domain kinase I (CTDK-I)
YGR209C*	TRX2	4.0	Thioredoxin
YOL100W	PKH2	3.7	Serine/threonine protein kinase
YMR022W*	QRI8	3.7	E2 ubiquitin-conjugation enzyme
YHR027C	RPN1	3.6	26S proteasome regulatory subunit
YIL113W*	SDP1	3.5	Stress-inducible dual specificity phosphatase
YOL148C*	SPT20	3.5	Member of the TBP class of SPT proteins
YDR139C	RUB1	3.4	Ubiquitin-like protein
YCL010C	SGF29	3.3	SAGA-associated factor
YCL052C	PBN1	3.3	Required for post-translational processing of Prb1p
YBR160W*	CDC28	3.1	Cyclin-dependent protein kinase
YPL120W*	VPS30	3.1	Involved in vacuolar protein sorting and autophagy

Table 1. Continued

ORF	Gene	Fold	Function
Energy			
YGR174C	CBP4	18.9	Ubiquinol–cytochrome *c* reductase assembly factor
YKL016C*	ATP7	9.7	Subunit of mitochondrial F1F0 ATP synthase
YEL012W*	UBC8	4.9	E2 ubiquitin-conjugating enzyme
YKL150W*	MCR1	4.7	Mitochondrial NADH-cytochrome *b*5 reductase
YMR089C*	YTA12	4.2	Protease of the SEC18/CDC48/PAS1 family
YIL070C	MAM33	3.6	Mitochondrial acidic matrix protein
YML110C	COQ5	3.4	Ubiquinone biosynthesis, methyltransferase
YGL008C*	PMA1	3.2	H^+-transporting P-type ATPase
YNL055C*	POR1	3.2	Mitochondrial porin
Transport			
YLL052C	AQY2	10.4	Water channel
YKL016C*	ATP7	9.6	Subunit of mitochondrial F1F0 ATP synthase
YNR006W	VPS27	9.0	Hydrophilic protein
YGL167C	PMR1	5.0	High affinity Ca^{2+}/$Mn2^+$ P-type ATPase
YKL150W*	MCR1	4.7	Mitochondrial NADH-cytochrome *b*5 reductase
YKL064W	MNR2	4.5	Manganese-resistant protein
YHR167W*	THP2	4.3	Subunit of the THO complex
YMR089C*	YTA12	4.2	Protease of the SEC18/CDC48/PAS1
YJL145W	SFH5	4.2	Phospholipid transporter
YMR319C	FET4	4.1	Low-affinity Fe(II) transporter
YPR032W	SRO7	4.0	Polarized exocytosis by regulating SNARE function
YGR209C*	TRX2	4.0	Thioredoxin
YML116W*	ATR1	3.9	Putative substrate-H^+ antiporter
YOR098C	NUP1	3.8	Nuclear pore complex (NPC) subunit
YHL036W*	MUP3	3.8	Low affinity methionine permease
YKR052C*	MRS4	3.2	Protein of the mitochondrial carrier family (MCF)
YNL246W*	VPS75	3.2	Protein involved in vacuolar protein sorting
YGL016W*	KAP122	3.2	Member of the karyopherin-β family
YGL008C*	PMA1	3.2	H^+-transporting P-type ATPase
YKR031C*	SPO14	3.2	Phospholipase D
YMR231W	PEP5	3.2	Vacuolar biogenesis protein
YOR329C	SCD5	3.2	Suppressor of clathrin deficiency

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Table 1. Continued

ORF	Gene	Fold	Function
YNL055C*	POR1	3.2	Mitochondrial porin
YPL120W*	VPS30	3.1	Involved in vacuolar protein sorting and autophagy
Others			
YJR137C*	ECM17	9.5	Involved in cell wall biogenesis and architecture
YGL053W	PRM8	6.0	Pheromone-regulated protein
YCR067C	SED4	5.4	ER membrane protein
YJR047C	ANB1	5.3	Translation initiation factor eIF-5A
YFR047C	BNA6	5.1	Quinolinate phosphoribosyl transferase
YCR024C	SLM5	4.9	Asparginyl-tRNA synthetase, mithochondrial
YGL174W	BUD13	4.4	Protein involved in bud-site selection
YMR159C	ATG16	3.7	Coiled-coil protein required for autophagy
YDR503C	LPP1	3.5	Lipid phosphate phosphatase
YOR212W	STE4	3.5	GTP-binding protein β-subunit
YDR405W	MRP20	3.5	Mitochondrial ribosomal protein, large subunit
YDR025W	RPS11A	3.4	Ribosomal protein S11.e
YER117W	RPL23B	3.4	Ribosomal protein L23.e
YFR041C	ERJ5	3.4	Endoplasmic reticulum located J-protein
YPL097W	MSY1	3.4	Tyrosyl-tRNA synthetase
YKL040C	NFU1	3.4	Iron homeostasis
YKL013C	ARC19	3.3	Subunit of the ARP2/3 complex
YHR121W	LSM12	3.3	Protein containing an Lsm domain and an AD domain
YLR450W	HMG2	3.2	3-Hydroxy-3-methylglutaryl-coenzyme A reductase 2
YIL093C	RSM25	3.0	Mitochondrial ribosomal protein, small subunit
Unknown/unclassified (45 ORFs)§			

Categories are derived from the Munich Information Center for Protein Sequences (MIPS) database.
* ORFs fall into more than two categories.
§ These ORFs can be found in Table 2.

Table 2. ORFs whose transcripts are not induced but whose protein expressions are induced by MMS ($n = 57$)

Transcription changes by MMS	ORF whose protein expression are induced by MMS
Induced	HUG1, PRX1, VPS27, YJL144W, POL30, YOR220W, YML131W, RNR3, TSA2, PRM8, YJR096W, UMP1, YKL086W, YFL044C, RXT3, GPX2, HSP12, YTH1, SED4, BNA6, PMR1, UBC8, GTT2, YLR201C, MCR1, YJL068C, TOA2, YIR036C, AMS1, MNR2, BUD13, YDR533C, THP2, CIN2, YTA12, YNL134C, CTK3, YDR262W, APA2, SRO7, RFA2, RNR4, TRX2, MPS2, YHR087W, YMR244C-A, YKL206C, ATR1, NUP1, TSA1, YJR085C, MUP3, YNL168C,, PKH2, QRI8, YHR192W, MAM33, RPN1, YNL194C, RBL2, YJU2, CAP1, STE4, VPS60, SDP1, MRP20, SPT20, COQ5, YFR041C, MSY1, SGF29, ARC19, YGL047W, YOR062C, HSP26, YGL085W, YDL119C, CSE2, YPR147C, YFR017C, PBN1, YHR121W, TOP2, KAP122, YNG2, PEP5, YMR184W, YMR099C, SCD5, POR1, CDC28, VPS30, YPR022C, ECM15, YIL087C, MED6, MSB4, YMR178W, RSM25, YLR118C
Not changed	CBP4, AQY2, ATP7, ECM17, YCL005W, SPO7, CWC24, YJR011C, YDL203C, YHL018W, SUB1, YLR271W, PMP1, UIP3, SVS1, TRM10, PFD1, MMS21, CDC1, YJL018W, YIH1, APG16, TRM9, SMC4, KIP1, LPP1, YBL036C, RUB1, YPL068C, HIR3,, NFU1, SLX4, CDC25, YGR126W, ASK1, MRS4, VPS75, ASF1, YKR075C, YLR412W, SPO14, RPB9, YGR058W, YPL108W, HMG2, PRP16, INM1, YAR028W, YOR199W, MNN9, MRPL24
Repressed	ANB1, SPE4, FET4, RPS11A, RPL23B,, PMA1

Transcription changes were based on the previous study of Jelinsky and Samson (1999).

may be involved in the induction of these 57 proteins. Jelinsky and Samson (1999) reported that 325 gene transcripts among 6200 ORFs were induced more than four-fold by MMS treatment. In terms of whether ORFs were inducible, 64% of our protein expression profiling data overlaps with previously reported transcription expression profiling data. The use of a different cut-off value also gives similar overlaps of two different approaches.

Among the newly identified ORFs, null mutants of three genes (*ASF1, RPB9* and *SLX4*) were reported to be sensitive to MMS (Chang *et al*., 2002). Null mutants of *ASF1* and *RPB9* are also sensitive to ionizing radiation, UV light and replication stresses. *slx4* mutant cells are sensitive only to MMS and not to the other DNA damage or replication stresses. Slx4 forms a complex with Slx1, in which Slx1-Slx4 is a second structure-specific endonuclease with endonuclease activity *in vitro* towards branched DNA substrates and a preference for simple-Y, 5′-flap or replication-fork-like structures (Fricke and Brill, 2003). Slx4

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becomes phosphorylated after DNA damage in a Mec1/Tel1-dependent manner and is required for the repair of DNA alkylation damage (Flott and Rouse, 2005). Asf1 is a histone chaperone which functions during both replication-coupled and replication-independent chromatin assembly. Asf1 also interacts with the Cac2 subunit of Caf-1 (Krawitz *et al*., 2002; Mello *et al*., 2002; Tyler *et al*., 2001) and stimulates histone deposition by Caf-1 in vitro (Sharp *et al*., 2001; Tyler *et al*., 1999). Asf1 functions with Hir to promote heterochromatic gene silencing and also contributes to genome stability during the S phase. Asf1 directly interacts with the DNA damage/replication checkpoint kinase Rad53 in a manner that is regulated by checkpoint activation (Emili *et al*., 2001; Hu *et al*., 2001). asf1Δ cells have multiple phenotypes, which suggests elevated levels of spontaneous DNA damage, including increased phosphorylation of Rad53, Rad9, Mrc1 and H2A (Hu *et al*., 2001; Prado *et al*., 2004; Ramey *et al*., 2004; Schwartz *et al*., 2003). Rpb9 is one subunit of RNA polymerase II (Pol II) that is not essential for cell viability and its deletion results in a mild sensitivity to temperature and relatively normal levels of transcription *in vivo* (Woychik *et al*., 1991). Rpb9 is located at the tip of the so-called 'jaws' of Pol II, which are thought to function by clamping the DNA downstream of the active site (Cramer *et al*., 2001; Gnatt *et al*., 2001) and to regulate transcription initiation and elongation (Awrey *et al*., 1997; Hemming *et al*., 2000; Hull *et al*., 1995; Van Mullem *et al*., 2002). Rpb9 also mediates a transcription-coupled repair (TCR) subpathway in *Saccharomyces cerevisiae* (Li and Smerdon, 2002). Sub1 is a transcription coactivator that facilitates transcription elongation by influencing enzymes that modify RNA polymerase II (Calvo and Manley, 2005), suggesting that DNA damage may affect the RNA polymerase II pathway. MMS21 is a SUMO ligase involved in chromosome organization and DNA repair, whose mutations are sensitive to MMS and show increased spontaneous mutations and mitotic recombinations.

For some highly inducible proteins, the basal protein levels are very low, which restricts accurate calculation of fold-induction values (Table 3). Among 19 proteins, 10 were previously identified as DNA damage-inducible (Jelinsky and Samson, 1999). To establish that the information obtained from FACS analysis was accurate, we chose at random 28 of the newly identified proteins for examination by conventional Western blot analysis. To minimize the effects of GFP tagging, we used different tagged yeast strains in immunoblot analysis. Yeast strains chromosomally intergrated TAP-tagged ORFs were exposed to MMS treatment and protein levels of TAP-tagged proteins were determined by immunoblot analysis. Before Western blotting, equal protein loads were monitored by staining of membranes with fast green stain (data not shown). We confirmed that all 28 proteins tested were induced by the MMS treatment (Figure 2).

We performed DNA damage-inducible protein screening by using the GFP-library and FACS analysis. This screening process indicated that 156 proteins could be identified as DNA damage-inducible proteins. Of the 100 proteins previously reported

Table 3. ORF whose protein expressions are significantly induced by MMS but the induction folds could not be determined

ORF	Gene	Function
YKR037C	SPC34	Outer kinetochore protein (part of Dam1 complex)
YGR161C	RTS3	Protein phosphatase type 2A
YLR091W*		Hypothetical ORF
YDR184C*	ATC1	Nuclear protein
YLR093C*	NYV1	V-SNARE component of the vacuolar SNARE complex involved in vesicle fusion
YBR008C	FLR1	Plasma membrane multidrug transporter
YOR228C		Hypothetical ORF
YHL026C*		Hypothetical ORF
YML064C*	TEM1	GTP-binding protein of the ras superfamily
YHR057C	CPR2	Peptidyl-prolyl *cis-trans*-isomerase (cyclophilin)
YOR052C		Hypothetical ORF
YER175C	TMT1	Trans-aconitate methyltransferase
YMR090W		Hypothetical ORF
YBR291C*	CTP1	Mitochondrial inner membrane citrate transporter
YMR060C	TOM37	Component of the mitochondrial outer membrane sorting and assembly machinery (SAM) complex
YPL064C*	CWC27	Component of a complex containing Cef1p
YOR251C*		Catalyses transfer of the sulphane atom of thiosulphate to cyanide to form sulphite and thiocyanate
YEL048C*		Hypothetical ORF
YOR320C*	GNT1	N-acetylglucosaminyltransferase

The intensity of the GFP signal in untreated cells was below an arbitrary threshold.

* ORFs whose transcriptions were previously reported not to be induced by MMS treatment.

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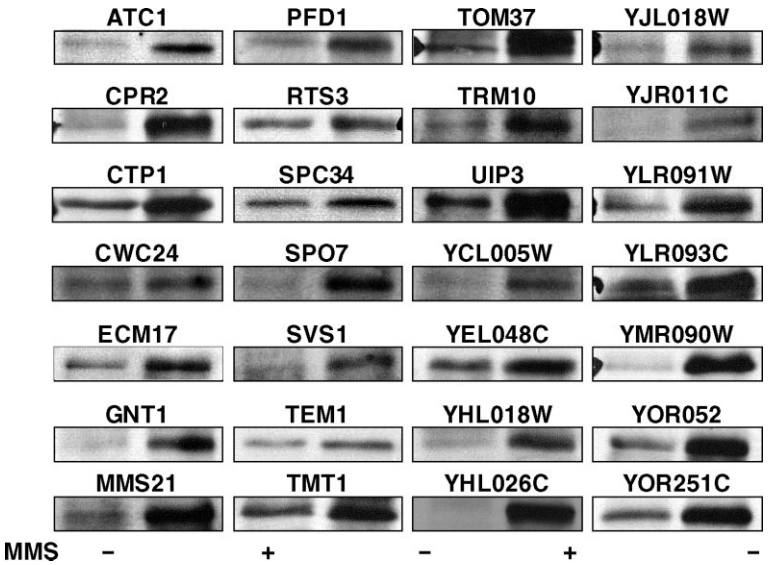

Figure 2. Immunoblot analysis of proteins whose transcription was not changed and only the protein levels were increased by MMS treatment. PFD1, subunit of prefoldin; TOM37, component of the mitochondrial outer membrane sorting and assembly machinery (SAM) complex; CPR2, peptidyl-prolyl *cis-trans*-isomerase; RTS3, protein phosphatase type 2A; TRM10, tRNA methyltransferase; CTP1, mitochondrial inner membrane citrate transporter; SPC34, outer kinetochore protein (part of Dam1 complex); SPO7, meiotic protein; ECM17, involved in cell wall biogenesis and architecture; SVS1, vanadate sensitive suppressor; GNT1, N-acetylglucosaminyltransferase; TEM1, GTP-binding protein of the ras superfamily; MMS21, SUMO ligase; TMT1, *trans*-aconitate methyltransferase

to be induced on a transcription level by MMS, 57 proteins were newly identified by this study. The screening approach that was used in the present study can validly identify new DNA damage-inducible proteins which do not demonstrate any changes at transcriptional levels. Further follow-up *in vivo* studies of these proteins may identify new regulation mechanisms of induction of these proteins. These latter studies could provide invaluable insights into understanding possible intracellular DNA damage responses and other stress response pathways.

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