

Roles of Rad23 protein in yeast nucleotide excision repair

Zhongwen Xie, Shuqian Liu, Yanbin Zhang and Zhigang Wang*

Graduate Center for Toxicology, University of Kentucky, Lexington, KY 40536, USA

Received July 6, 2004; Revised September 20, 2004; Accepted October 22, 2004

ABSTRACT

Nucleotide excision repair (NER) removes many different types of DNA lesions. Most NER proteins are indispensable for repair. In contrast, the yeast Rad23 represents a class of accessory NER proteins, without which NER activity is reduced but not eliminated. In mammals, the complex of HR23B (Rad23 homolog) and XPC (yeast Rad4 homolog) has been suggested to function in the damage recognition step of NER. However, the precise function of Rad23 or HR23B in NER remains unknown. Recently, it was suggested that the primary function of RAD23 protein in NER is its stabilization of XPC protein. Here, we tested the significance of Rad23-mediated Rad4 stabilization in NER, and analyzed the repair and biochemical activities of purified yeast Rad23 protein. Cellular Rad4 was indeed stabilized by Rad23 in the absence of DNA damage. Persistent overexpression of Rad4 in *rad23* mutant cells, however, largely failed to complement the ultraviolet sensitivity of the mutant. Consistently, deficient NER in *rad23* mutant cell extracts could not be complemented by purified Rad4 protein *in vitro*. In contrast, partial complementation was observed with purified Rad23 protein. Specific complementation to the level of wild-type repair was achieved by adding purified Rad23 together with small amounts of Rad4 protein to *rad23* mutant cell extracts. Purified Rad23 protein was unable to bind to DNA, but stimulated the binding activity of purified Rad4 protein to *N*-acetyl-2-aminofluorene-damaged DNA. These results support two roles of Rad23 protein in NER: (i) its direct participation in the repair biochemistry, possibly due to its stimulatory activity on Rad4-mediated damage binding/recognition; and (ii) its stabilization of cellular Rad4 protein.

INTRODUCTION

DNA is frequently damaged by many environmental agents. Nucleotide excision repair (NER) is an important mechanism for removing a wide spectrum of different DNA lesions. Many bulky DNA adducts are specifically repaired by NER in eukaryotes, such as *N*-acetyl-2-aminofluorene (AAF) adducts,

cisplatin intra-strand crosslinks, polycyclic aromatic hydrocarbon adducts, and the major ultraviolet (UV) lesions cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts (1–7). Therefore, NER constitutes a crucial defense mechanism against DNA damage-induced cytotoxicity, mutagenesis and carcinogenesis. Compromising NER activity will lead to increased cellular sensitivity to DNA-damaging agents, increased mutation frequency and a high risk of carcinogenesis, as exhibited by the human hereditary disease xeroderma pigmentosum (XP) (8).

Cellular NER consists of two subpathways: global genome repair and transcription-coupled repair (9,10). Transcription-coupled NER is specific to the repair of actively transcribed genes and specifically involves at least RNA polymerase II, CSA and CSB. On the other hand, global genome NER is responsible for the repair of the untranscribed regions of the genome and the untranscribed strand of an actively expressed protein-coding gene. Conceptually, NER can be divided into five distinct biochemical steps: damage recognition, incision, excision, repair synthesis and DNA ligation. *In vivo* genetics and *in vitro* biochemistry with cell-free repair systems and purified NER proteins have contributed a great deal to the understanding of eukaryotic NER. *In vitro* NER systems established to date in eukaryotes specifically reflect the global genome repair subpathway (1,5,11–13). Two categories of NER proteins are known. Most of the NER proteins are indispensable for repair, such as the repair/transcription factor TFIIH, and the yeast Rad4 and Rad14, which corresponds to the human XPC and XPA, respectively. The second category of NER proteins plays accessory roles in repair, without which cells exhibit moderate rather than severe sensitivity to DNA-damaging agents. Rad23 is such an accessory NER protein in yeast. Its mammalian homologs are HR23A and HR23B (14). Rad23 (HR23) strongly interacts with Rad4 (XPC) (14–16). Some studies have suggested that the XPC/HR23B complex functions in the damage recognition step of NER (17,18). However, the role of HR23B in the complex-mediated damage binding/recognition is not known.

Yeast *rad23* deletion mutant cells are moderately sensitive to DNA-damaging agents (19). Both transcription-coupled and global genomic NER in *rad23* mutant cells are significantly reduced, but not totally abolished (20). Consistently, *in vitro* NER in *rad23* deletion mutant extracts is also deficient (15). Clearly, proficient NER requires Rad23 function. Rad23 protein contains multiple functional domains: an N-terminal ubiquitin-like (UBL) domain, a Rad4-interaction domain and two ubiquitin-associated (UBA) domains (21–23). The

*To whom correspondence should be addressed. Tel: +1 859 323 5784; Fax: +1 859 323 1059; Email: zwang@uky.edu

UBA domains are not required for NER activity of Rad23 (23). Thus, in addition to its role in NER, this protein most likely plays other functions in cells, such as the suggested cell cycle progression (24,25). Consistent with multi-functionality of RAD23, knockout mice deleted of both the *HR23A* and the *HR23B* genes lead to embryonic lethality (26).

It has been shown that Rad23 interacts with the 26S proteasome and the UBL domain is required for this interaction (27,28). The 26S proteasome, consisting of a 20S core particle and two copies of a 19S regulatory complex, is a large protein complex involved in the degradation of proteins targeted by the ubiquitin pathway (29). It was further shown that the 19S regulatory complex negatively modulates NER in yeast cells (30,31). More recently, Lommel *et al.* (32) found that Rad4 protein transiently accumulates following DNA damage and that Rad23 enhances Rad4 stability following UV radiation of yeast cells. It was suggested that Rad23 protects Rad4 from degradation by the proteasome in cells (32). A similar XPC stabilization effect by *HR23A/B* was reported by Ng *et al.* (26) most recently in cultured mouse cells even without UV radiation. These observations led Ng *et al.* (26) to conclude that the primary function of RAD23 protein in NER is its stabilization of XPC protein.

To better understand the role of Rad23 protein in NER, we have used the yeast model system to test the significance of Rad23-mediated Rad4 stabilization in NER and further analyzed the repair and biochemical activities of purified Rad23 protein. In this report, we show that (i) decreased Rad4 stability is not the major cause of deficient NER in *rad23* mutant cells; (ii) Rad23 protein directly participates in NER; and (iii) Rad23 protein stimulates the binding activity of Rad4 on damaged DNA. These results support two roles of Rad23 protein in NER: its direct participation in the repair biochemistry, possibly due to its stimulatory activity on Rad4-mediated damage binding/recognition; and its stabilization of cellular Rad4 protein.

MATERIALS AND METHODS

Materials

Purified yeast Rad2 protein was obtained from Enzymax (Lexington, KY), which was purified from *Escherichia coli* cells overexpressing the yeast *RAD2* gene. The *Pfu* DNA polymerase was from Stratagene (La Jolla, CA). Protease inhibitors, alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG), alkaline phosphatase-conjugated anti-rat IgG, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium were obtained from Sigma Chemicals (St. Louis, MO). A mouse monoclonal antibody against the His₆ tag was purchased from Qiagen (Valencia, CA). A rat monoclonal anti-HA antibody was from Boehringer Mannheim (Indianapolis, IN). *N*-acetoxy-*N*-2-acetyl-aminofluorene (AAAF, the activated form of AAF) was obtained from the Midwest Research Institute (Kansas City, MO). All oligonucleotides were synthesized by Operon (Alameda, CA).

Strains

The yeast *Saccharomyces cerevisiae* wild-type strains used were SX46A (12) and W303-1B (15). The yeast *S.cerevisiae*

mutant strains used were BJ2168 Δ rad2 (*MATa RAD2::URA3 leu2 trp1 ura3-52 pep4-3 prb1-1122 prc1-407*), SX46A Δ rad4 (*MATa RAD4::URA3 ade2 his3-532 ura3-52 trp1-289*), SX46A Δ rad14 (15) and MGSC101 (*rad23 Δ ::RUA3*) (15). While SX46A Δ rad4 and SX46A Δ rad14 are isogenic to SX46A, MGSC101 is isogenic to W303-1B.

Plasmid constructions

Yeast expression plasmid vectors pEGU6, pEGTh6 and pEGLha were derived from Yeplac195, Yeplac112 and Yeplac181 (33), respectively. All three vectors contained the 2 μ m origin for multi-copy plasmid replication and the yeast-inducible *GALI1/10* promoter. While both pEGU6 and pEGTh6 contained 6 His codons for protein N-terminal tagging, they contained the yeast *URA3* gene and the yeast *TRP1* gene, respectively, for plasmid selection. A sequence coding for the HA tag at the protein N-terminus and the yeast *LEU2* gene for plasmid selection were contained in pEGLha. The yeast *RAD4* gene was cloned into the BamHI and PstI sites of the vector pEGTh6, yielding pEGTh6-RAD4. The yeast *RAD23* gene was amplified from yeast DNA by PCR with the *Pfu* DNA polymerase, using the primers 5'-CGGGATC-CATGGTTAGCTTAACCTTTAAAAATTTTC-3' and 5'-ACGCGTCGACATCAACACTTCTGGAA-3'. The resulting 1.3 kb DNA fragment was cleaved with BamHI and Sall restriction endonucleases and cloned into the corresponding sites of pEGU6 and pEGLha, generating pEGU6-RAD23 and pEGLha-RAD23, respectively. The yeast *RAD14* gene was amplified from yeast DNA by PCR with the *Pfu* DNA polymerase, using the primers 5'-GAAGATCTATGCAGAACTTGAATGGTGG-3' and 5'-GAACTGCAGAGGCCCATGTGGCC-3'. The resulting 1 kb DNA fragment was cleaved with BglII and PstI restriction endonucleases and cloned into the BamHI and PstI sites of pEGTh6, yielding pEGTh6-RAD14.

Damaged DNA

To prepare pUC18 DNA containing AAF adducts, the plasmid (100 μ g) was incubated at 37°C for 3 h in 1 ml of TE buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA) containing 3 μ M AAF and 20% ethanol. After adding NaCl to 0.5 M, the modified DNA was then purified by 5–20% sucrose gradient centrifugation at 28 000 r.p.m. for 17 h at 4°C in a Beckman SW41Ti rotor. Fractions of 0.5 ml each were collected from the bottom of the gradient and 5 μ l aliquots were analyzed by electrophoresis on a 1% agarose gel to locate the DNA. Fractions containing supercoiled DNA were pooled, precipitated in ethanol, dissolved in TE buffer and stored at –20°C.

To prepare AAF-adducted oligonucleotide DNA, 2 nmol of the 79mer oligonucleotide 5'-GGAATTCGGAATTACAGGCTCTAACACCGTCTCATCTTCGCTCGTCCACTTTTTTTTCG-3' was incubated with 200 nmol of *N*-AAAF at 37°C in the dark for 3 h in 100 μ l of TE buffer containing 20% ethanol. Free AAF was removed by extracting the reaction mixture five times with water-saturated ether. Then, damaged and undamaged oligonucleotides were separated by electrophoresis on a 20% denaturing polyacrylamide gel. AAF-modified DNA migrated slower on the gel and was sliced out of the gel. The gel slices were soaked in 150 μ l water at room temperature for 4 h.

Finally, AAF-damaged DNA was recovered using GenElute DNA spin column (Sigma). To obtain duplex oligonucleotide, equal molar amounts of undamaged or the AAF-damaged 79mer oligonucleotide and its 79mer complementary strand were mixed and annealed by incubating for 5 min at 85°C in TES (10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 100 mM NaCl) buffer followed by cooling slowly to room temperature.

Preparation of yeast cell-free extracts

Yeast cell-free extracts used for *in vitro* NER were prepared according to our previously described method (12). Yeast whole cell extracts containing overexpressed Rad4 and/or Rad23 were prepared as follows. Yeast SX46A Δ rad4 cells containing pEGTh6-RAD23, or *rad23* mutant cells (MGSC101) containing pEGTh6-RAD4 alone or both pEGTh6-RAD4 and pEGLha-RAD23 were grown at 30°C overnight in minimum medium containing 2% sucrose and the required amino acids. At an OD₆₀₀ of ~1, protein expression from the plasmids was induced by diluting the culture 10-fold in 100 ml of YP (2% Bacto-peptone, 1% yeast extract) medium supplemented with 2% galactose and 0.5% sucrose. After growing for 13 h at 30°C, the cells were collected by centrifugation and were resuspended in 800 μ l of a buffer containing 20 mM Hepes-KOH, pH 7.6, 10 mM MgSO₄, 10 mM EGTA, 20% glycerol, 5 mM DTT and protease inhibitors (34). The cells were homogenized at 4°C with Zirconium beads in a mini-Beadbeater, using 4 pulses of 1 min each with 2 min pause between pulses. Clear whole cell extracts were obtained after centrifugation at 14 000 r.p.m. for 15 min at 4°C in a microcentrifuge.

Analysis of Rad4 protein stability in yeast cells

Yeast *rad23* mutant cells (MGSC101) containing pEGTh6-RAD4 or both pEGTh6-RAD4 and pEGLha-RAD23 were grown in minimum medium containing 2% sucrose and the required amino acids at 30°C overnight. At an OD₆₀₀ of ~1, protein expression from the plasmids was induced by diluting the culture 10-fold in 300 ml of YP medium supplemented with 2% galactose and 0.5% sucrose. After growing for 13 h at 30°C, a 50 ml aliquot of the culture was collected by centrifugation and stored at -80°C. The remaining culture was centrifuged and the cells were washed three times with YP medium. Then, the cells were resuspended in 250 ml of YP medium supplemented with 2% glucose, and continued to grow at 30°C. Aliquots of 50 ml each were removed at 1, 3, 5, 10 and 24 h after the medium change. The cells in each aliquot were collected by centrifugation and stored at -80°C. Finally, whole cell extracts were prepared as described above and stored at -80°C.

Measurement of UV sensitivity

Plasmids pEGTh6-RAD4, pEGLha-RAD23, pEGTh6-RAD14 and the vector pEGTh6 were used to individually transform various yeast strains as indicated. Yeast cells were grown at 30°C in minimum media containing 2% sucrose and the required amino acids. At an OD₆₀₀ of ~1, protein expression from the plasmids was induced by diluting the culture 10-fold in 50 ml of YP medium supplemented with 2% galactose and 0.5% sucrose (YPG medium). After growing for 13 h at 30°C, the cells were collected by centrifugation. Appropriately

diluted cells were plated onto YPG plates, and the uncovered plates were irradiated with short wave UV light from a germicidal UV lamp at the indicated doses. Surviving colonies were scored after 3–4 days of incubation in the dark at 30°C. UV survival was calculated by dividing surviving colonies after UV treatment by those without UV treatment.

In vitro NER

In vitro NER was performed as described by Wang *et al.* (1,12,35). Briefly, a standard NER reaction mixture (50 μ l) contained 200 ng each of damaged pUC18 DNA and undamaged pGEM3Zf DNA, 45 mM Hepes-KOH (pH 7.8), 7.4 mM MgCl₂, 0.9 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20 μ M each dATP, dGTP, and dTTP, 4 μ M dCTP, 1 μ Ci of [α -³²P]dCTP (3000 Ci/mmol), 40 mM phosphocreatine (disodium salt), 2.5 μ g of creatine phosphokinase, 4% glycerol, 100 μ g/ml BSA, 5% polyethylene glycol 8000 and 250 μ g of yeast cell-free extracts. After incubation at 26°C for 2 h, EDTA and RNase A were added to 20 mM and 20 μ g/ml, respectively, and incubated at 37°C for 10 min. SDS and proteinase K were added to 0.5% and 200 μ g/ml, respectively, and incubated at 37°C for 30 min. Plasmid DNA was purified by phenol and then by chloroform extractions, and linearized with the HindIII restriction endonuclease. DNA was separated by electrophoresis on a 1% agarose gel and repair synthesis was visualized by autoradiography.

Electrophoretic mobility shift assay (EMSA)

First, the AAF-damaged 79mer oligonucleotide was 5'-labeled with T4 polynucleotide kinase and [γ -³²P]ATP (3000 Ci/mmol). The labeled oligonucleotide was then mixed with its complementary strand (79mer) in equal molar amounts and annealed to form duplex DNA as described above. A standard DNA binding reaction mixture (10 μ l) contained 10 fmol of the ³²P-labeled and damaged duplex DNA, various amounts of unlabeled competitor DNA as indicated, 20 mM Tris-HCl, pH 7.5, 5 mM Mg₂Cl, 1 mM DTT, 100 μ g/ml BSA, and purified yeast Rad4 or Rad23 protein or both as indicated. After incubation at 30°C for 30 min, 2 μ l of a loading dye was added, which contained 100 mM Tris-HCl, pH 7.5, 0.05% bromophenol blue and 50% glycerol. DNA was separated by electrophoresis on a 4.5% non-denaturing polyacrylamide gel at 4°C and visualized by a phosphorImager.

Protein purification

Yeast SX46A Δ rad4 cells containing pEGTh6-RAD4 or pEGU6-RAD23 were grown in minimum medium containing 2% sucrose and the required amino acids at 30°C for 2 days. Overexpression of the protein was induced by diluting the culture 10-fold in 16 l of YP medium supplemented with 2% galactose and 0.5% sucrose. After growing for 12 h at 30°C, the cells were collected by centrifugation and washed with water. Then, the cells were resuspended in an extraction buffer containing 50 mM Tris-HCl, pH 7.5, 1 M KCl, 10% sucrose, 5 mM β -mercaptoethanol and protease inhibitors (34), and were homogenized on ice with Zirconium beads in a Beadbeater for 15 pulses of 30 s each with 2 min pause between pulses. After centrifugation at 33 000 r.p.m. for 2 h at 4°C in a Beckman type 50.2Ti rotor, the clarified extract (~100 ml) was loaded onto a HiTrap chelating column

charged with NiSO₄ (2 × 5 ml; Amersham Pharmacia Biotech, Piscataway, NJ). The column was washed with 240 ml of Ni buffer A (50 mM Tris-HCl, pH 7.5, 1 M KCl, 10% glycerol, 5 mM β-mercaptoethanol and protease inhibitors) containing 10 mM imidazole, followed by a second wash with 240 ml of Ni buffer A containing 35 mM imidazole. Bound proteins were eluted with a linear gradient of 35–250 mM imidazole in Ni buffer A. The His₆-tagged Rad4 and Rad23 proteins were identified by western blot analysis using a mouse monoclonal antibody specific to the His₆ tag. Fractions containing the Rad4 or Rad23 protein were pooled, concentrated with PEG 10000 and desalted through 5 × 5 ml Sephadex G-25 columns in FPLC buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol and protease inhibitors) containing 50 mM KCl (for Rad23) or 100 mM KCl (for Rad4). The resulting sample (~20 ml) was loaded onto a FPLC Mono Q HR5/5 column. After washing the column with 10 ml of FPLC buffer A, bound Rad23 or Rad4 protein was eluted with a 30 ml linear gradient of 50–500 mM KCl or 100–500 mM KCl, respectively, in FPLC buffer A. Rad23 protein was eluted at ~275 mM KCl, and Rad4 protein was eluted at ~375 mM KCl in separate purifications. During purification, both silver staining of SDS-polyacrylamide gels and western blot analysis were used to identify Rad4 or Rad23 protein.

RESULTS

Rad4 protein is stabilized by Rad23 in yeast cells

It was reported that yeast Rad4 protein is unstable in *rad23* mutant cells following UV radiation (32). To determine whether Rad23 is able to stabilize Rad4 protein in the absence of DNA damage, we examined the stability of Rad4 under normal

growth conditions. The *rad23* deletion mutant cells were transformed with either a single plasmid expressing Rad4 or two plasmids expressing Rad4 and Rad23, respectively. To facilitate protein detection, Rad4 was tagged with His₆ and Rad23 was tagged with the HA epitope at the protein N-terminus. Both *RAD4* and *RAD23* genes were under the control of the yeast *GALI/10* promoter. Gene expression from this promoter is suppressed by 2% glucose but induced by 2% galactose in the growth medium. Without induction, Rad4 and Rad23 expression from the plasmids were undetectable by western blot analysis in cell extracts (Figure 1A, lanes 1 and 3). Following galactose induction of the yeast culture, both Rad4 and Rad23 proteins were readily detected (Figure 1A, lanes 2 and 4). The steady state level of Rad4, which reflects the balance between protein synthesis and protein degradation, was slightly lower in the absence of Rad23 (compare Figure 1A, lanes 2 and 4). To directly examine the stability of Rad4 protein in yeast cells, we first induced gene expression with galactose. Then, Rad4 expression or Rad4 and Rad23 expressions were turned off by transferring yeast cells back to the medium without galactose but containing 2% glucose. Aliquots of the culture were removed at various times for cell extract preparation. Finally, Rad4 protein in the cell extracts were determined by western blot analysis. As shown in Figure 1B (lanes 2–7), in the presence of Rad23 protein, ~50% of Rad4 remained 5 h after turning off the gene expression from the *GALI/10* promoter. In contrast, Rad4 protein became non-detectable 3 h after turning off the gene expression from the *GALI/10* promoter in *rad23* mutant cells (Figure 1B, lanes 8–13). These results show that Rad23 stabilizes Rad4 in yeast cells. This Rad4 stabilization by Rad23 does not require exogenously induced DNA damage.

Since Rad4 forms a tight complex with Rad23 (15,36), it is possible that Rad4 protein alone may be intrinsically unstable, thus contributing to the quick disappearance of Rad4 in *rad23*

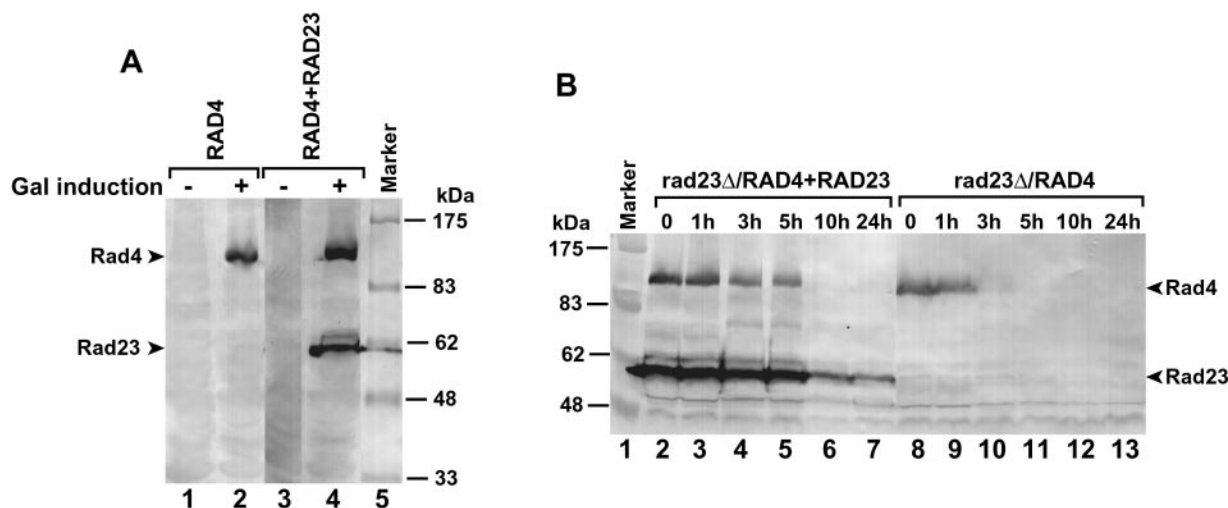


Figure 1. Stability of Rad4 protein in yeast cells. (A) Yeast *rad23* deletion mutant cells containing pEGTh6-RAD4 (lanes 1 and 2) or both pEGTh6-RAD4 and pEGLha-RAD23 (lanes 3 and 4) were grown at 30°C without (lanes 1 and 3) or with 13 h induction by 2% galactose (lanes 2 and 4). Equal amounts of the whole cell extracts (50 μg extract protein each) were separated by electrophoresis on a 10% SDS-polyacrylamide gel. Expression of the His₆-tagged Rad4 and HA-tagged Rad23 proteins were examined by western blot analysis using two monoclonal antibodies with each specific to the respective tag. In separate experiments, the His₆ antibody and the HA antibody did not show non-specific cross-reaction against the HA-tagged Rad23 and the His₆-tagged Rad4, respectively (data not shown). Lane 5, protein size markers. (B) Following 13 h induction of Rad4/Rad23 (lanes 2–7) or Rad4 (lanes 8–13), galactose was replaced by 2% glucose via medium change to repress expression of the tagged Rad4 or Rad4/Rad23 proteins. Aliquots of the culture were removed at various times as indicated 0–24 h after medium change. The tagged Rad4 and Rad23 proteins in the whole cell extracts were examined by western blot analysis as in (A). Lane 1, protein size markers.

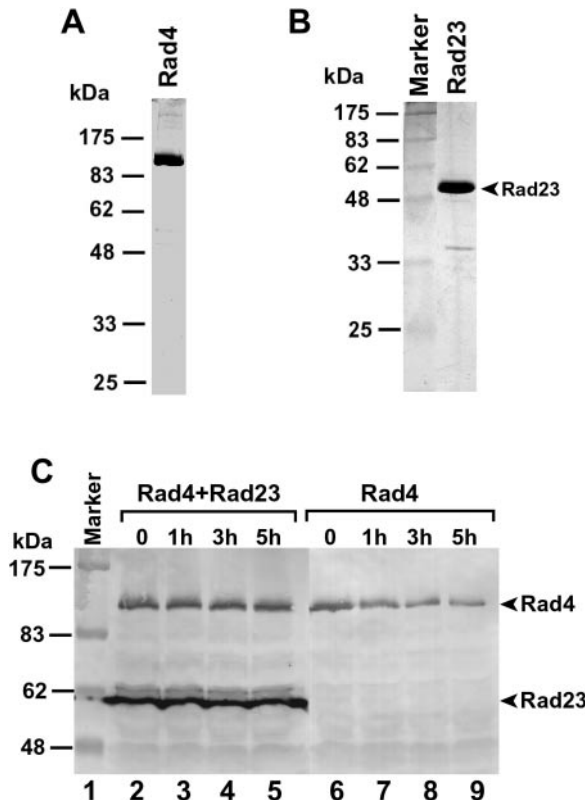


Figure 2. Stability of purified Rad4 protein in yeast cell-free extracts. (A) Purified yeast Rad4 protein. (B) Purified yeast Rad23 protein. The His₆-tagged Rad4 or Rad23 was separated by electrophoresis on a 15% SDS-polyacrylamide gel, followed by western blot analysis using a monoclonal antibody specific to the His₆ tag. (C) Purified Rad4 protein (255 ng) (lanes 2–5) or purified Rad4 (255 ng) and Rad23 proteins (122 ng) (lanes 6–9) were added to 250 μ g of *rad23* mutant cell extracts and incubated at 26°C under standard NER reaction buffer conditions except for omitting polyethylene glycol, DNA and [α -³²P]dCTP to avoid *in vitro* repair. At various times as indicated, aliquots of 12.5 μ l each were removed and separated by electrophoresis on a 10% SDS-polyacrylamide gel, followed by western blot analysis using a monoclonal antibody specific to the His₆ tag. Protein size makers in kDa are indicated on the left.

mutant cells. To examine this possibility, we purified Rad4 (Figure 2A) and Rad23 (Figure 2B) proteins separately. To ensure that our Rad23 preparation was absolutely devoid of Rad4, Rad23 was purified from *rad4* deletion mutant cells, following overexpression from pEGUh6-RAD23. We did not notice any abnormal instability of either Rad4 or Rad23 protein during purification. We then added the purified Rad4 alone or the purified Rad4 and Rad23 together to cell-free extracts of *rad23* mutant cells. Aliquots of the extracts were removed at various incubation times. The stability of the purified protein in the extracts was determined by western blot analysis. As shown in Figure 2C and compared to Figure 1B, purified Rad4 was relatively stable in the yeast cell-free extracts with or without Rad23. It should be noted that the small protein tags used in these experiments did not significantly affect the protein function, based on the following observations. First, the tagged Rad4 and Rad23 fully complemented UV sensitivity of the respective mutant strains (Figure 3A) (data not shown). Second, the tagged Rad4 and Rad23 proteins also formed a protein complex, as indicated by

their co-purification sequentially through Ni-affinity and Resource Q columns following their co-expression in yeast cells (data not shown). These results show that Rad4 protein is not associated with an intrinsic instability. Thus, the quick disappearance of Rad4 in *rad23* mutant cells is probably a result of active cellular protein degradation.

Effect of Rad4 overexpression on UV sensitivity of *rad23* mutant cells

Recently, Ng *et al.* (26) concluded that the primary function of RAD23 in NER is to stabilize XPC protein (Rad4 homolog) in mammals. Since Rad23 indeed stabilizes Rad4, we then tested this possibility in yeast. Cells lacking Rad23 are sensitive to UV radiation due to deficient NER (19) (Figure 3A). If the primary function of Rad23 is to stabilize Rad4, it is predicted that persistent overexpression of Rad4 protein should lead to complementation of the UV-sensitive phenotype of *rad23* mutant cells. The mutant cells were transformed with a *RAD4* overexpression plasmid under the control of the strong *GAL1/10* promoter. Rad4 overexpression was achieved by induction with 2% galactose in the culture medium (Figure 1A). Following Rad4 overexpression, the cells were irradiated with UV and grown in plates containing 2% galactose for continued Rad4 induction. As shown in Figure 3A, only a partial complementation for UV resistance was observed. The UV sensitivity of *rad23* mutant cells remained largely unaffected by the overexpressed Rad4 protein. The small enhancement of UV resistance was specific to Rad4 overexpression in *rad23* mutant cells, because this effect was not observed when Rad14 was overexpressed in *rad23* mutant cells (Figure 3B), or when Rad4 was overexpressed in *rad14* mutant cells (Figure 3C). Rad4 overexpression also had no effect on UV resistance of wild-type yeast cells (Figure 3A). These results are inconsistent with the notion that the primary function of Rad23 is to stabilize Rad4.

Rad23 protein directly participates in NER

To directly determine whether decreased Rad4 stability is the major cause of the deficient NER in *rad23* mutant cells, we performed NER assays in yeast cell-free extracts, using plasmid DNA containing AAF adducts. We have previously shown that under the conditions used, AAF DNA adducts are repaired specifically by the NER pathway in yeast cell-free extracts (12,15). NER was monitored by radiolabeling the repair patch during DNA repair synthesis.

Consistent with our previous results (15), NER was deficient in *rad23* mutant cell extracts (Figure 4A, lane 2), as compared to the proficient repair in wild-type cell extracts (Figure 4A, lane 1). We then attempted to complement the deficient repair in *rad23* mutant cell extracts with purified Rad4 and Rad23 proteins. As shown in Figure 4A (lanes 3–6), purified Rad4 was unable to complement deficient NER of *rad23* mutant extracts even at high protein concentrations. Purified Rad23 protein only partially complemented deficient repair of *rad23* mutant extracts (Figure 4A, lanes 7–10). In contrast, addition of purified Rad23 and Rad4 together restored the deficient repair to the wild-type level (Figure 4A, lanes 11–14). Combining 10 ng of Rad4 with 50 ng of Rad23 was sufficient to achieve full complementation (Figure 4A, lane 11). The complementation activity of purified Rad4 and Rad23 was specific

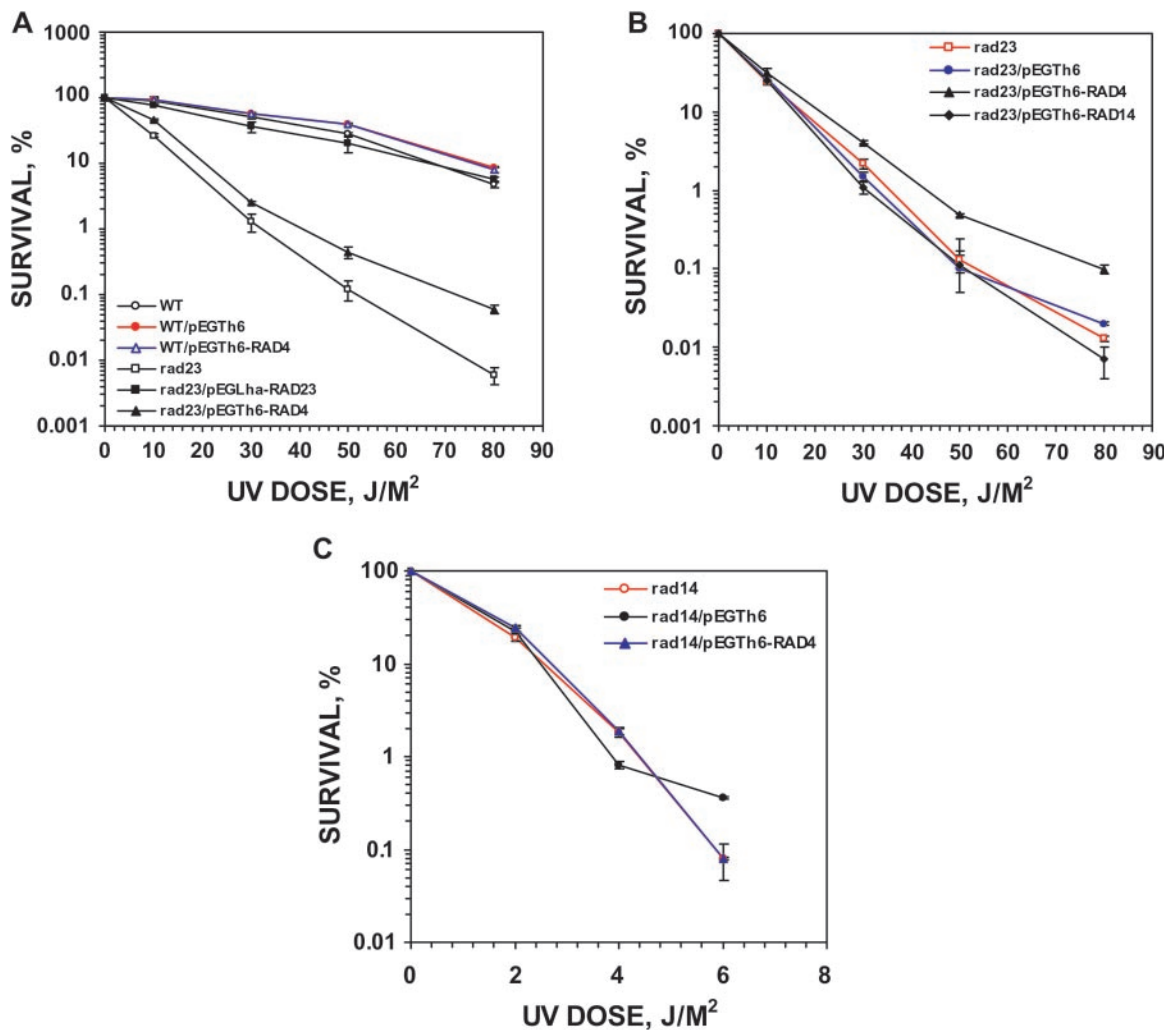


Figure 3. Effect of Rad4 overexpression on UV sensitivity of *rad23* mutant cells. Yeast cells were initially grown in minimum medium containing 2% sucrose. Rad4 or Rad14 overexpression from pEGTh6-RAD4 and pEGTh6-RAD14, respectively, was achieved by growing cells in YP medium containing 0.5% sucrose and 2% galactose (induction medium) for 13 h at 30°C. After dilution, the cells were plated onto the induction medium plates. The uncovered plates were irradiated with UV light at the indicated doses. Surviving colonies were counted after incubation at 30°C for 3–4 days. Survival rates are expressed relative to those of non-irradiated cells. Results are averages of triplicate experiments with the SDs shown as error bars. (A) Effect of Rad4 overexpression on UV sensitivity of *rad23* mutant cells. Yeast strains are the wild-type W303-1B (WT) (open circle), W303-1B containing the empty vector (WT/pEGTh6) (closed circle in red), W303-1B containing Rad4 overexpression plasmid (WT/pEGTh6-RAD4) (open triangle in blue), the *rad23* mutant MGSC101 (*rad23*) (open square), MGSC101 containing Rad23 expression plasmid (*rad23*/pEGLha-RAD23) (closed square) and MGSC101 containing Rad4 overexpression plasmid (*rad23*/pEGTh6-RAD4) (closed triangle). (B) Effect of Rad14 overexpression on UV sensitivity of *rad23* mutant cells. Yeast strains are the *rad23* mutant MGSC101 (*rad23*) (open square in red), MGSC101 containing the empty vector (*rad23*/pEGTh6) (closed circle in blue), MGSC101 containing Rad4 overexpression plasmid (*rad23*/pEGTh6-RAD4) (closed triangle) and MGSC101 containing Rad14 overexpression plasmid (*rad23*/pEGTh6-RAD14) (closed diamond). (C) Effect of Rad4 overexpression on UV sensitivity of *rad14* mutant cells. Yeast strains are the *rad14* mutant SX46AΔ*rad14* (*rad14*) (open circle in red), SX46AΔ*rad14* containing the empty vector (*rad14*/pEGTh6) (closed circle) and SX46AΔ*rad14* containing Rad4 overexpression plasmid (*rad14*/pEGTh6-RAD4) (closed triangle in blue).

to *rad23* mutant extracts, because deficient NER in *rad2* mutant extracts was complemented by purified Rad2 (Figure 4B, lanes 3–6), but could not be complemented by purified Rad4 and Rad23 (Figure 4B, lanes 7–10).

In separate experiments, we also attempted to complement the deficient repair in *rad23* mutant cell extracts with cell-free extracts containing overexpressed Rad4 and/or Rad23. In these experiments, Rad4 protein was overexpressed in *rad23* mutant cells, and Rad23 protein was overexpressed in *rad4* mutant cells. The respective cell-free extracts were prepared and added to the *rad23* mutant cell extracts for *in vitro* NER. Again, neither overexpressed Rad4 cell extract

nor overexpressed Rad23 cell extract (10–100 μg) alone could fully complement deficient NER of *rad23* mutant extracts. In contrast, addition of small amounts of an extract (3 μg) containing both overexpressed Rad4 and Rad23 proteins fully complemented deficient NER of *rad23* mutant extracts (data not shown).

Together, these results show that Rad23 protein directly participates in NER, and that decreased Rad4 stability is not the major cause of deficient NER in *rad23* mutant cells. Furthermore, these results suggest that Rad4 is the only NER protein being significantly stabilized by Rad23 in yeast cells.

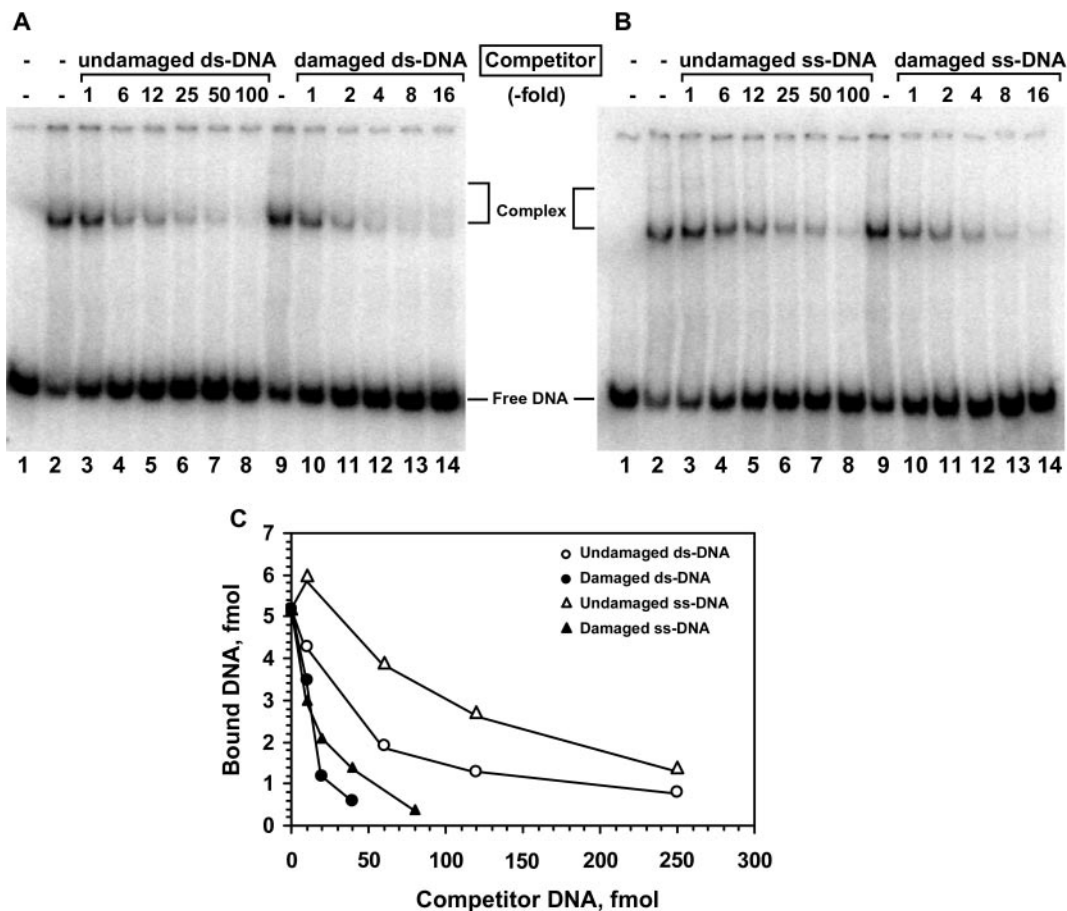


Figure 6. Higher affinity of Rad4/Rad23 for damaged over undamaged DNA. A 79mer duplex DNA containing a 5' 32 P-labeled and AAF-damaged strand was used for standard DNA-binding assays using an EMSA method as described in Materials and Methods. DNA-binding assays were performed using 10 ng of purified yeast Rad4 protein (115 fmol) and 27 ng of purified yeast Rad23 (629 fmol) in the absence of any competitor DNA (lanes 2 and 9) or in the presence of increasing amounts of undamaged DNA (1- to 100-fold molar excess) (lanes 3–8) and AAF-damaged DNA (1- to 16-fold molar excess) (lanes 10–14). The competitor DNA was not labeled with 32 P. Lane 1, reaction without the purified proteins. (A) Undamaged or AAF-damaged 79mer duplex DNA as the binding competitor. (B) Undamaged or AAF-damaged 79mer single-stranded DNA as the binding competitor. (C) Quantitation of results in (A) and (B).

yeast cells. Since transcription of the *RAD23* gene is UV inducible (38), we asked whether Rad23 exerts a similar cellular effect on Rad4 in the absence of exogenous DNA damage. This proved to be the case. Apparently, the Rad23 function in stabilizing Rad4 is well conserved in mammals. It was shown recently that XPC protein, which is the homolog of Rad4, is also stabilized in mouse cells by HR23A and HR23B, two homologs of Rad23 (26). Lommel *et al.* (32) suggested that Rad23 stabilizes Rad4 by protecting it from proteasome-mediated degradation. Supporting this notion, Rad4 protein is stabilized in proteasome-deficient mutant cells (32), and removal of UV-induced CPDs was reported to be enhanced in these mutant cells (30). In our studies, we found that although Rad4 was unstable in *rad23* mutant cells, the purified Rad4 protein was relatively stable in *rad23* mutant cell extracts *in vitro*. Thus, Rad4 protein by itself is not associated with an intrinsic instability. Our results are consistent with the conclusion of Lommel *et al.* (32) that cellular Rad4 is actively degraded by the 26S proteasome, and its quick turnover is protected by Rad23 protein.

Since purified Rad4 and Rad23 together are necessary and sufficient for full complementation of deficient NER of *rad23*

mutant cell extracts, it is clear that Rad4 is the only NER protein that is significantly stabilized by Rad23 in cells, at least for the global genome repair. It was observed that Rad4 and XPC proteins transiently accumulate in yeast and mammalian cells, respectively, following DNA damage (26,32). This has been proposed as an important mechanism of NER regulation (26). However, since persistent overexpression of Rad4 protein in wild-type yeast cells did not yield an effect on cellular resistance to UV radiation, it is unlikely that damage-induced Rad4 accumulation may represent a significant regulatory mechanism for repair. Rad4 accumulation may simply reflect its enhanced stabilization by higher levels of Rad23, as the *RAD23* gene is damage inducible in yeast (38).

XPC stabilization by HR23A/B led Ng *et al.* (26) to conclude that this is the primary function of RAD23 protein. Such a mechanism predicts that deficient NER in *rad23* mutant cells should be largely complemented by persistent high levels of Rad4 protein in the cell through gene overexpression. This prediction is not supported by our results in yeast. First, persistent overexpression of Rad4 protein only slightly enhanced the UV resistance of *rad23* mutant cells, in contrast to full complementation by expressing the *RAD23* gene. Thus,

deficient NER in *rad23* mutant cells is not corrected to a large extent by high levels of Rad4 protein. Second, deficient *in vitro* NER in *rad23* mutant cell extracts could not be effectively complemented by purified Rad4 protein. Consistent with our results, Lommel *et al.* (32) reported that overexpression of Rad4 or lack of the proteasome function could not suppress the deficient removal of UV-induced CPDs in *rad23* mutant cells. On the other hand, we observed that Rad4 overexpression slightly enhanced the UV resistance of *rad23* mutant cells, and that purified Rad23 protein alone could not fully complement deficient NER *rad23* mutant cell extracts. Clearly, the Rad4 protein concentration in *rad23* mutant cells is limiting relative to other NER proteins for repair. Hence, stabilizing Rad4 protein is an important but not the only function of Rad23 protein in yeast NER. Another important function of Rad23 protein is its direct participation in NER, as demonstrated by the requirement for both purified Rad23 and Rad4 proteins to fully complement the deficient NER of *rad23* mutant cell extracts. Since *rad23* mutant cells are only modestly sensitive to UV radiation unlike other mutant cells such as *rad4* and *rad14*, Rad23 protein can only play an accessory role rather than an indispensable role in the biochemical pathway of NER. We found that while it is unable to bind DNA, Rad23 protein stimulates the DNA-binding activity of Rad4 protein. This is the only biochemical activity observed for Rad23 protein in NER. Thus, we postulate that stimulation on Rad4 binding to damaged DNA represents the major mechanism of the direct participation of Rad23 protein in NER. Definitive proof of this model awaits extensive structure-function studies of this protein.

Although the precise mechanism by which Rad23 stimulates DNA binding by Rad4 is not clear at present, physical interaction between these two proteins may be involved. Indeed, Rad23 strongly interacts with Rad4 in the yeast and the mammalian systems (14–16). Intriguingly, a protein complex consisting of XPC, HR23B and CEN4 was isolated from human cells, in which the CEN4 component directly interacts with XPC (39). The role of CEN4 in this complex and whether this protein contributes to NER remains unknown. Since the stimulatory activity of Rad23 on Rad4 DNA binding was observed with purified proteins *in vitro*, it is unlikely that the stimulatory mechanism involves protein modification during damage recognition by Rad4. In addition to Rad4, Rad23 also interacts with several other proteins, one of which is the 3-methyladenine DNA glycosylase involved in base excision repair (40). Consistent with our model that stimulating Rad4 binding to damaged DNA accounts for a major role of Rad23 in NER, the human RAD23 proteins additionally stimulate the activity of the 3-methyladenine DNA glycosylase *in vitro* (40). Our conclusions that yeast Rad23 plays dual functions by stimulating Rad4-mediated damage binding/recognition and by stabilizing Rad4 in cells may provide a general model for understanding RAD23 protein in NER of other eukaryotes.

ACKNOWLEDGEMENTS

We thank R. Daniel Gietz for the yeast expression plasmids Yeplac112, Yeplac195 and Yeplac181; Errol Friedberg for

the yeast strains SX46A, BJ2168 Δ rad2, SX46A Δ rad4 and SX46A Δ rad14; and Jaap Brouwer for the yeast strains W303-1B and MGSC101. This work was supported by a NIH grant CA67978.

REFERENCES

1. Wang,Z., Wu,X. and Friedberg,E.C. (1993) Nucleotide-excision repair of DNA in cell-free extracts of the yeast *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **90**, 4907–4911.
2. Wang,Z., Svejstrup,J.Q., Feaver,W.J., Wu,X., Kornberg,R.D. and Friedberg,E.C. (1994) Transcription factor b (TFIIH) is required during nucleotide excision repair in yeast. *Nature*, **368**, 74–76.
3. Braithwaite,E., Wu,X. and Wang,Z. (1998) Repair of DNA lesions induced by polycyclic aromatic hydrocarbons in human cell-free extracts: involvement of two excision repair mechanisms *in vitro*. *Carcinogenesis*, **19**, 1239–1246.
4. Braithwaite,E., Wu,X. and Wang,Z. (1999) Repair of DNA lesions: mechanisms and relative repair efficiencies. *Mutat. Res.*, **424**, 207–219.
5. Abouessekhra,A., Biggerstaff,M., Shivji,M.K.K., Vilpo,J.A., Moncollin,V., Podust,V.N., Protic,M., Hubscher,U., Egly,J.-M. and Wood,R.D. (1995) Mammalian DNA nucleotide excision repair reconstituted with purified protein components. *Cell*, **80**, 859–868.
6. Huang,J.C., Svoboda,D.L., Reardon,J.T. and Sancar,A. (1992) Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5' and the 6th phosphodiester bond 3' to the photodimer. *Proc. Natl Acad. Sci. USA*, **89**, 3664–3668.
7. Kusumoto,R., Masutani,C., Sugawara,K., Iwai,S., Araki,M., Uchida,A., Mizukoshi,T. and Hanaoka,F. (2001) Diversity of the damage recognition step in the global genomic nucleotide excision repair *in vitro*. *Mutat. Res.*, **485**, 219–227.
8. Cleaver,J.E. and Kraemer,K.H. (1989) In Scriver,C.R., Beaudet,A.L., Sly,W.S. and Valle,D. (eds.), *The Metabolic Basis of Inherited Disease*, 6th edn. McGraw-Hill Book Co., NY, pp. 2949–2971.
9. Hanawalt,P.C. (1994) Transcription-coupled repair and human disease. *Science*, **266**, 1957–1958.
10. de Laat,W.L., Jaspers,N.G. and Hoeijmakers,J.H. (1999) Molecular mechanism of nucleotide excision repair. *Genes Dev.*, **13**, 768–785.
11. Wood,R.D., Robins,P. and Lindahl,T. (1988) Complementation of the Xeroderma pigmentosum DNA repair defect in cell-free extracts. *Cell*, **53**, 97–106.
12. Wang,Z., Wu,X. and Friedberg,E.C. (1996) A yeast whole cell extract supports nucleotide excision repair and RNA polymerase II transcription *in vitro*. *Mutat. Res.*, **364**, 33–41.
13. Mu,D., Park,C.-H., Matsunaga,T., Hsu,D.S., Reardon,J.T. and Sancar,A. (1995) Reconstitution of human DNA repair excision nuclease in a highly defined system. *J. Biol. Chem.*, **270**, 2415–2418.
14. Masutani,C., Sugawara,K., Yanigisawa,J., Soniyama,T., Ui,M., Enomoto,T., Takio,K., Tanaka,K., van der Spek,P.J., Bootsma,D. *et al.* (1994) Purification and cloning of a nucleotide excision repair complex involving the xeroderma pigmentosum group C protein and a human homologue of yeast RAD23. *EMBO J.*, **13**, 1831–1843.
15. Wang,Z., Wei,S., Reed,S.H., Wu,X., Svejstrup,J.Q., Feaver,W.J., Kornberg,R.D. and Friedberg,E.C. (1997) The *RAD7*, *RAD16* and *RAD23* genes of *S.cerevisiae*: requirement for transcription-independent nucleotide excision repair *in vitro* and interactions between the gene products. *Mol. Cell. Biol.*, **17**, 635–643.
16. Guzder,S.N., Sung,P., Prakash,L. and Prakash,S. (1998) Affinity of yeast nucleotide excision repair factor 2, consisting of the Rad4 and Rad23 proteins, for ultraviolet damaged DNA. *J. Biol. Chem.*, **273**, 31541–31546.
17. Sugawara,K., Ng,J.M., Masutani,C., Iwai,S., van der Spek,P.J., Eker,A.P., Hanaoka,F., Bootsma,D. and Hoeijmakers,J.H. (1998) Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. *Mol. Cell*, **2**, 223–232.
18. Batty,D., Rappic'Otrin,V., Levine,A.S. and Wood,R.D. (2000) Stable binding of human XPC complex to irradiated DNA confers strong discrimination for damaged sites. *J. Mol. Biol.*, **300**, 275–290.
19. Watkins,J.F., Sung,P., Prakash,L. and Prakash,S. (1993) The *Saccharomyces cerevisiae* DNA repair gene RAD23 encodes a nuclear protein containing a ubiquitin-like domain required for biological function. *Mol. Cell. Biol.*, **13**, 7757–7773.

20. Mueller, J.P. and Smerdon, M.J. (1996) Rad23 is required for transcription-coupled repair and efficient overall repair in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **16**, 2361–2368.
21. Friedberg, E.C., Walker, G.C. and Siede, W. (1995) *DNA Repair and Mutagenesis*. American Society of Microbiology Press, Washington, DC.
22. Prakash, S. and Prakash, L. (2000) Nucleotide excision repair in yeast. *Mutat. Res.*, **451**, 13–24.
23. Bertolaet, B.L., Clarke, D.J., Wolff, M., Watson, M.H., Henze, M., Divita, G. and Reed, S.I. (2001) UBA domains of DNA damage-inducible proteins interact with ubiquitin. *Nature Struct. Biol.*, **8**, 417–422.
24. Biggins, S., Ivanovska, I. and Rose, M.D. (1996) Yeast ubiquitin-like genes are involved in duplication of the microtubule organizing center. *J. Cell. Biol.*, **133**, 1331–1346.
25. Clarke, D.J., Mondesert, G., Segal, M., Bertolaet, B.L., Jensen, S., Wolff, M., Henze, M. and Reed, S.I. (2001) Dosage suppressors of pds1 implicate ubiquitin-associated domains in checkpoint control. *Mol. Cell. Biol.*, **21**, 1997–2007.
26. Ng, J.M., Vermeulen, W., van der Horst, G.T., Bergink, S., Sugawara, K., Vrieling, H. and Hoeijmakers, J.H. (2003) A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein. *Genes Dev.*, **17**, 1630–1645.
27. Schaubert, C., Chen, L., Tongaonkar, P., Vega, I., Lambertson, D., Potts, W. and Madura, K. (1998) Rad23 links DNA repair to the ubiquitin/proteasome pathway. *Nature*, **391**, 715–718.
28. Russell, S.J., Reed, S.H., Huang, W., Friedberg, E.C. and Johnston, S.A. (1999) The 19S regulatory complex of the proteasome functions independently of proteolysis in nucleotide excision repair. *Mol. Cell*, **3**, 687–695.
29. Coux, O., Tanaka, K. and Goldberg, A.L. (1996) Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.*, **65**, 801–847.
30. Lommel, L., Chen, L., Madura, K. and Sweder, K. (2000) The 26S proteasome negatively regulates the level of overall genomic nucleotide excision repair. *Nucleic Acids Res.*, **28**, 4839–4845.
31. Gillette, T.G., Huang, W., Russell, S.J., Reed, S.H., Johnston, S.A. and Friedberg, E.C. (2001) The 19S complex of the proteasome regulates nucleotide excision repair in yeast. *Genes Dev.*, **15**, 1528–1539.
32. Lommel, L., Ortolan, T., Chen, L., Madura, K. and Sweder, K.S. (2002) Proteolysis of a nucleotide excision repair protein by the 26S proteasome. *Curr. Genet.*, **42**, 9–20.
33. Gietz, R.D. and Sugino, A. (1988) New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene*, **74**, 527–534.
34. Xin, H., Lin, W., Sumanasekera, W., Zhang, Y., Wu, X. and Wang, Z. (2000) The human *RAD18* gene product interacts with HHR6A and HHR6B. *Nucleic Acids Res.*, **28**, 2847–2854.
35. Wang, Z., Wu, X. and Friedberg, E.C. (1992) Excision repair of DNA in nuclear extracts from the yeast *Saccharomyces cerevisiae*. *Biochemistry*, **31**, 3694–3702.
36. Guzder, S.N., Habraken, Y., Sung, P., Prakash, L. and Prakash, S. (1995) Reconstitution of yeast nucleotide excision repair with purified Rad proteins, replication protein A, and transcription factor TFIIH. *J. Biol. Chem.*, **270**, 12973–12976.
37. Jansen, L.E., Verhage, R.A. and Brouwer, J. (1998) Preferential binding of yeast Rad4.Rad23 complex to damaged DNA. *J. Biol. Chem.*, **273**, 33111–33114.
38. Madura, K. and Prakash, S. (1990) Transcript levels of the *Saccharomyces cerevisiae* DNA repair gene *RAD23* increase in response to UV light and in meiosis but remain constant in the mitotic cell cycle. *Nucleic Acids Res.*, **18**, 4737–4742.
39. Araki, M., Masutani, C., Takemura, M., Uchida, A., Sugawara, K., Kondoh, J., Ohkuma, Y. and Hanaoka, F. (2001) Centrosome protein centrin 2/caltractin 1 is part of the xeroderma pigmentosum group C complex that initiates global genome nucleotide excision repair. *J. Biol. Chem.*, **276**, 18665–18672.
40. Miao, F., Bouziane, M., Dammann, R., Masutani, C., Hanaoka, F., Pfeifer, G. and O'Connor, T.R. (2000) 3-Methyladenine-DNA glycosylase (MPG protein) interacts with human RAD23 proteins. *J. Biol. Chem.*, **275**, 28433–28438.