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Ubiquitin Ligase Activity of TFIIH and the Transcriptional Response to DNA Damage

Short Article

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Summary

Core transcription factor (TF) IIH purified from yeast possesses an E3 ubiquitin (Ub) ligase activity, which resides, at least in part, in a RING finger (RNF) domain of the Ssl1 subunit. Yeast strains mutated in the Ssl1 RNF domain are sensitive to ultraviolet (UV) light and to methyl methanesulfonate (MMS). This increased sensitivity to DNA-damaging agents does not reflect a deficiency in nucleotide excision repair. Rather, it correlates with reduced transcriptional induction of genes involved in DNA repair, suggesting that the E3 Ub ligase activity of TFIIH mediates the transcriptional response to DNA damage.

Introduction

A five subunit "core" of general transcription factor TFIIH is essential both for transcription initiation and for DNA repair (Svejstrup et al., 1996). The core includes a DNA helicase and associates with a second helicase and with a cyclin-dependent protein kinase complex to form the factor involved in transcription. Alternatively, the core associates with the second helicase and with a set of five Rad proteins to form a "repairosome" especially competent for nucleotide excision DNA repair.

We now report a third catalytic function of TFIIH, an E3 Ub ligase activity. Transcription proteins, such as RNA polymerase II, are targets of Ub ligases, but no ubiquitylation activity has previously been identified within the transcription machinery. Indeed, a growing body of evidence suggests the involvement of the ubiquitin/proteasome system in transcription and DNA repair in both proteolytic and nonproteolytic pathways, but few of the E3 Ub ligases involved are known (Conaway et al., 2002; McBride et al., 2003; Muratani and Tansey, 2003).

Our findings further illuminate the relationship between transcription and DNA repair. The connection through TFIIH was limited until now to the common subunits involved, which could reflect little beyond the requirement for helicase activities for the two processes. Our analysis of the functional consequences of mutations in the TFIIH E3 ligase suggests a more intimate connection, a regulatory role of TFIIH in the transcription of DNA damage-response genes.

Results

Our study was prompted by the observation of RNF motifs in the Ssl1/p44 (yeast/human) subunit of core TFIIH and in the Tfb3/MAT1 subunit of the cyclin-dependent protein kinase complex (Fribourg et al., 2000; Gervais et al., 2001). Inasmuch as RNF motifs are commonly indicative of E3 Ub ligase activity (Joazeiro and Weissman, 2000), we assayed highly purified preparations of yeast core TFIIH for such activity. Assays were performed in a ubiquitylation system lacking only E3 protein. Core TFIIH was purified from strains bearing either a hexahistidine tag on Tfb1 or a TAP tag on Tfb4 (Chang and Kornberg, 2000; Takagi et al., 2003). Both preparations contained only the five core subunits (Figure 1A), with no Tfb3 or other TFIIH subunits detectable by mass spectrometry or by immunoblotting (Takagi et al., 2003), and both supported the formation of Ub conjugates (Figures 1B, lane 4 and Figure 1D, lanes 5–8), dependent on all components of the ubiquitylation system (E1, E2, E3, Ub, and Mg•ATP; Figure 1C). Both core TFIIH preparations therefore possessed E3 ligase activity.

To confirm that E3 activity is intrinsic to core TFIIH (and not due to a trace contaminant of the preparations) and to identify the core subunit responsible for the activity, we expressed the subunits individually as GST fusion proteins in *E. coli*. GST-Ssl1 exhibited E3 activity in the ubiquitylation assay (Figure 2C, lanes 2–4), whereas GST-Tfb1, GST-Tfb2, or GST-Tfb4 did not. The E3-specific activity of the GST-Ssl1 fusion protein was less than that of core TFIIH (Figure S1 available online with this article), suggesting a stimulatory effect of other core TFIIH subunits. The assays with core TFIIH (Figure 1) revealed that at least part of the reaction products are unanchored Ub chains whose synthesis may require the participation of another subunit in addition to Ssl1 to provide, for example, a noncovalent Ub binding surface (see Mms2/Ubc13 and E2-25K [Merkley and Shaw, 2004; Moraes et al., 2001; VanDemark et al., 2001]). Or Ssl1 might function simi-

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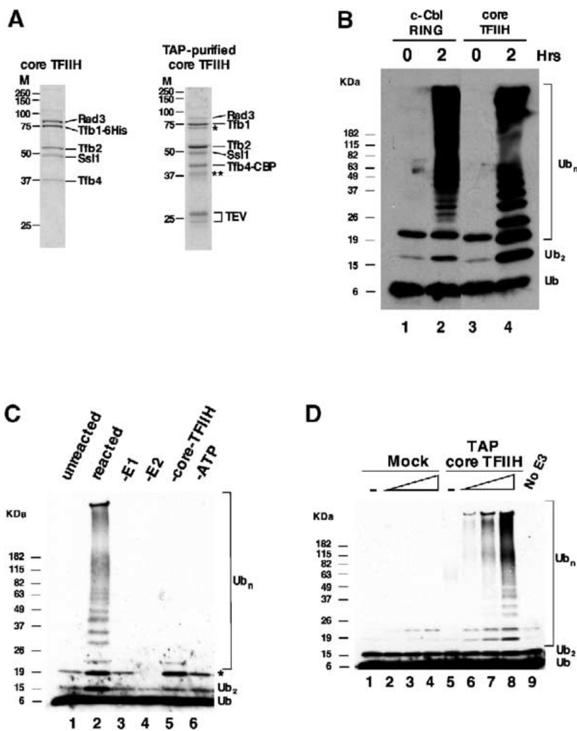

Figure 1. Yeast Core TFIIH Exhibits E3 Ligase Activity

(A) SDS-PAGE of highly purified *S. cerevisiae* core TFIIH preparations used in this study. A phenyl 5-WP fraction (Chang and Kornberg, 2000) (left) and an IgG column fraction (Takagi et al., 2003) (right) were electrophoresed in a 10% gel, followed by staining with Coomassie blue. Some Rad3 has been lost from the IgG column fraction, as occasionally noted for core TFIIH preparations in the past (Svejstrup et al., 1994). *Contaminant from the TEV preparation. **Unknown contaminant.

(B) Assays of E3 ligase activity (using Ubc4 as the E2) were performed with 1.8 μg of GST-c-Cbl and 0.22 μg of core TFIIH for 2 hr at 24°C. Ub conjugates were resolved by reducing SDS-PAGE and detected by immunoblotting with anti-Ub antibody. GST-c-Cbl RNF provided a positive control (Joazeiro et al., 1999). Abbreviations: Ub, ubiquitin; Ub$_2$, di-Ub; and Ub$_n$, Ub conjugates.

(C) Components required for the E3 ligase activity of core TFIIH. Ubiquitylation assays were performed in a complete system (lane 2) or in the absence of E1 (lane 3), E2/Ubc4 (lane 4), E3/core TFIIH (lane 5), or ATP (lane 6). Synthesis of Ub conjugates was detected as in (B) with the use of 0.1 μg of core TFIIH. The asterisk indicates a crossreactive band derived from the E2/Ubc4 preparation that overlaps with a Ub-modified reaction product.

(D) E3 ligase assays of core TFIIH (0.4 mg/ml) purified from a TAP-tagged yeast strain and of the product of a mock purification from an untagged strain, in the amounts indicated (1, 2, or 4 μl). Unreacted samples (reaction time <15 s; lanes 1 and 5) and a negative control (no addition of core TFIIH or mock preparation; lane 9) are also shown. Ub conjugates were detected as in (B).

larly to the RNF protein Rbx1, whose E3 activity is dependent on interaction with another subunit of a multiprotein complex (Skowyra et al., 1999). As Ssl1 has been shown to interact directly with the Tfb4 subunit of core TFIIH (Y.T., H.K., and R.D.K., unpublished data), we investigated the influence of this subunit upon E3 ligase activity. GST-Tfb4, itself inert in the ubiquitylation assay (Figure 2C, lanes 5–7), enhanced the E3 activity of GST-Ssl1 (Figure 2C, lanes 8–10), whereas GST alone did not (data not shown).

The involvement of the RNF domain in the E3 activity of GST-Ssl1 was tested by mutagenesis. The RNF domain is located in the C-terminal region of the protein and is highly conserved among Ssl1 homologs (Fribourg et al., 2000), including a conserved hydrophobic residue (Ile444) at a position where hydrophobic residues in other RNF E3s mediate critical interaction with the E2 component of the ubiquitylation system (Zheng et al., 2000). Substitution of alanine at positions in human p44 corresponding to conserved cysteines 403 and 406 of the yeast Ssl1 protein (Figure 2B) interfere with the binding of a zinc ion and so presumably disrupt RING finger structure (Fribourg et al., 2000). We generated mutant forms of Ssl1 bearing both substitutions, C403A and C406A. The mutant proteins were inactive in ubiquitylation in vitro (Figure 2D, lanes 3–5). The behavior of the mutants in vivo was investigated by replacing the *S. cerevisiae* chromosomal *SSL1* gene with plasmid-born mutant genes. All the mutant yeast strains were viable (Figure 3) although temperature-sensitive (data not shown). Thus, an intact Ssl1 RNF is not essential for cell growth, so it is dispensable for TFIIH complex assembly and does not play a general role in RNA polymerase II transcription. Consistent with this, the mutation in human p44 corresponding to C403A does not affect TFIIH complex assembly in insect cells or activity in transcription in vitro (Tremeau-Bravard et al., 2001). Furthermore, neither of the yeast mutants was defective in transcription in vitro (Figure 4A). In contrast with these results, mutations in other TFIIH subunits impairing TFIIH helicase and kinase activities interfere with both cell growth and transcription (Svejstrup et al., 1996).

The lack of requirement for the Ssl1 RNF for global gene transcription was confirmed by two further findings. First, *ssl1* RNF mutant strains grew indistinguishably from wild-type (wt) in the presence of 6-azauracil and mycophenolic acid (data not shown), inhibitors of transcription elongation (Riles et al., 2004; Wind and Reines, 2000). Second, in gene expression profiles, only 1% of genes in the C403A mutant and 0.3% of genes in the C406A mutant differed in expression level from wt (Tables S1 and S2).

By contrast, the Ssl1 RNF evidently functions in, and is required for, DNA repair: both *ssl1* mutant strains showed increased sensitivity to UV radiation and to treatment with the DNA-damaging agent MMS (Figure 3). This is perhaps unsurprising, in view of the well-established role of TFIIH in nucleotide excision repair (NER). Two lines of evidence, however, argue against a role of the Ssl1 RNF in NER. The first came from the phenotypes of double mutants bearing both amino acid substitutions in the Ssl1 RNF and a deletion of the *RAD14* gene known to abrogate NER. The double mutant strains C403A/*rad14Δ* and C406A/*rad14Δ* were significantly more UV sensitive than *rad14Δ* alone (Figure 3A). The *ssl1* mutations are thus epistatic to *rad14Δ* and so must lie in a different pathway. A second line of evidence against the involvement of the Ssl1 RNF motif in NER came from assays of NER in vitro. NER was monitored by the excision of single-stranded fragments 20–30 residues in length from DNA modified with a bulky N-acetoxy-2-acetylaminofluorene (AAF) adduct. Extracts of *ssl1* mutant strains showed almost as much

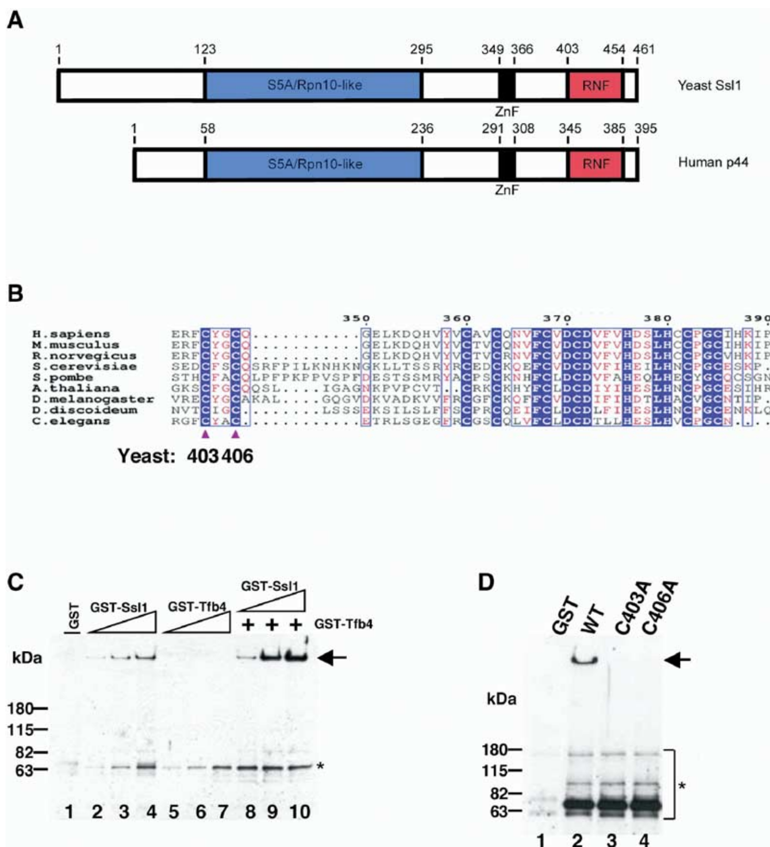

Figure 2. E3 Ligase Activity Resides in Ssl1 and Requires an Intact RNF Motif

(A) Domain organization of *S. cerevisiae* Ssl1 and of its human homolog p44. ZnF, zinc finger motif.

(B) Sequence alignment of the C termini of *S. cerevisiae* Ssl1 and its homologs (*M. musculus* [AA183802], *R. norvegicus* [XP_215466], *S. pombe* [c1682], *A. thaliana* [AC005322], *D. melanogaster* [AC005720], *D. discoideum* [ACO50835], *C. elegans* [Z30662]), derived from multiple sequence alignment with hierarchical clustering method as described (Corpet, 1988). Similar and identically conserved residues are boxed and shaded. Locations of alanine substitution mutations (403 and 406) are indicated at the bottom. Abbreviations: cysteine, C; histidine, H; phenylalanine, F; aspartic acid, D; leucine, L; and glycine, G.

(C) GST, GST-Ssl1, and GST-Tfb4 proteins were assayed for E3 ligase activity alone or in combination. GST (2 μg, lane 1), increasing amounts of protein (0.5, 1.0, or 2.0 μg; lanes 2–4, 5–7, and 8–10), and 2 μg of Tfb4 (lanes 8–10) were used as indicated. Ub conjugates were detected as in Figure 1B. The asterisk indicates a crossreacting protein that is also present in untreated samples.

(D) Effect of RNF cysteine mutations on E3 ligase activity. GST (lane 1), GST-Ssl1 (lane 2), GST-Ssl1 C403A (lane 3), and GST-Ssl1 C406A (lane 4) were assayed for E3 ligase activity (1 μg of GST or GST fusion protein in all cases). Ub conjugates were detected as in Figure 1B. The asterisk indicates crossreacting proteins that are also present in untreated samples.

activity as wt, whereas extracts of the *rad14* strain or of another strain defective in NER due to a point mutation in the Rad3 helicase (*rad3-21*) (Song et al., 1990) were much less active (Figure 4B). Consistent with these two lines of evidence, the *ssl1* mutant strains were much less sensitive to UV radiation than *rad4Δ* or *rad3-21* (Figure 3A) and more sensitive to MMS than the NER-defective strains (Figure 3B), a plausible finding because damage due to MMS is repaired by pathways such as base excision repair (BER) rather than NER.

An altogether different role of the Ssl1 RNF motif in DNA repair was suggested by gene expression profiles. The *ssl1* mutant strains were impaired in the transcriptional response to DNA damage, well characterized in *S. cerevisiae* (Gasch et al., 2001). In wt yeast, 251 genes exhibited a transcriptional response upon treatment with MMS (a change in expression of at least 2-fold, based on data with a p value < 0.01; Figure 5 and Table S3). In the C403A and C406A mutants, the transcriptional response of ~71% (179/251) and ~28% (71/251) of these genes, respectively, was reduced by at least 50% (Figure 5, Table 1, and Table S3). This diminished response was observed regardless of the stringency of the analysis (fold change and p value parameters; data not shown). The larger number of genes affected in the C403A mutant, which encompassed almost all those affected in the C406A mutant (Figure 5 and Table S3), was consistent with the greater sensitivity of the C403A mutant to MMS (Figure 3B). Among the genes affected were several whose products are directly involved in DNA repair (Table 1), such as the *MAG1* gene, which encodes 3-methyl adenine DNA glycosylase, a component of the BER pathway. Genes induced in wt, but not *ssl1* mutant strains, also included some identified in the *Saccharomyces* Genome Database as involved in the "response to stress." The *ssl1* mutant strains were not, however, generally deficient in this regard, because they displayed normal resistance to salt and osmotic stress (Figure 3C).

Discussion

What might be the target of the Ssl1 E3 ligase? RNA polymerase II comes first to mind, as it is known to stall at sites of DNA damage and undergo ubiquitylation and degradation (Beaudenon et al., 1999; Bregman et al., 1996). The E3 ligase involved has been identified, however, as the product of the *RSP5* gene, a HECT domain E3 ligase that is recruited to damage sites by a complex of Def1 and the transcription-coupled repair factor Rad26 (Huibregtse et al., 1997; Reid and Svejstrup, 2004). Other possible targets of the Ssl1 E3 ligase include transcriptional activator proteins. The ubiquitin system controls the level and the nuclear localization of many activators. In some cases, sites of ubiquitylation ("degrons") overlap with regions required for activation ("transcriptional activation domains"), and the two processes are evidently intertwined (Muratani and Tansey,

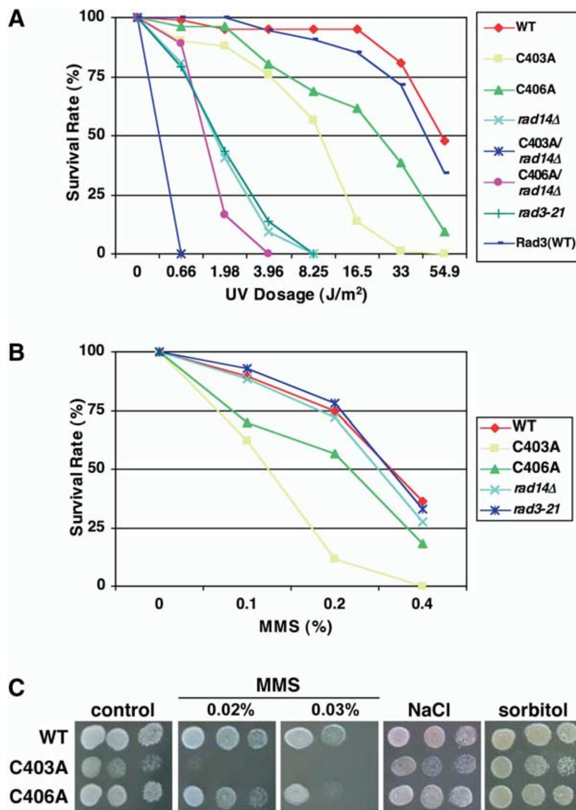

Figure 3. Mutations that Abolish E3 Ligase Activity of Ssl1 In Vitro Render Yeast More Sensitive to DNA-Damaging Agents In Vivo

(A) Yeast cells spotted onto YPD plates were exposed to UV radiation at the levels indicated and then grown in the dark for two days at 30°C. Survival rates are plotted. The experiments were performed in duplicate. Error bars were omitted for simplicity.

(B) Yeast cells were exposed to methanesulfonate (MMS) for 30 min at 30°C, spotted onto SC-Leu plates, and grown for two days at 30°C. Survival rates are plotted. The experiments were performed in duplicate. Error bars were omitted for simplicity. Wt, wild-type. Strain names: C403A and C406A, Ssl1 RNF mutants; *rad14Δ*, *RAD14* deletion mutant; C403A/*rad14Δ* and C406A/*rad14Δ*, Ssl1 RNF and *RAD14* deletion double mutants; *rad3-21*, *RAD3* helicase mutant; and Rad3(Wt), wild-type strain.

(C) Isogenic derivatives of the strain PDY2, with the genotypes indicated on the left, were spotted (4 μl) with serial dilution onto YPD plates containing 0.02% or 0.03% MMS, 0.8 M NaCl, or 1.2 M sorbitol. Control is a YPD plate without any addition. Yeast cell densities were normalized by A_{600} (2.0, 0.2, or 0.02) before spotting. Pictures were taken after 2 days (control and MMS plates) or 3 days (NaCl and sorbitol) incubation at 30°C.

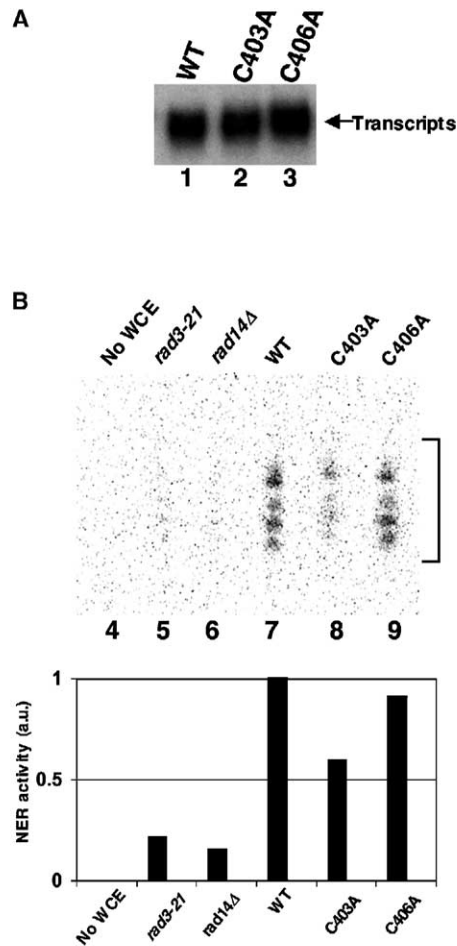

Figure 4. Ssl1 RNF Mutations Affect Neither Transcription nor Nucleotide Excision Repair Activities

(A) Whole-cell extracts (WCE) from wt (lane 1), and Ssl1 mutant strains C403A (lane 2) and C406A (lanes 3) were tested for promoter-dependent transcription in vitro. Transcripts (~360 bp) separated by 6% denaturing PAGE and revealed by autoradiography are indicated.

(B) WCEs from wt and Ssl1 RNF mutants strains were assayed for NER with an AAF-modified, ^{32}P-labeled DNA. Single-stranded DNA fragments 20–30 residues in length produced by NER were separated by 8% denaturing PAGE (top, lanes 1–6) and quantified by PhosphorImager analysis (bottom).

2003). Ubiquitylation of an activator of DNA-damage genes by the Ssl1 E3 ligase would perfectly explain our findings and will be an object of future investigation.

The connection between transcription and the ubiquitin system through Ssl1 may extend beyond the RNF domain. A central region of Ssl1 exhibits homology to the VWA domain present in the N-terminal half of the proteosome subunit Rpn10/S5a (Figure 2A). This region of Rpn10 interacts with other subunits of the proteasome and facilitates the degradation of a number of proteasome substrates (Verma et al., 2004). Association of the transcription machinery with the proteosome has been reported, but the mechanism of recruitment has not been determined (Muratani and Tansey, 2003). Other E3 ligases are known to associate with the proteasome, and the Ssl1 E3 ligase might perform this role in transcription.

TFIIH is highly conserved between *S. cerevisiae* and humans: all subunits show significant sequence homology, and the three-dimensional structure is conserved as well (Chang and Kornberg, 2000; Schultz et al., 2000). In particular, both RNF and Rpn10/S5a-homology domains of Ssl1 are similar in sequence to the corresponding regions of the human homolog p44 (Figure 2A). Mutations of human TFIIH are associated with xeroderma pigmentosum, Cockayne's syndrome, and trichothiodystrophy (Bootsma and Hoeijmakers, 1993;

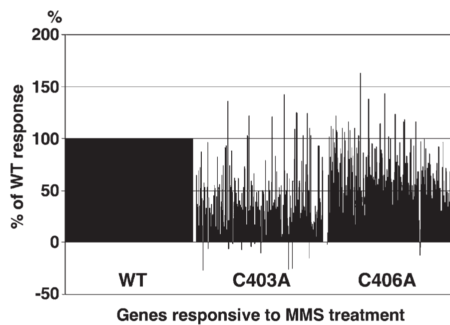

Figure 5. Whole Genome Analysis Reveals Defective Transcriptional Response to MMS Treatment of Ssl1 RNF Mutant Strains

The transcriptional response to MMS treatment is shown for Ssl1 RNF mutant strains, C403A, and C406A, relative to the response in wt, of genes whose transcription was altered more than 2-fold by MMS treatment in wt. Each bar represents the response of one gene.

Giglia-Mari et al., 2004; Lehmann, 2001). It will be of interest to learn whether p44 possesses E3 ligase activity, and whether defects in this activity are associated with human disease as well.

Experimental Procedures

Ubiquitylation Assays

In vitro ubiquitylation was performed as described (Joazeiro et al., 1999), with the use of His-Ubc4 and core TFIIH or GST fusion proteins (0.1–1.8 μg). Core TFIIH was isolated from *S. cerevisiae* with the use of either a hexahistidine tag (Chang and Kornberg, 2000) or a TAP tag as described (Takagi et al., 2003). GST fusion proteins were expressed and isolated as described (Takagi et al., 2003). The GST-Ssl1 mutants C403A and C406A were made with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and were verified by sequencing. Ub immunoblot analysis was performed with either a monoclonal (Ubi-1, Zymed) or a polyclonal antibody (Z0458, Dako).

Construction of Yeast Strains Bearing Ssl1 Mutations and Rad14 Deletion Mutations

An Eag I-HindIII fragment from pSSL1 recovered from the yeast strain PDY2 (Estruch and Cole, 2003) was subcloned into pRS315. Mutations were introduced into pRS315/Ssl1 with the QuikChange Site-Directed Mutagenesis Kit and were verified by sequencing. Plasmids bearing wt or mutant *SSL1* were introduced into PDY2 by shuffling, as described (Burke et al., 2000). A *RAD14* deletion strain, rad14Δ (YT121), was derived from PDY2 by replacing the *RAD14* open reading frame with a PCR-generated *TRP1* cassette. Plasmids bearing wt or Ssl1 RNF mutants were introduced into a rad14 deletion strain by shuffling, yielding rad14Δ and Ssl1 RNF double mutant strains, C403A/rad14Δ and C406A/rad14Δ.

Sensitivity to DNA-Damaging Agents and Stress

UV sensitivity was assayed as described (Feaver et al., 1999). Briefly, yeast grown to saturation in YPD medium were spotted onto YPD plates at ∼50 or 500 cells/spot. The plates were exposed to UV radiation and grown in the dark at 30°C for two days. These experiments were performed in duplicate. MMS sensitivity was assayed as described (Lee et al., 2002) except that cells were exposed to MMS for 30 min. Growth under stress was on YPD plates containing the indicated concentrations of NaCl or sorbitol. Yeast strains were grown in YPD overnight, adjusted in concentration on the basis of the A_{600}, and spotted on the plates with 10-fold serial dilution in duplicate.

In Vitro Transcription Assay

Whole-cell extracts were prepared from wt and Ssl1 RNF mutant yeast strains and assayed for transcription essentially as described (Woontner and Jaehning, 1990) except that yeast cells were broken by the blender method (Takagi et al., 2003), and pGCN4 (Kim et al., 1994) was used as DNA template.

NER Assay

NER assays were performed as described (Wang et al., 2003) except with reaction conditions as in Kong and Svejstrup, (2002) and with N-acetoxy-2-AAF- instead of cisplatin-modified DNA. AAF-modified DNA was prepared essentially as described (Koehl et al., 1989). Briefly, 13mer DNA (5′-CCTCTCGCCTCTC-3′) was treated with a 4-fold molar excess of N-acetoxy-N-2-AAF (N-Ac-O-AAF) in 2 mM citrate (pH 7.2) and 5% ethanol for 1 hr at 37°C. The reaction mixture was extracted three times with chloroform to remove unbound fluorene derivative, and the resulting product 13mer-AAF monoadduct was purified by HPLC on a C18 column. The 13mer-AAF was labeled with ^{32}P at the 5′ end and incorporated in duplex DNA by ligation with the following five oligonucleotides, essentially as described (Wang et al., 2003): 5′-GCTTCTGCACAGAGTGTC GGTTGCACCCTCAAGGACATCACCGCGTGCCTGGTGTATATC-3′, 5′-GGACTCAATTTGCCAGACCCCAACCTAGTGGAACAACAGCGA CCCGCGCTCAAGCGGAG-3′, 5′-CTCCGCTTGAGCGCGGGTCGC TGTTGTTCCACTAGGTTGGGGTCTG-3′, 5′-GCAAATTGAGTCCGA GAGGCGAGAGGGATATACAC-3′, and 5′-CAGGCACGCGGTGATG TCCTTGAGGGTGCAACCGACACTCTGTGCAGAAGC-3′. The six oligonucleotides were annealed, ligated, and fractionated by denaturing PAGE. The full-length product strands were isolated and reannealed.

Microarray Analysis

Yeast was grown in YPD medium at 30°C to an A_{600} of 0.2 and treated with MMS or not for 30 min at 30°C. Cells were collected by centrifugation, frozen in liquid nitrogen, and stored at −80°C. Total RNA was prepared with Trizol reagent (Invitrogen) and further purified with an RNeasy Mini Kit (Qiagen). Total RNA was used to prepare cDNA, which was used as a template to produce biotinylated cRNA, which was fragmented and hybridized to Affymetrix

Table 1. Induction of Genes Involved in DNA Repair after MMS Treatment Is Deficient in Ssl1 RNF Mutant Strains

	Wt	C403A		C406A	
Genes Involved in DNA Repair	Change in Expression (%)	Change in Expression (%)	Response Relative to Wt	Change in Expression (%)	Response Relative to Wt
RAD59	120.4%	*56.9%*	*0.47*	n/a	n/a
RAD54,XRS1	126.4%	92.4%	0.73	*69.8%*	*0.55*
RFA1,BUF2,FUN3,RPA1	138.9%	91.8%	0.66	*76.3%*	*0.55*
DIN7,DIN3	160.1%	*93.8%*	*0.59*	153.2%	0.96
MMS5, MAG1	285.6%	*136.4%*	*0.48*	172.9%	0.61

Wt and Ssl1 RNF mutant strains were treated or not with 0.03% MMS for 30 min in YPD medium at 30°C. Genes that changed in expression by 100% or more ($p < 0.01$) in the wt strain and that are annotated under the gene ontology process "DNA repair" by the Saccharomyces Genome Database are listed. In cases of 40% or greater difference in induction after MMS treatment between mutants and wt, entries are italicized. n/a, not available (i.e., $p > 0.01$).

S98 yeast microarrays. Data were processed with Resolver software, version 4.0 (Rosetta Biosoftware). Resolver filters, for inclusion of differentially expressed genes, were absolute fold changes of at least two and p values < 0.01 by using Resolver's ratio ANOVA function. Resolver ANOVA analysis is similar to standard ANOVA but uses two inputs, expression measurement quantity and estimated error of measurement quantity (see http://www.rosettabio.com/publications/default.htm for additional references).

Supplemental Data

Supplemental Data include one figure and three tables and are available online with this article at http://www.molecule.org/cgi/content/full/18/2/237/DC1/.

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Accession Numbers

The genechip data were deposited in the database GEO (Gene Expression Omnibus). Accession number for the dataset is GSE2343.