

# Cellular Ubiquitination and Proteasomal Functions Positively Modulate Mammalian Nucleotide Excision Repair

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The ubiquitin-proteasome pathway is fundamental to synchronized continuation of many cellular processes, for example, cell-cycle progression, stress response, and cell differentiation. Recent studies have shown that the ubiquitin-proteasome pathway functions in the regulation of nucleotide excision repair (NER) in yeast. In order to investigate the role of the ubiquitin-proteasome pathway in the NER of mammalian cells, global genomic repair (GGR), and transcription-coupled repair (TCR) were examined in a mouse ts20 cell line that harbors a temperature-sensitive ubiquitin-activating enzyme (E1). We found that E1 inactivation-induced ubiquitination deficiency decreased both GGR and TCR, indicating that the ubiquitination system is involved in the optimization of entire NER machinery in mammalian cells. We specifically inhibited the function of 19S proteasome subunit by overexpressing 19S regulatory complex hSug1 or its mutant protein hSug1mk in repair competent human fibroblast, OSU-2, cells and compared their capacity for NER. The results showed that 19S regulatory complex positively modulates NER in cells. In addition, we treated OSU-2 cells with the inhibitors of 20S subunit function, MG132 and lactacystin, and demonstrated that the catalytic activity of 20S subunit is also required for efficient NER. Moreover, the UV-induced recruitment of repair factor xeroderma pigmentosum protein C (XPC) to damage sites was negatively affected by treatment of repair competent cells with MG132. Taken together, we conclude that the ubiquitin-proteasome pathway has a positive regulatory role for optimal NER capacity in mammalian cells and appears to act through facilitating the recruitment of repair factors to DNA damage sites. © 2004 Wiley-Liss, Inc.

**Key words:** nucleotide excision repair; ubiquitin-proteasome pathway; UV radiation; DNA damage; mammalian cells; XPC

## INTRODUCTION

Nucleotide excision repair (NER) is a pathway that removes many bulky chemical adducts, as well as UV-induced photoproducts from cellular DNA. Through a multi-step repair process, DNA damage is recognized and incisions are made so that the damage is removed as part of an oligonucleotide. Finally, the resulting gap is filled by DNA polymerase and ligase [1,2]. The biochemical mechanism of NER has been extensively characterized. However, the regulation of NER is still poorly understood. In the last few years, the ubiquitin-proteasome system has become increasingly recognized as a controller of numerous physiological processes. A growing body of evidence suggests that the ubiquitin-proteasome pathway is involved in DNA repair (for review, see [3]).

Ubiquitin, a 76-amino acid peptide, is linked to the targeted proteins by the sequential action of three enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin-protein ligase (E3) [4]. Usually, the ubiquitinated proteins are handed over to the large 26S proteasome

for degradation [5]. Yet some ubiquitinated, especially mono-ubiquitinated, proteins are not targeted to the proteasome. Instead, singular ubiquitination of protein moieties serves as a regulatory function independent of proteolysis [6–8].

The 26S proteasome consists of two different subunits, the 19S regulatory complex and the 20S catalytic core unit [9]. The 19S regulatory complex is comprised of at least 18 subunits, including six

Abbreviations: NER, nucleotide excision repair; GGR, global genomic repair; TCR, transcription-coupled repair; XPC, xeroderma pigmentosum protein C; CPD, cyclobutane pyrimidine dimer; 6-4PP, pyrimidine (6-4) pyrimidone photoproduct; TS, transcribed strand; NTS, nontranscribed strand; PBS, phosphate-buffered saline; NGS, normal goat serum; DAPI, 4'-6'-diamidino-2-phenylindole; DDB2, damaged-DNA binding complex subunit 2.

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ATPases belonging to AAA (ATPase associated with a variety of cellular activities) family. These ATPases are thought to unfold substrate proteins in an ATPase-dependent manner [10]. It has been suggested that this activity may also be adapted for the disassembly or rearrangement of protein complexes without proteolysis [11]. The eukaryotic 20S catalytic core unit contains 14 different subunits arranged into four stacked, seven-member rings that form a hollow cylinder [12]. The two inner rings are each composed of seven different  $\beta$ -subunits and two outer rings are each made up of seven different  $\alpha$ -subunits. The proteolytic active sites face the interior of the cylinder and are thus sequestered from the cellular environment, defining the proteasome as a "self-compartmentalizing" protease [13,14].

The role of ubiquitination system in NER has been suggested by finding that Rad23, a component of NER machinery, has an ubiquitin-like domain. Deletion of the ubiquitin-like domain impairs the DNA repair function of RAD23 and this domain can be functionally substituted by the authentic ubiquitin sequence [15]. Furthermore, Ikehata et al. [16] have speculated that ubiquitin activating enzyme E1 might also be involved in the repair of UV-induced DNA damage. They found that the mouse mutant cells, expressing a temperature-sensitive E1, exhibit decreased cell viability and increased mutagenicity following UV irradiation and growth at nonpermissive temperatures. However, the removal of UV-induced DNA damage was not determined, and the role of ubiquitination system in eukaryotic NER remains undefined. Regarding the participation of proteasome system, Schaubert et al. [17] have reported that Rad23 interacts with the 26S proteasome through an amino-terminal ubiquitin-like domain, whereas the carboxy terminus of Rad23 binds to the Rad4 DNA repair protein. This offers a potential link between the DNA repair and proteasome pathway. However, recently published works have identified critical discrepancies for the possible role of 26S proteasome in NER of yeast system [18–21]. Due to the paucity of clear evidence for the cross-talk between 26S proteasome and NER in mammalian cells, our studies have attempted to demonstrate the possible relationship between these two key cellular processes. We have examined the participation of both ubiquitin and proteasome with different mammalian cell systems and found that induction of ubiquitination deficiency causes a decrease in both global genomic repair (GGR) and transcription-coupled repair (TCR) in mouse cells. Inhibition of the regulatory and the catalytic functions of proteasome in the repair competent human fibroblasts also results in inefficient removal of UV-induced DNA lesions. Overall data indicated that the normal function of the ubiquitin-proteasome pathway positively regulates the NER in mammalian cells, at least in part, by facilitating the recruitment of

xeroderma pigmentosum protein C (XPC) repair factor to DNA damage sites.

## MATERIALS AND METHODS

### Cell Culture and Treatments

Mouse embryo fibroblast ts20 (thermosensitive for E1 ubiquitin-activating enzyme) and its parental cell line A31N were kindly provided by Dr. Harvey L. Ozer (UMDNJ-New Jersey Medical School). Both cell lines were cultured in 50% F-10 + 50% DMEM medium containing 10% fetal calf serum and antibiotics at 32°C in a humidified atmosphere of 5% CO<sub>2</sub> [22]. When needed, cells were transferred to the restrictive temperature (39°C) 16 h prior to the treatment and beginning of experiments. Normal human fibroblasts (OSU-2) were established in culture as described earlier [23]. For individual experiments, proteasome proteolysis inhibitors, MG132, or lactacystin (Calbiochem, La Jolla, CA final concentration, 10  $\mu$ M), were added to the cell culture 1 h before UV irradiation and maintained in the culture media after UV irradiation. UV irradiation was performed from a germicidal lamp at a dose rate of 1.0 J/m<sup>2</sup>/s as measured by a Kettering model 65 radiometer (Cole Palmer Instrument Co., Vernon Hill, IL). There was no apparent loss of cells up to 24 h following 20 J/m<sup>2</sup> UV irradiation in all monolayer cultures used in this study.

### Gene Constructs

The full-length *hSug1* cDNA was cut out from pGEX-*hSug1* (generously provided by Dr. Jeffrey Kudlow, The University of Alabama at Birmingham) with *BamH* I and *Sal* I and then ligated into the eukaryotic *myc*-tagged expression vector pCMV-Tag3B (Stratagene, La Jolla, CA). The full-length *hSug1* cDNA along with the *myc*-Tag was PCR-amplified from pCMV-*hSug1*, and then inserted into the *Sal* I and *Not* I sites of the eukaryotic high expression vector pVR1012 to get the construct pVR1012-*myc-hSug1*. It has been found that mutation of the lysine residue at position 196 eliminates *hSug1*'s both ATPase and helicase activities, and the resulting ATPase and helicase mutant of *hSug1* acts as a dominant negative mutant gene [24,25]. Accordingly, we used the recombinant PCR technique to replace the lysine residue (AAG) at position 196 with a methionine residue (ATG) to get the *hSug1* dominant negative mutant (*hSug1mk*). Both of *hSug1* plus *myc*-Tag cDNA and *hSug1mk* plus *myc*-Tag cDNA sequences were confirmed by DNA sequencing of the target constructs.

### Transient Transfection of OSU-2 Cells for Gene Overexpression

Transfection was performed using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's

instructions. Briefly, exponentially growing cells were seeded in a 60-mm dish at a density of  $4 \times 10^5$  cells 24 h prior to construct transfection. Cells were transfected with either plasmids or parental vector for 48 h, UV irradiated at  $20 \text{ J/m}^2$ , and allowed to repair for desired period of time. The cell samples were collected and lysed for either protein or DNA analysis.

#### Quantitation of Cyclobutane Pyrimidine Dimer (CPD) and Pyrimidine (6-4) Pyrimidone Photoproduct (6-4PP) by Immuno-Slot Blot Assay

The amount of dimers in DNA were quantitated with noncompetitive immuno-slot blot assay, essentially as earlier described [23,26]. Briefly, after UV exposure ( $20 \text{ J/m}^2$ ) and desired incubation periods, cells were recovered by trypsinization and immediately lysed for DNA isolation. The same amount of DNA samples were loaded on nitrocellulose membranes and the amount of CPD or 6-4PP was detected with corresponding polyclonal anti-CPD antibody (UV-3) [27] or monoclonal anti-6-4PP (64M-2) antibody (Provided by Dr. Tsukasa Matsunaga, Kanazawa University, Japan). The intensity of each band was determined by laser densitometric scanning and the amount of damage remaining, compared with the initially induced DNA damage, was used to calculate the repair rates in different cell types.

#### Strand-Specific DNA Repair Analysis by Southern Blot Analysis

Strand-specific DNA repair of UV irradiation-induced CPD damage was examined separately within the transcribed strand (TS) and nontranscribed strand (NTS) of *EcoR* I restriction fragments of hypoxanthine guanine phosphoribosyltransferase (*hprt*) gene and *p53* gene as described earlier [28–30]. Briefly, after UV treatment ( $20 \text{ J/m}^2$ ) and repair for desired time, total DNA of the cells were isolated and equal amounts ( $20 \mu\text{g}$ ) of DNA sample were treated with either T4 endonuclease-V or enzyme buffer alone and incubated at  $37^\circ\text{C}$  for 1 h. After incubation, the DNA was purified by extraction with phenol/chloroform and precipitated with ethanol. The samples were then dissolved in denaturing buffer and incubated at  $37^\circ\text{C}$  for 30–45 min to denature the DNA followed by electrophoresis in a neutral agarose gel (0.7%) for 18–20 h. The DNA in the gel was partially depurinated, neutralized, and transferred to a nylon membrane. Membranes were hybridized in 10 mL of solution containing 50% (v/v) formamide,  $6 \times \text{SSC}$ , 0.5% SDS, 5% dextran sulfate, denatured salmon sperm DNA ( $100 \mu\text{g/mL}$ ), and  $1-2 \times 10^8$  c.p.m. of  $^{32}\text{P}$ -labeled, single-stranded, exon-specific probes generated by asymmetric PCR [31]. After 20–22 h hybridization at  $42^\circ\text{C}$ , the membranes were washed to a final stringency at  $62^\circ\text{C}$  in  $1 \times \text{SSC}/1\%$  SDS and exposed to a phosphorimager screen. The ratio of full-length restriction fragment in the endonuclease-treated and untreated samples was

determined by quantifying the individual band intensities upon imaging and processing by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The average number of UV-lesions per fragment was calculated using Poisson distribution [32].

#### Western Blot Analysis

The cells were lysed in SDS lysis buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and protease inhibitors) and boiled for 10 min. The proteins were quantitated, separated by SDS-PAGE, and the immunoblot analysis was performed as described earlier by using chemiluminescent detection [29]. The following antibodies and dilution were used: monoclonal anti-*myc* (Roche Molecular Biochemicals; 1:1000); monoclonal anti-p53 (DO-1 and Pab 240, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:200); monoclonal antiactin (Neomarkers, Fremont, CA; 1:500); polyclonal anti-XPC (1:1000).

#### Local UV Irradiation and Immunofluorescent Staining

To perform micropore UV irradiation, the cells grown on glass coverslips were washed with prewarmed phosphate-buffered saline (PBS) and UV irradiated as described [33]. For immunofluorescent staining, cells grown on coverslips were washed twice with cold PBS and subsequently fixed and permeabilized in buffer containing freshly made 2% paraformaldehyde and 0.5% Triton X-100 in PBS at  $4^\circ\text{C}$  for 30 min. The cells were blocked with normal goat serum (NGS), incubated with the primary antibody against XPC (rabbit anti-XPC at 1:200) in PBS with 1.5% NGS, and stained with a secondary antibody (FITC conjugated goat antirabbit IgG at 1:200 dilution). After first step staining, the cells were treated with 2 M HCl at  $37^\circ\text{C}$  for 10 min to denature the DNA, followed by a PBS rinse to remove HCl. The slides were incubated with 1:1000 dilution of mouse anti-CPD antibody (TDM-2) followed by a secondary antibody (Texas Red conjugated goat antimouse IgG at 1:400 dilution) in PBS with 1.5% NGS. The cells, after washing with PBS containing 0.1% Tween-20, were mounted in an antifade containing medium with  $0.75 \mu\text{g/mL}$  of 4'-6'-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) as a DNA counter stain. Fluorescence images were obtained with a Nikon Fluorescence Microscope E800 (Nikon, Tokyo, Japan) fitted with appropriate filters for DAPI, FITC, and Texas Red. The digital images were captured with a cooled CCD camera and processed with the help of its SPOT software (Diagnostic Instruments, Inc., Sterling Heights, MI).

## RESULTS

### E1 Enzyme Inactivation Decreases the Rates of Both GGR and TCR

Several lines of evidence have suggested that ubiquitin system might be involved in DNA repair.

However, insufficient evidence exists to show that ubiquitination is required for the optimal NER, especially in mammalian cells. In order to understand the participatory role of ubiquitination in NER and obtain further evidence in mammalian cells, ts20 cell line, a thermosensitive (ts) mutant derived from Balb/c 3T3 mouse embryo fibroblasts, was utilized in the initial experiments. During its culture, E1 ubiquitin activating enzyme is active at permissive temperature (32°C), whereas E1 is inactive and the corresponding ubiquitination function is compromised at restrictive temperature (39°C) [22]. We first examined the stabilization of p53 protein to ensure E1 inactivation at the restrictive temperature. Our result shows that the temperature shift entailed the stabilization-linked accumulation of p53 in ts20 cells but not in parent A31N cells (data not shown). This data confirmed the earlier observations, which indicated the E1 mediated sensitization and blockage of p53 degradation of ts20 cells at restrictive temperature [22,34]. We then assessed the capacity of ts20 and its parental A31N cells to remove UV-induced CPD at permissive and restrictive temperatures. Through slot-blot analysis with anti-CPD antibody, we determined the total CPD amount at each time point and found that there was no difference in the initial amount of CPD induced by 20 J/m<sup>2</sup> UV at 32 and 39°C, indicating that the temperature does not affect the CPD induction. As shown in Figure 1, the removal of CPD from entire

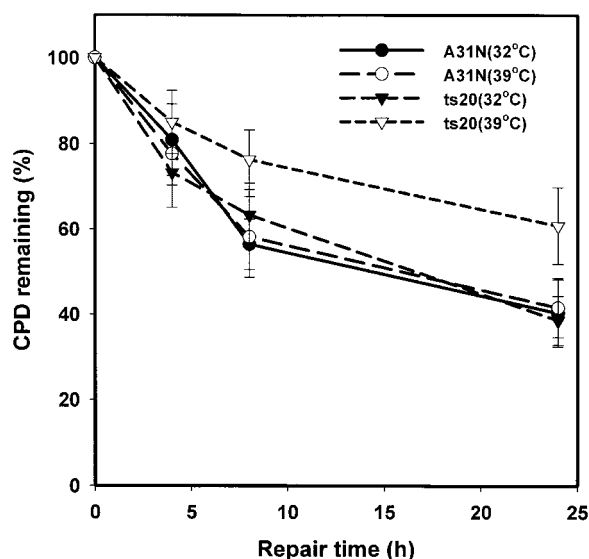


Figure 1. The removal of UV-induced cyclobutane pyrimidine dimer (CPD) from the whole genome was decreased in E1 thermosensitive mutant ts20 cells at the nonpermissive temperature. ts20 and its parent cell line A31N were cultured at 32°C or shifted to 39°C for 16 h, UV irradiated at 20 J/m<sup>2</sup> and further incubated for indicated time periods at 32 or 39°C. The cells were recovered at indicated time and high-molecular weight DNA was isolated. Identical amounts of DNA from various samples were subjected to immunoslot blot analysis to quantitate the amount of CPD by using anti-CPD antibody. Each experiment was run in triplicate and the data plotted are mean  $\pm$  SEM.

genomic DNA in ts20 cells occurred at a faster initial rate upon incubation at the permissive 32°C and only 40% CPD remained in genomic DNA at 24 h following UV irradiation. However, the removal of CPD in the same cells at the nonpermissive 39°C was demonstrably slower at various times following UV irradiation, especially at 24 h, at which time point 65% CPD still remained, following UV irradiation. This indicates that E1 inactivation contributes to compromised NER and that ubiquitination is required for the optimal NER in mammalian cells. In order to distinguish the specific involvement of ubiquitination pathway in two NER subpathways, we further examined the strand-specific repair of UV-induced CPD in both strands of 18 kb *EcoR* I fragment of *hprt* and 16 kb *EcoR* I fragment of *p53* genes in ts20 and its parent cell line A31N at 32 or 39°C. As shown in Figure 2A and B, successful DNA repair is reflected by the restoration of the full-length DNA restriction fragment following T4 endonuclease treatment and probing for characteristic *hprt* or *p53* gene fragments. The rates of CPD repair, analyzed from scans of various autoradiograms, are shown in Figure 2C and D. For the repair of *hprt* gene (Figure 2A and C), restoration of *hprt* TS in ts20 cells at 32°C was fast and reached 80% at 24 h following UV irradiation. However, the repair of TS at 39°C showed considerable decrease and only 60% of CPD was repaired at 24 h following UV irradiation. Similarly, the repair of NTS in ts20 cells was dramatically slower at 39°C than that at 32°C with respective repair rates of 15% and 73% at 24 h following UV irradiation. Analogous repair patterns were observed at *p53* gene locus (Figures 2B and D). However, the differential repair emanating from impaired ubiquitination function was distinctly pronounced for both the TS and NTS of *p53* gene. This data indicates that the ubiquitination function is required in NER for both NER subpathways, presumably having a greater impact on GGR. The strand-specific repair of both *p53* and *hprt* genes was also analyzed in H38.5 cells, which is generated by the transfection of ts20 cells with human genomic DNA containing a wild-type *E1* gene. The rates of CPD removal, in H38.5 cells at 39°C, were similar to those in ts20 cell at 32°C (data not shown). This further confirmed that E1 is required for efficient NER.

#### Overexpression of 19S Subunits hSug1 and hSug1mk Compromised the Optimal NER

Normally, inside cells, the ubiquitinated proteins are targeted for degradation by 26S proteasome. However, a portion of overall protein ubiquitination is independent of proteolysis and serves a regulatory function. To explore whether the role of ubiquitination pathway in NER is related to 26S proteasome, we determined the influence of 26S proteasome on NER. It is well known that 26S proteasome contains

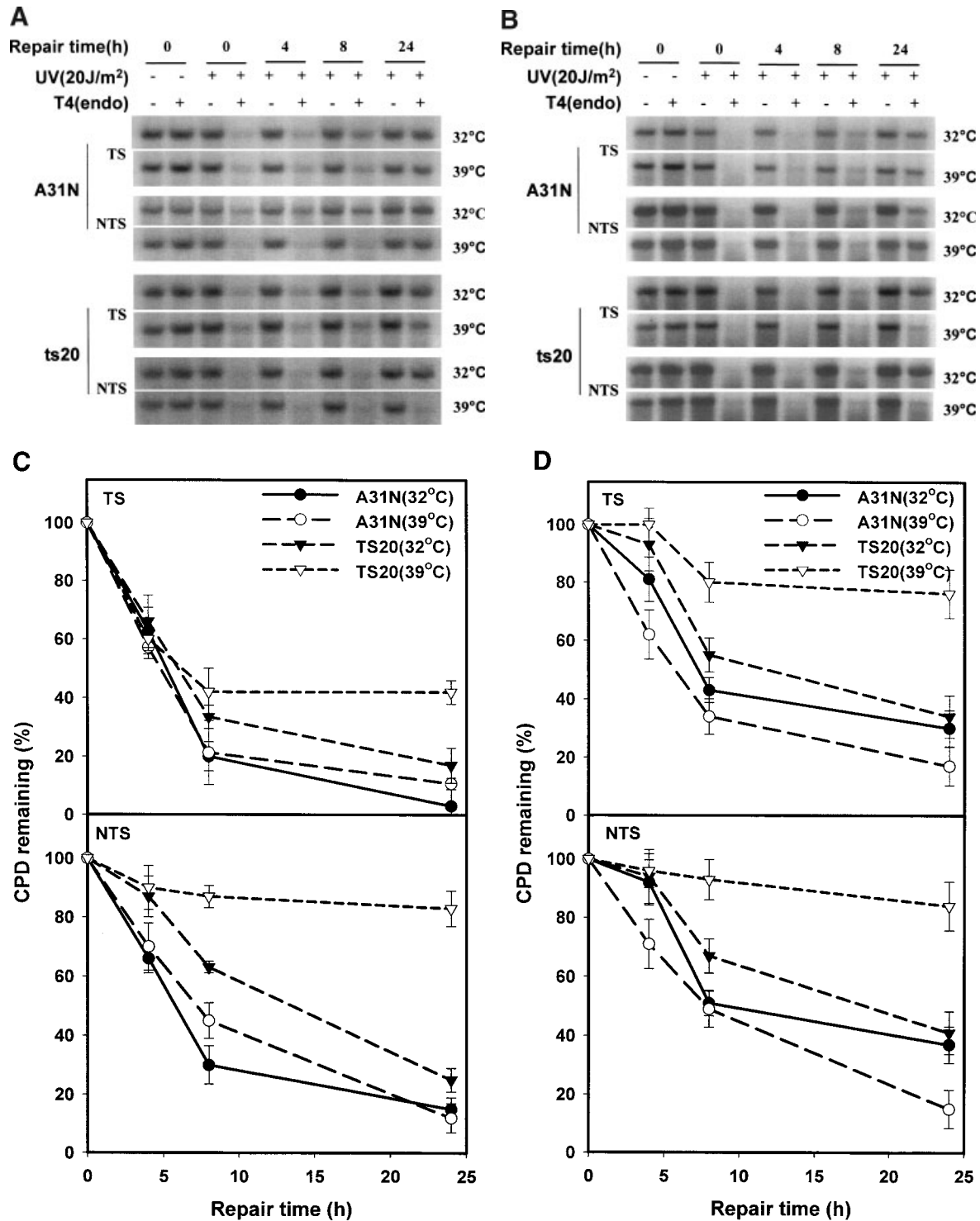


Figure 2. Strand-specific DNA repair of CPD from human *hprt* and *p53* genes in A31N and ts20 cells after 20 J/m<sup>2</sup> UV-irradiation. (A and B) Autoradiograms represent the extent of removal of CPD in the transcribed strand (TS) and nontranscribed strand (NTS) of 18 kb *EcoR* I fragment of the human *hprt* (A) and 16 kb *EcoR* I fragment of *p53* (B) genes. DNA, isolated from untreated confluent cells (first two lanes of each panel) or from cells incubated for indicated time periods after UV-irradiation, was digested with *EcoR* I. Samples of 20  $\mu$ g *EcoR* I digested DNA were then treated (+) or mock-treated (-) with T4-endonuclease-V, electrophoresed under formamide denaturing conditions, Southern transferred to nylon membrane

and hybridized with *hprt* or *p53* strand-specific <sup>32</sup>P-labeled DNA probes. (C and D) Repair profiles for the rate of removal of CPD within the TS and NTS of the human *hprt* (C) and *p53* (D) gene. The frequency of induction of CPD and their rates of removal were determined as the reappearance of the full-length restriction fragments in the T4 endonuclease-V treated and untreated samples upon quantitation by phosphorimager analysis. The average number of lesions (endonuclease-sensitive sites) per fragment was calculated using the Poisson equation. Data of the graphs represent the averages of three independent experiments.

two functionally distinguishable multiprotein complexes, a 19S regulatory and a 20S catalytic core complex. For studying the function of 19S regulatory complex in NER, our strategy relied on overexpressing a component subunit of 19S complex to compete with the endogenous 19S complex for binding of the substrates. For example, hSug1, which is one of the six known mammalian proteasomal ATPase, has been identified as an integral component of the 26S proteasome in that it copurifies with the proteasome in both conventional and nickel-chelate affinity chromatography [35]. Endogenous hSug1 protein primarily exists in proteasome as part of the 19S complex and as such participates in its proteolytic function. Figure 3A shows that the overexpression of either the wild-type or mutant hSug1 increased the steady-state level of p53, indicating that non-complexed forms of both hSug1 decreased the 19S-mediated degradation of p53. In another separate experiment, we found that overexpression of either wild-type hSug1 or mutant hSug1mk inhibited the degradation of GAL4-VP16 fusion protein [36]. Subsequently, we compared the repair efficiency of UV-induced CPD in NER-competent human fibroblast (OSU-2) cells overexpressing either wild-type hSug1 or mutant hSug1mk. As shown in Figure 3B, both mock-transfected and empty vector transfected OSU-2 cells exhibit efficient repair of UV induced CPD. About 70% of CPD was eliminated from the genome during 24 h following UV irradiation. On the contrary, the removal of CPD in OSU-2 cells overexpressing the wild-type hSug1 exhibited a significant decrease. Only 35% of CPD was removed within 24 h postirradiation. Similarly, overexpression of dominant negative mutant hSug1mk compromised the removal of CPD to the same extent as the wild-type hSug1. The data indicated that overexpression of hSug1 or hSug1mk exerts a negative regulatory influence on mammalian NER. It may be noted that the decreased repair was evident even under the conditions of augmented cellular p53 levels which is known to positively impact the GGR of UV induced lesions in cells [29,37–39].

#### The Catalytic Function of 20S Proteasomal Core Complex is Required for the Efficient NER in Mammalian Cells

Although the results described above revealed the modulation of repair by ubiquitin-proteasome pathway, they do not offer clues about the involvement of proteolysis function in the regulation of mammalian NER. Recent studies have shown that apart from proteasomal proteolysis-associated function, the ubiquitin-proteasome pathway could act in different cellular processes via proteolysis-independent mechanisms [6–8]. To investigate such involvement in DNA repair process, we treated NER-proficient OSU-2 cells with proteasomal proteolysis inhibitors, MG132 or lactacystin, and analyzed the removal of UV-induced CPD and 6-4PP. First, the inhibitory

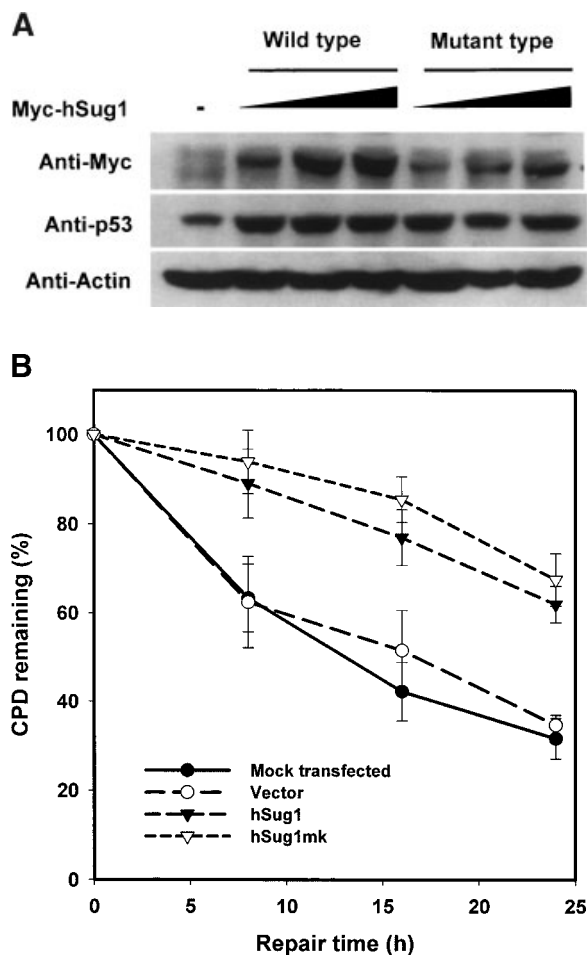


Figure 3. Overexpression of hSug1 or hSug1mk impaired the proteasome function for degradation of p53 and decreased the rate of nucleotide excision repair (NER). (A) OSU-2 cells were transiently transfected with either hSug1 or hSug1mk and cultured further for 48 h. The cells were lysed at desired times and the protein samples were subjected to SDS-PAGE. The ectopic expression of myc tagged hSug1 or hSug1mk and the steady-state level of endogenous p53 were detected with corresponding antibodies. (B) Immunoblot quantification of CPD removal in hSug1 or hSug1mk overexpressed OSU-2 cells. After transfection with hSug1, hSug1mk, or empty vector for 48 h, OSU-2 cells were UV irradiated at 20 J/m<sup>2</sup>, and allowed to repair for indicated period of time. DNA was quantitated and identical amount of DNA were subjected to immunoblot to quantitate remaining CPD with anti-CPD antibody. Graph represents the average of at least three biological experiments.

effect on protein degradation occurring through proteasomal proteolysis was established in treated OSU-2 cells. It was found that the steady-state level of p53 increased significantly after treatment with either MG132 or lactacystin (data not shown). Under the same treatment conditions, we then examined the repair of UV-induced CPD (Figure 4A) and 6-4PP (Figure 4B). In the absence of proteasome inhibitors, OSU-2 exhibited about 40% removal of CPD 24 h following UV irradiation. However, upon treatment with either MG132 or lactacystin, the cells exhibited a considerable decrease in the rate as well as extent of CPD repair. For example, less than 20% CPD were

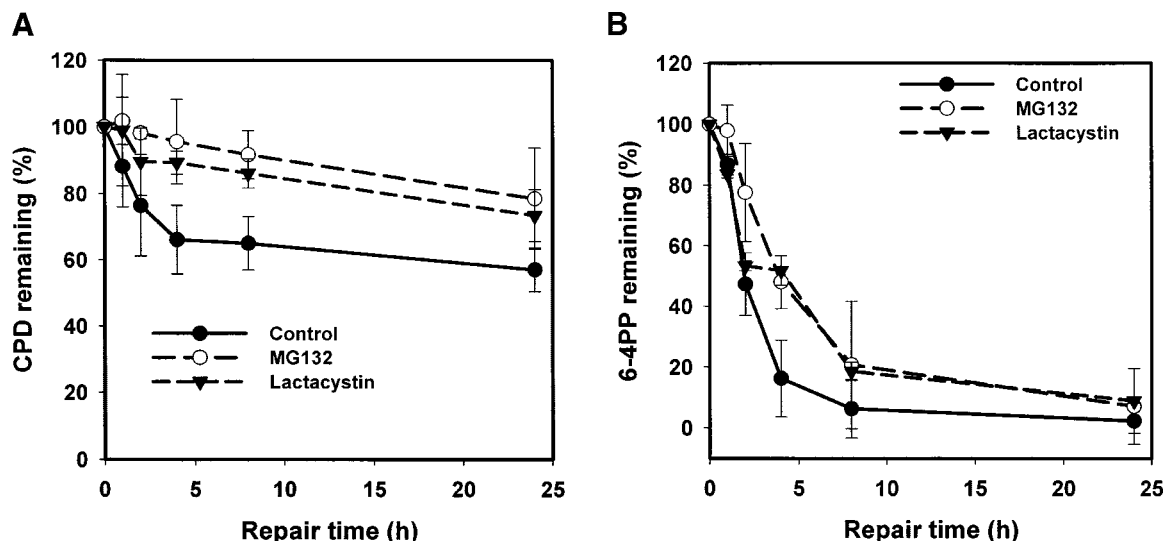


Figure 4. Proteasome inhibitor treatment compromised the removal of UV-induced DNA damage. Confluent OSU-2 cells were treated with either MG132 or lactacystin (both at final concentration of 10  $\mu$ M), or mock treated 1 h prior to UV irradiation at 20 J/m<sup>2</sup>. The cells were either lysed immediately or further cultured in the medium

containing same amount of proteasome inhibitor for desired times and then lysed for DNA isolation. Same amount of DNA was loaded on nitrocellulose membrane and detected with either anti-CPD (A) or anti-6-4PP (B) antibody. Graphs of triplicate data show the initial or remaining CPD or 6-4PP in whole genome.

removed after 24 h of irradiation in inhibitor treated cells. Similarly, the level of 6-4PP removal showed a decrease identical to that of CPD, albeit the relative rate of 6-4PP repair was faster than CPD. Decreased repair of 6-4PP lesions was observed with both MG132 and lactacystin. These results indicated that proteasomal proteolysis is required for efficient NER of different photolesions.

#### MG132 Treatment Inhibits UV-Induced XPC Recruitment to DNA Damage Site

During repair of mammalian genome, GGR-specific recognition factor XPC, recruited to DNA damage site from the dispersed nuclear areas, is followed by sequential assembly of TFIIH, XPA, RPA, XPG, and XPF [40–42]. The initial recruitment of XPC is a crucial event of GGR for productive assembly of all other necessary factors and for repair to commence at target sites in repair-proficient cells [40]. Using micropore local UV irradiation in combination with immunofluorescence microscopy, we examined the recruitment of XPC to UV irradiated nuclear spots of cells grown in presence of MG132. As shown in Figure 5, irradiation through filters with micropore (5  $\mu$ m in diameter) generates DNA damage confined to the subnuclear areas, which can be visualized by specific antibody to CPD. The staining of CPD and XPC, with their specific primary antibodies and secondary antibodies conjugated with different fluorescent markers, makes it possible to observe the DNA damage as well as repair factor localization simultaneously. In the cells without MG132 treatment, the XPC specific signal intensified in the area with CPD, indicating that XPC protein in the nucleus is relocated from the shielded nuclear

areas and recruited to DNA damage sites. We analyzed about 400 cells and counted the total number of CPD foci as well as XPC foci in each sample. Upon calculating the ratio of XPC foci to CPD foci, we found that about 80% and 58% CPD sites that exhibited XPC recruitment after 30 min and 1 h following UV irradiation, respectively (Figures 5A and B). This is consistent with our results reported earlier [33]. Treatment of cells with MG132 did not affect the formation of CPD locally, but drastically impacted the recruitment of XPC to damage sites. The ratios of XPC to total CPD foci were only 37% and 34% at 30 min and 1 h following UV irradiation, respectively (Figures 5A and B). In order to rule out the possibility that the inefficient XPC recruitment was not due to qualitative or quantitative changes of repair factors, we examined the effect of MG132 on the level of XPC before and after UV irradiation of OSU-2 cells. As shown in Figure 6, neither UV irradiation nor MG132 treatment affected the level of XPC protein. Moreover, MG132 treatment did not induce polyubiquitination of XPC protein. These data indicate that proteolysis or modification of XPC is not critical for NER. We also assessed the influence of MG132 on the recruitment of another repair factor, XPB, a subunit of TFIIH, which is common to both GGR and TCR subpathways. UV damage-specific recruitment of XPB to irradiated nuclear spots decreased significantly upon treatment of cell with MG132 (data not shown). These results provide additional support to our suggestion that proteasomal proteolysis actively participates in facilitating the recruitment of repair factors to initiate the NER of DNA damage.

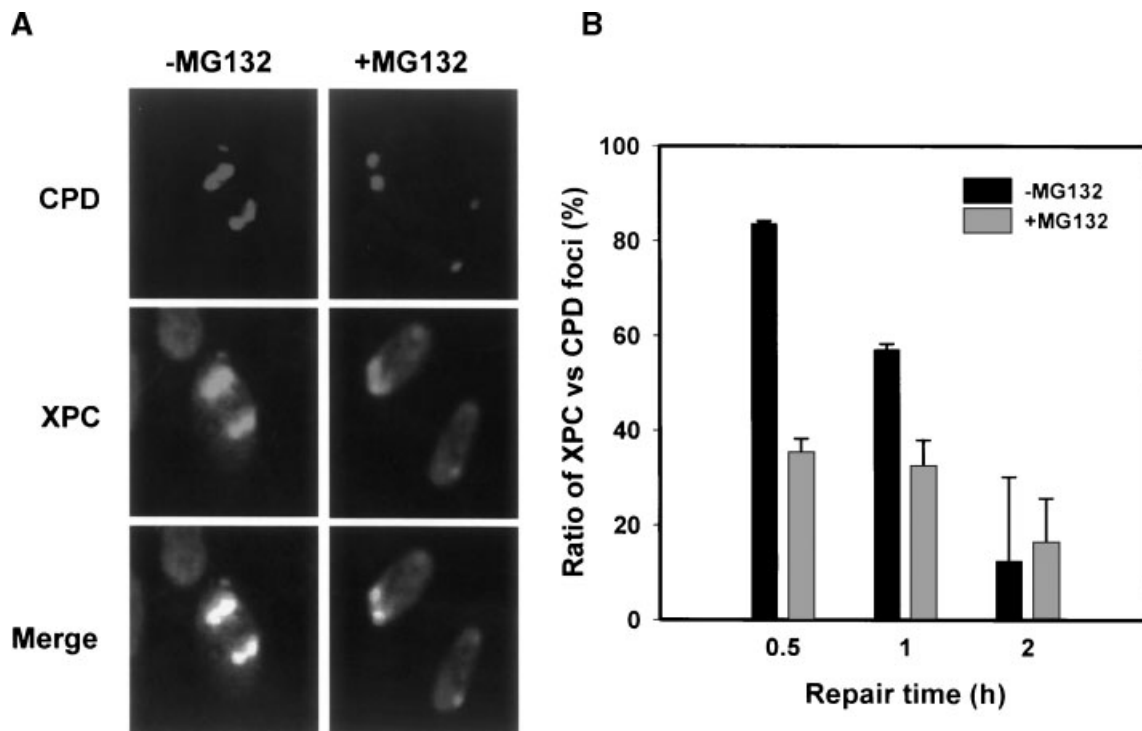


Figure 5. Proteasome inhibitor treatment decreased the recruitment of xeroderma pigmentosum protein C (XPC) to UV-induced damage site. OSU-2 cells were treated with 10  $\mu$ M MG132 or mock treated for 1 h and subjected to UV irradiation at 40 J/m<sup>2</sup> through an isopore polycarbonate filter cover (5  $\mu$ m). The cells on coverslips were fixed at indicated times after irradiation and doubly stained to

decorate with mouse anti-CPD and rabbit anti-XPC antibodies. (A) The immunofluorescent photomicrographs of focally damaged nuclear DNA and recruitment of XPC in OSU-2 cells treated with MG132 or mock treated; (B) The ratio of XPC foci to the total CPD foci determined from evaluation of at least 400 independent cells in the photomicrograph.

## DISCUSSION

### Specific Inhibitors Modulate Ubiquitination, Protein Degradation, and NER in Mammalian Cells

The thermosensitive E1 ubiquitin-activating enzyme cell mutant is a very useful tool to study the function of ubiquitination in mammalian cell system. Ikehata et al. [16] have shown that incubation of mouse cells, expressing a temperature-sensitive

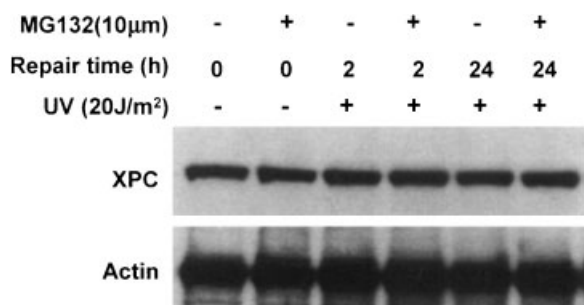


Figure 6. Proteasome inhibitor treatment did not affect the level of XPC protein. Confluent OSU-2 cells were treated with MG132 at a final concentration of 10  $\mu$ M, or mock treated 1 h prior to UV irradiation at 20 J/m<sup>2</sup>. The cells were lysed after 2 or 24 h incubation in the medium containing same amount of proteasome inhibitor. Similar protein concentration was loaded for SDS-PAGE and XPC as well as actin protein was detected with their specific antibodies.

E1 ubiquitin-activating enzyme, at nonpermissive temperature induces UV sensitivity and mutagenesis. This suggests that ubiquitin-conjugation system contributes to DNA repair in mammalian cells. In this study, we used the thermosensitive E1 mutant cell line for determining the actual UV-induced CPD removal and have compared it directly in both E1-activate and E1-inactivate cells. Furthermore, we also assessed the removal of CPD in separated strands of specific genes to estimate the efficiency of both GGR and TCR. It is realized, however, that the ts20 cell line might represent a leaky phenotype with regards to E1 inactivation upon transfer of cells from permissive to nonpermissive temperature [34]. Thus, the data generated has the potential to underestimate the contribution of ubiquitination to NER. Despite such eventuality, this approach provides an effective tool for the direct assessment of the role of ubiquitination in mammalian cells. Therefore, combination with direct detection of CPD removal, our cumulative results provide clear evidence that ubiquitination system is not only involved in mammalian cell NER, but plays a modulatory role in both GGR and TCR subpathways of genomic maintenance.

In this study, we also analyzed the independent contributions of 19S regulatory complex and 20S

catalytic core subunit of 26S proteasome for the removal of UV-induced CPD. In order to specifically inhibit the function of 19S, we overexpressed the 19S subunit hSug1 and its dominant negative mutant hSug1mk in human cells. Because expression of both wild-type and mutant hSug1 inhibited the function of protein degradation through proteasome, we believe that overexpressed hSug1 not only competes with endogenous hSug1 (as part of the 19S regulatory complex) for binding to hSug1 substrates, but also disrupts the proper stoichiometry of the 19S subunit and thereby abolishes 19S proteasomal function. Under this condition, the decreased NER efficiency can be explained via the impaired function of 19S regulatory complex. The availability of proteasome inhibitors now allows a rapid analysis of the possible contribution of proteasomal proteolysis in intact cells [43]. The most widely used are the peptide aldehydes, such as MG132. These agents are substrate analogues and potent transition-state inhibitors primarily of the chymotrypsin-like activity of the proteasome [44,45]. However, the peptide aldehydes also inhibit certain lysosomal cysteine protease and the calpains. Therefore, it is important when using these agents for study of proteasome function in cells to also show that similar biological effects occur with other more specific proteasome inhibitors, for example, lactacystin that do not affect these other proteases [43]. Lactacystin is a well characterized and highly specific inhibitor of peptide and protein degradation by the 20S proteasome [46,47]. In this study, we employed both inhibitors to affect the catalytic function of 20S in human cells and obtained similar results towards the inhibition of p53 degradation as well as removal of CPD and 6-4PP. Therefore, the data convincingly demonstrate that the catalytic function of 20S subunit is required for the efficient NER.

#### Ubiquitination is Involved in the Optimal GGR and TCR in Mammalian Cells

The first report of a possible relationship between DNA repair and ubiquitination process was made in 1987. Rad6 protein, required for postreplication DNA repair, was shown to possess a ubiquitin conjugating (E2) activity [48]. Ever since ubiquitin has often been implicated in DNA repair through mechanisms that, interestingly, often seem unrelated to protein degradation. Watkins et al. [15] reported that, besides Rad6, the product of *Saccharomyces cerevisiae Rad23* gene, which is involved in NER of UV-damaged DNA, contains a Ub-like domain at its amino terminus. Deletion of the Ub-like domain of Rad23 impairs its DNA repair function. Furthermore, this domain can be functionally substituted by the authentic ubiquitin sequence. These earlier studies indicated a link between the independent processes of ubiquitination and NER. In addition, the studies reporting high UV sensitivity of *S. cerevisiae*

upon replacement of ubiquitin gene with an UbK63R mutant (substituting lysine 63 residue of ubiquitin), defined a new role of ubiquitin in DNA repair [49]. Demonstration of increased UV sensitivity of E1-inactive temperature-sensitive cells upon growth at nonpermissive temperatures, for the first time, suggested the potential involvement of ubiquitination in NER of mammalian cells [16]. Studies described here, provide further evidence that ubiquitination system is involved in both GGR and TCR pathways in mammalian cell.

Despite considerable evidence supporting the participation of ubiquitination system in enhancing NER, the mechanism remains unclear. In so far as the process of NER is concerned, three possible mechanisms can be envisioned for ubiquitination to modulate NER. First, ubiquitination could physically modify the individual NER factors and cause their activation through a proteolysis-independent function. Although ubiquitin-modified forms of six essential NER factors have not so far been identified upon UV irradiation of mammalian cells, this possibility remains open for consideration. Second, ubiquitination might change the NER response through an effect on chromatin remodeling, for example, a role for histone ubiquitination in DNA repair has been suggested in numerous reports [50]. Ubiquitinated H2A and H2B are the most abundant ubiquitin conjugates in higher eukaryotes and disruption of histone ubiquitination is known to lead to defective DNA repair [51]. The proposed roles for histone ubiquitination, however, are not only controversial [50] but complex, extending from chromatin uncoiling and gene transcription to gene silencing [52]. Third, ubiquitination could affect NER via proteolysis function of the ubiquitin-proteasome system, for example, by removing the stalled and dysfunctional repair enzyme complexes from a damaged genome.

#### Ubiquitin-Proteasome Pathway is Required in NER Through its Proteasome Proteolytic Function in Mammalian Cells

In this study, we found that either ubiquitination or 26S proteasome including 19S regulatory complex and the catalytic function of 20S proteasomal core complex is indispensable for optimal NER. Therefore, the ubiquitin-proteasome pathway is involved in optimal NER at least through its proteolytic function. It has been reported that UV radiation can induce ubiquitination and proteasomal degradation of the large subunit of mammalian RNA polymerase II (RNA Pol II), which is stalled on UV-induced DNA damage and serves as a signal for assembling damage recognition factors in TCR [53,54]. The ubiquitination and degradation of stalled RNA Pol II could alter its attachment to the DNA template so that repair factors can gain easier access to lesions in genome. In support of the similar role of

ubiquitin-dependent degradation pathway in NER, a recent publication has shown that UV irradiation triggers ubiquitin-dependent degradation of p21 (WAF1), and such degradation is essential for optimal DNA repair [55]. Moreover, Rad4 has been shown to be ubiquitinated and Rad23 controls this process in *S. cerevisiae*. The interaction of Rad23 with Rad4 is not only to control degradation of Rad4, but also to assist in assembling the NER incision complex at UV-induced CPD [20]. A recent report also showed that degradation of ectopically expressed human XPC-GFP in mouse cells occurs via ubiquitin proteasome dependent proteolysis [56]. So, following the damage recognition and even the completion of DNA repair, specific repair proteins would be consumed through proteasomal degradation to allow regulated assembly/disassembly of NER factors [20]. Another NER factor, damaged DNA-binding protein subunit 2 (DDB2), is shown to degrade in response to UV-irradiation by the proteasome [57]. Moreover, DDB2 and CSA, which are involved in GGR and TCR, respectively, are each integrated into nearly identical complexes via interaction with DDB1. Both complexes contain cullin 4A and Roc1, which display ubiquitin ligase activity. They also contain the COP9 signalosome (CSN), a known regulator of cullin-based ubiquitin ligase [58]. So, the degradation of DDB2 might be occurring through ubiquitin-proteasome pathway. DDB2 is considered an important repair factor in GGR of CPD and the binding of DDB2 with CPD is essential for efficient recruitment of XPC [59]. During the repair of CPD, DDB2 is thought to be the first factor to bind CPD. XPC/hHR23B is postulated to recognize DDB2 bound to DNA and presumably replaces it at the lesion site [60]. Accordingly, the degradation of DDB2 at the damage site will be a prerequisite to make space for XPC binding. Our finding that the recruitment of XPC to damage site is compromised by inhibition of proteasome provides new evidence that the ubiquitin-proteasome pathway is involved in the access of repair factor to damage sites. Although Bulteau et al. [61] have shown that UV-A and UV-B irradiation had an inhibitory effect on proteasome function, as assessed by the degradation of specific fluorogenic substrates, through the generation of endogenous inhibitors (e.g., 4-hydroxy-2-nonenal modified proteins), this inhibitory effect is not as completed as it is with drugs that target proteasome enzymatic activity (e.g., MG132). Moreover, given UV-C irradiation also decreases the proteasome peptidase activity, this inhibition is not sufficient to influence the normal process of NER. For example, following UV irradiation, DDB2 and p21 have been found to be degraded by proteasome [55,57].

Limited studies on the role of 26S proteasome in NER, primarily in yeast system, are not in complete agreement. Data of Russell et al. [19] suggest that inhibition of proteasomal ATPase diminishes NER

activity in vitro and increases UV sensitivity in vivo. Nevertheless, blockage of protein degradation by the proteasome was shown to have no effect on the efficiency of NER. They concluded that ATPase of the 19S regulatory complex is required for full activity of the NER machinery but by a mechanism other than involving protein degradation by the 26S proteasome. Gillette et al. [18] demonstrated in vivo that 19S can negatively regulate the rate of NER in yeast by point mutations in the conserved ATPase domain of several subunits of the 19S regulatory complex, and this kind of negative regulation is independent of proteolysis. In contrast, Lommel et al. [21] showed that the conditional mutations in the 19S regulatory subunit of the 26S proteasome result in increased NER in vivo in yeast. Repair of both TS and NTS of an RNA Pol II-transcribed gene was increased in the absence of proteasome function, suggesting that proteolysis played a negative role in NER. Our studies demonstrated that in mammalian cells, both 19S and 20S subunits of 26S proteasome positively regulate the efficiency of NER, and the inhibition of 20S catalytic activity decreased the recruitment of NER factors to damage sites. Some discordance in results could possibly be due to the use of different systems. Although NER is conserved from yeast to man, various DNA repair components of UV responses and the regulation of NER factors in yeast and man are known to be different [62].

In summary, we find that ubiquitin as well as 19S or 20S subunits of proteasome positively regulate NER in mammalian cells. Based on our data, we speculate that one of the plausible mechanism of ubiquitin-proteasome pathway mediated NER regulation is through proteolytic function. Moreover, our finding of proteasome inhibitors decreasing the recruitment of NER factors, suggest that the ubiquitin-proteasome pathway could facilitate the access of NER factors to UV-lesions buried in chromatin. Lastly, the regulatory influence exerted by the ubiquitin-proteasome pathway on DNA repair, while a conserved feature among eukaryotic cells from yeast to human, must be occurring through different mechanisms.

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