

Ubiquitylation-independent degradation of Xeroderma pigmentosum group C protein is required for efficient nucleotide excision repair

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ABSTRACT

The Xeroderma Pigmentosum group C (XPC) protein is indispensable to global genomic repair (GGR), a subpathway of nucleotide excision repair (NER), and plays an important role in the initial damage recognition. XPC can be modified by both ubiquitin and SUMO in response to UV irradiation of cells. Here, we show that XPC undergoes degradation upon UV irradiation, and this is independent of protein ubiquitylation. The subunits of DDB-Cul4A E3 ligase differentially regulate UV-induced XPC degradation, e.g DDB2 is required and promotes, whereas DDB1 and Cul4A protect the protein degradation. Mutation of XPC K655 to alanine abolishes both UV-induced XPC modification and degradation. XPC degradation is necessary for recruiting XPG and efficient NER. The overall results provide crucial insights regarding the fate and role of XPC protein in the initiation of excision repair.

INTRODUCTION

Living cells could, at any moment, suffer DNA damage. If damage is left unrepaired, consequent genomic instability can compromise cell survival. Nucleotide excision repair (NER) is a versatile repair pathway that can eliminate a wide variety of lesions, e.g. UV-induced photolesions including cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP), from the genome of UV exposed cells (1). NER includes two distinct subpathways, global genomic repair (GGR) which removes lesions from the entire genome, whereas transcription coupled repair (TCR) eliminates the DNA damage in the transcribed strand of actively transcribing genes (2). An autosomal recessive disorder, Xeroderma Pigmentosum (XP) exhibits

impaired NER activity. XP patients are classified into seven groups (XP-A to -G), and the defects of corresponding seven genes (*XPA* to *XPG*) are responsible for the missing NER activity in these XP patients. It is becoming increasingly clear and accepted that NER in mammalian cells is mediated by the sequential assembly of repair proteins at the site of the DNA lesion, rather than by the action of a pre-assembled repairosome (3–5). XPC–hHR23B complex is most likely the initial damage recognition factor when the lesions are situated in the transcriptionally inactive genome or non-transcribed strand of actively transcribed genes (5,6), whereas XPA–RPA serves an equally critical function in verifying the presence of the DNA lesion (7). In addition, due to its high affinity for UV-damaged DNA, the damaged DNA binding protein (DDB) complex has also been implicated in the damage recognition step of GGR. DDB is a heterodimer of DDB1 and DDB2 components. Studies on the role of DDB2 in NER have raised some concerns (8). Nevertheless, accumulating evidence has confirmed that DDB2 is undeniably involved in GGR. For example, several studies have shown that the cells from some XP-E patients or DDB2-deficient Chinese hamster V79 cells have a partial deficiency in NER (9–11). Microinjection of the purified DDB complex into XP-E cells reversed the NER defect (12–14). Since NER can be reconstituted with purified components and damaged DNA in the absence of DDB (15,16), DDB is believed to be relevant only to the NER within the chromatin context. Our previous studies as well as work of other laboratories have clearly shown that DDB2 is a key factor in regulating GGR of CPD, most likely through the recruitment of XPC to the DNA damage sites (17–19).

XPC is a 940-amino acid protein, and harbors domains that can bind to damaged DNA and repair factors, e.g. hHR23B, XPB and Centrin 2 (20–22). XPC always exists in a bound form with hHR23B and Centrin 2 in cells. This protein complex actively participates in the process of NER (21,23,24). Although hHR23B contains

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two ubiquitin-associated domains and one ubiquitin-like domain, it can stabilize XPC and enhance the binding between XPC and damaged DNA (25). XPC protein can be modified upon UV irradiation, the modifications include ubiquitylation and sumoylation (26,27). Interestingly, the ubiquitylation of XPC does not lead to its degradation, but increases the binding of XPC to damaged DNA (26). While the role of sumoylated XPC is still unclear, it was speculated to protect XPC from degradation (27). DDB2 is required for the UV-induced XPC modifications. Among the modifications, the UV-induced XPC ubiquitylation is regulated by DDB-Cul4A E3 ubiquitin ligase complex comprised of DDB1, DDB2, Cul4A, Roc1 and COP9 signalosome (28). DDB-Cul4A complex can ubiquitylate both DDB2 and XPC, but the fates of ubiquitylated DDB2 and XPC appear to be quite different, ubiquitylated DDB2, but not XPC, is subjected to proteasomal degradation (26).

XPC expression can be induced following UV irradiation through transcriptional activation (29). Furthermore, overexpressed exogenous XPC has been found to be intrinsically unstable and is degraded by the proteasome (30). Nevertheless, the association with hHR23B protein partly stabilizes these XPC *in vivo*. Similarly, tagged Rad4, the homolog of XPC in yeast, is found to be actively degraded by the 26S proteasome, and the turnover is protected by Rad23 protein (31,32). However, the endogenous mouse XPC protein is shown to be stable, with a half life of over 6 h (33), and the endogenous Rad4 in yeast is also stable in the absence of UV light, but is degraded following UV irradiation (34). Our previous study suggested that human XPC undergo degradation following UV irradiation (27), and this degradation precedes the XPC induction observed later in the process. In this report, we have demonstrated that XPC is indeed degraded by 26S proteasome upon UV irradiation, and this degradation is independent of ubiquitylation. Furthermore, we provide evidence showing that the subunits of DDB-Cul4A complex differentially affect the UV-induced XPC degradation. Additionally, we have found that K655 residue of XPC protein is intimately involved in the UV-induced modifications as well as degradation of XPC. Elimination of UV-induced XPC degradation impairs the efficient NER of CPD through an effect on the recruitment of XPG to damaged DNA sites.

MATERIALS AND METHODS

Cell culture and UV irradiation

Normal human fibroblasts OSU-2 cells, established and maintained in culture as described earlier (35), Li-Fraumeni Syndrome fibroblast strain designated 041 cells (kindly provided by Dr Michael Tainsky, MD Anderson Cancer center, Houston, TX, USA), XP-C (GM15983), HeLa cells with over-expressed FLAG and HA-tagged DDB2 (HeLa-DDB2 cells) (a gift of Dr Yoshihiro Nakatani, Dana-Farber Cancer Institute, Boston, MA, USA) were grown in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics. XP-A

(GM04312) and XPA complemented cells (XP-A + XPA, GM15876), XP-F (GM08437) and XPF complemented cells (XP-F + XPF, kindly provided by Dr Gan Wang, Wayne State University, Detroit, MI, USA), XP-G (XP3BR-SV) and XPG complemented cells (XP-G + XPG, kindly provided by Dr Karlene Cimprich, Stanford University, Stanford, CA, USA) and XP-E (GM01389) cells were grown in MEM supplemented with 10% FCS and antibiotics. Mouse embryo fibroblast ts20 (thermosensitive for E1 ubiquitin-activating enzyme) and its parental cell line A31N (Kindly provided by Dr Harvey L. Ozer, UMDNJ-New Jersey Medical School) were cultured in 50% F-10 + 50% DMEM medium containing 10% FCS and antibiotics. HeLa cells with over-expressed FLAG-HA-DDB2 and V5-His-XPC (HeLa-DDB2-XPC cells) were generated in our lab and cultured in DMEM containing 500 µg/ml G418 (36). All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ except A31N and ts20 cells, which are maintained at 32°C. For overall UV exposure, the cells were washed with PBS, irradiated with varying UV doses and incubated in suitable medium for the desired time period. The irradiation was performed with a germicidal lamp at a dose rate of 0.8 J/m²/s as measured by a Kettering model 65 radiometer (Cole Palmer Instrument Co., Vernon Hill, IL, USA).

Site-directed mutagenesis, plasmid construction and transfection

XPC-V5-His and DDB1-V5-His plasmids were generated in our lab. pXPC3 plasmid containing XPC with N-terminal 1–117 amino acids deletion (Δ 1–117) was kindly provided by Dr Randy Legerski (The University of Texas MD Anderson Cancer Center, Houston, TX, USA). Cul4A-c-Myc plasmid was kindly provided by Dr Yue Xiong (University of North Carolina, Chapel Hill, NC, USA). DDB2-FLAG plasmid (kindly provided by Dr Gilbert Chu, Stanford University, Stanford, CA, USA) was used to generate point mutants R273H and K244E, and XPC-V5-His plasmid was used to generate point mutants K655A and K917A by QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The plasmids were transfected into cells either by FuGene 6 (Roche, Indianapolis, IN, USA) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. To generate stably transfected cell lines, G418 (500 µg/ml) was added to the medium for selection and resistant colonies confirmed by western blotting.

Western blot analysis

The cells were trypsinized and washed once with PBS. The cell pellets were lysed by boiling for 10 min in a sample buffer (2% SDS, 10% glycerol, 10 mM DTT, 62 mM Tris-HCl pH 6.8, protease inhibitor cocktail). Protein samples were loaded on 8–16% Tris-Glycine gels (Invitrogen) and separated by PAGE. The proteins were then transferred to nitrocellulose membrane, blocked by 5% milk and immunoprobed. The antibodies used were, rabbit anti-XPC and rabbit anti-DDB2

(generated in our lab) (27), rabbit anti-Cul4A and rabbit anti-DDB1 (a gift from Dr Yue Xiong), goat anti-Lamin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-hHR23B (BD Bioscience, San Jose, CA, USA), rabbit anti-V5 (Bethyl Laboratories, Montgomery, TX, USA) and mouse anti-c-Myc (Invitrogen).

Local UV irradiation and immunofluorescence

XP-C cells growing on glass coverslips were transfected with XPC-V5-His plasmid for 48 h. The cells were then washed with PBS and UV irradiated through an isopore polycarbonate filter (Millipore, Bedford, MA, USA), containing pores of a 5 μm in diameter, as described previously (37). The cells were then double stained with rabbit anti-XPC and mouse anti-CPD (TDM-2, MBL International, Woburn, MA, USA), or rabbit anti-XPC and mouse anti-XPA (Lab Vision, Fremont, CA, USA), or rabbit anti-XPC and mouse anti-XPG (Lab Vision) or mouse anti-V5 (to visualize XPC, Invitrogen) and rabbit anti-XPB (Santa Cruz). Fluorescence images were obtained with a Nikon Fluorescence Microscope E80i (Nikon, Tokyo, Japan) fitted with appropriate filters for FITC and Texas Red. The digital images were then captured with a cooled CCD camera and processed with the help of its SPOT software (Diagnostic Instruments, Sterling Heights, MI, USA).

GST pull down assay

GST and the fusion protein GST-hSug1 were expressed in *Escherichia coli* strain DH5 α transformed with either pGEX4T-1 or pGEX-hSug1 (kindly provided by Dr Andrew Paterson, The University of Alabama at Birmingham, Birmingham, AL, USA). After purification, GST and GST-hSug1 were separately incubated with glutathione Sepharose 4B beads (Amersham Bioscience, Uppsala, Sweden) at 4°C for 2 h in PBS. The nuclear extract from OSU-2 cells were prepared by incubating OSU-2 cells in nuclear extract (NE) buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, protease inhibitor cocktail) for 20 min and NE was collected by centrifugation. GST or GST-hSug1 bound beads were incubated with either NE, or purified recombinant XPC (a gift of Dr Yue Zou, East Tennessee State University, Johnson City, TN, USA) at 4°C for 2 h in NE buffer. After washing five times with NE buffer, the beads were boiled in 2 \times SDS loading buffer for 5 min and the supernatant was subjected to western blot analysis.

siRNA transfection

Cul4A and DDB1 siRNA oligonucleotides were synthesized by Dharmacon (Lafayette, CO, USA) in a purified and annealed duplex form. The sequences targeting Cul4A and DDB1 were 5'-GAACAGCGAUCGUAACAAUU-3' and 5'-UAACAUGAGAACUCUUGUC-3', respectively. Specific and control siRNA transfections were performed with Lipofectamine 2000 (Invitrogen) according to manufacture's instruction.

Immuno-slot blot analysis

The amount of CPD in DNA was quantified with non-competitive immuno-slot blot assay. Briefly, XP-C cells in 100 mm plates were transiently co-transfected with DDB2 and either empty vector, wild type or K655A XPC mutant. Twenty-four hours post-transfection, cells were split into 60 mm plates and grown for an additional 24 h. After UV exposure (10 J/m²) and desired incubation periods, cells were recovered by trypsinization and immediately lysed for DNA isolation. The identical amounts of DNA samples were loaded on nitrocellulose membranes and the amount of CPD was detected with monoclonal anti-CPD antibody (TDM-2). 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 relative repair rates.

RESULTS

XPC is degraded following UV irradiation

Our previous studies have indicated that the level of XPC in cells decreases upon UV irradiation (27). To further confirm this phenomenon, we compared the decay rates of XPC in UV- or mock-irradiated normal human fibroblast, OSU-2 cells pre-treated with cycloheximide (CHX), an inhibitor of *de novo* protein synthesis. Figure 1A shows that in the absence of new XPC synthesis, XPC protein exhibits a high decay rate following UV irradiation, and the distinct pattern of XPC degradation could be observed as early as 30 min after UV treatment (lanes 5–8). To rule out the possibility that the decreased XPC level at 125 kDa is due to the conversion of XPC to slower migrating modified forms, we over exposed the film to show the corresponding levels of various XPC bands. As shown in Figure 1A, the modification of XPC is not fully obvious at 2 h time point and yet the level of XPC at 125 kDa is lower than that seen at 1 h time point. We then scanned all bands of XPC and quantified the total XPC amount and normalized by Lamin B level. As shown in Figure 1A, total XPC amount did decrease with the elongation of incubation time following UV irradiation. This result indicates that the decrease of XPC observed after UV irradiation is most likely due to the protein degradation. Moreover, we treated the cells with proteasome inhibitor MG132 prior to UV irradiation to test the involvement of 26S proteasome in this UV-induced XPC degradation. We found that the UV-induced decrease of XPC levels is promptly inhibited in the presence of MG132 (Figure 1B, lanes 1 and 2 versus 3 and 4). A similar result regarding the effect of MG132 on the immediate fate of XPC was also seen with repair-deficient XP-A cells (Supplementary Figure 1). These combined data indicate that UV irradiation causes XPC protein degradation *via* proteasome-mediated proteolysis. Moreover, Figure 1B showed that when protein degradation is inhibited by treatment with MG132, more XPC was detected in UV-treated cells than that in mock-treated cells (lane 3 versus 4). However, when we further inhibited

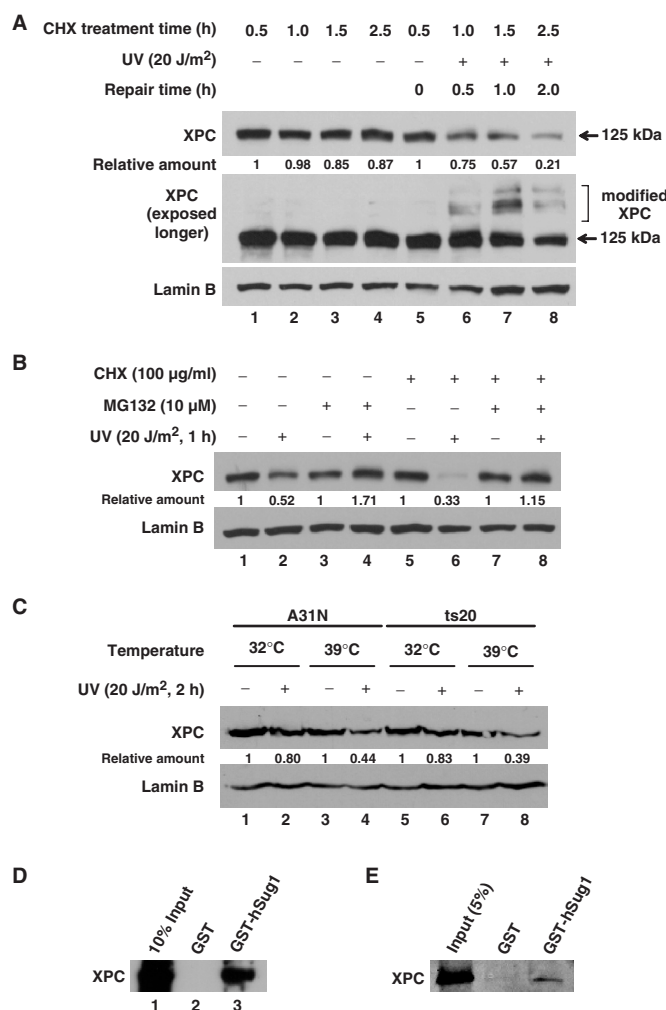


Figure 1. XPC is degraded independent of ubiquitylation upon UV irradiation. (A) OSU-2 cells were treated with 100 µg/ml of CHX 0.5 h prior to UV irradiation at 20 J/m² or mock treatment. Cells were further incubated in the medium containing CHX for different time periods. The whole cell lysates were subjected to western blot analysis using anti-XPC antibody. The same membrane was also immunoblotted for Lamin B as a loading control. (B) OSU-2 cells were treated with either MG132 (10 µM) or CHX (100 µg/ml) or both for 1 h prior to UV irradiation at 20 J/m² or mock treatment and the cells were incubated in the same medium for another 1 h. The cell lysates were subjected to immunoblotting as described above. (C) A31N and ts20 cells were cultured for 16 h at 32°C or 39°C, UV irradiated at 20 J/m², and cultured for another 1 h at the same temperatures. The whole cell lysates were subjected to immunoblotting and the level of XPC was detected with anti-XPC antibody. (D and E) The cell lysates from OSU-2 cells (D) or purified recombinant XPC protein (E) were incubated with recombinant GST or GST-hSug1 proteins bound to GST beads. The protein bound to the beads was subjected to immunoblotting using anti-XPC antibody. Relative amount of total XPC at various times post-UV or mock-treatment were quantified relative to the respective unirradiated levels and normalized by Lamin B controls.

protein synthesis by treatment with CHX, this UV-induced XPC increase did not occur (lane 7 versus 8). These results indicate that both degradation and induction of XPC protein occurs in tandem within UV irradiated cells.

UV-induced XPC degradation is independent of ubiquitylation

Previous studies have implied that UV-induced ubiquitylation of XPC is reversible and does not serve as a signal for degradation (26). Therefore, we reasoned that UV-induced XPC degradation observed in our experiments might be independent of ubiquitylation. To test this hypothesis, we analyzed the UV-induced changes of *in vivo* XPC levels in mammalian cells capable of conditional inactivation of E1 enzyme. Ts20 cells growing at permissive 32°C, thus harboring normal E1 activity, exhibit a small extent of XPC degradation upon irradiation (Figure 1C, lane 5 versus 6). However, when E1 is inactivated by transferring cultures to non-permissive 39°C (38,39), these cells showed considerable XPC degradation following irradiation (lane 7 versus 8). Exactly the same XPC degradation response is seen in parent control A31N cells at both permissive and non-permissive temperatures (Figure 1C, lanes 1–4). In essence, the ubiquitylation defect failed to impinge on the protein degradation. These *in vivo* data clearly indicate that UV-induced XPC degradation is independent of ubiquitylation and suggest a direct interaction of XPC with proteasome. To substantiate this idea of direct ubiquitylation-independent interaction, the GST pull down assay was conducted with the whole cell lysates prepared from OSU-2 fibroblasts. We found that recombinant hSug1, a subunit of 19S proteasome, physically binds to XPC protein (Figure 1D). The interaction was further tested with purified recombinant XPC and hSug1, and the result shows that XPC protein can bind to hSug1 directly (Figure 1E). Taken together, we believe that UV-induced XPC degradation is independent of ubiquitylation and that XPC can bind to 26S proteasome through direct interaction with hSug1.

NER process does not affect UV-induced XPC degradation

To explore the relationship between XPC degradation and the NER process, we examined the kinetics of UV-induced XPC degradation in normal human fibroblast as well as human cell lines belonging to different XP complementation groups and the corresponding cell lines corrected for the cognate repair deficiency. As shown in Figure 2A, normal human fibroblast, OSU-2 cells, showed a significant decrease in XPC levels at 1 h, followed by an increase until it again reached the control levels at ~8 h after UV irradiation. Meanwhile, all three XP-A, XP-F and XP-G cell lines exhibit the typical XPC degradation upon UV irradiation (Figure 2B–D), indicating that UV-induced XPC degradation is not affected by the absence of any of these essential repair factors and is independent of the productive cellular excision repair process. In addition, XP-A and XP-F cells exhibit a similar XPC dynamics as that of repair-proficient OSU-2 cells, characterized by a prompt decrease at 1 and 2 h followed by restoration beginning at 4 h following UV irradiation (Figure 2B and D, lanes 1–5). On the contrary, XP-G cells demonstrated continued XPC degradation without any detectable recovery of XPC at later intervals (Figure 2C, lanes 1–5). Nevertheless, ectopic expression of XPG

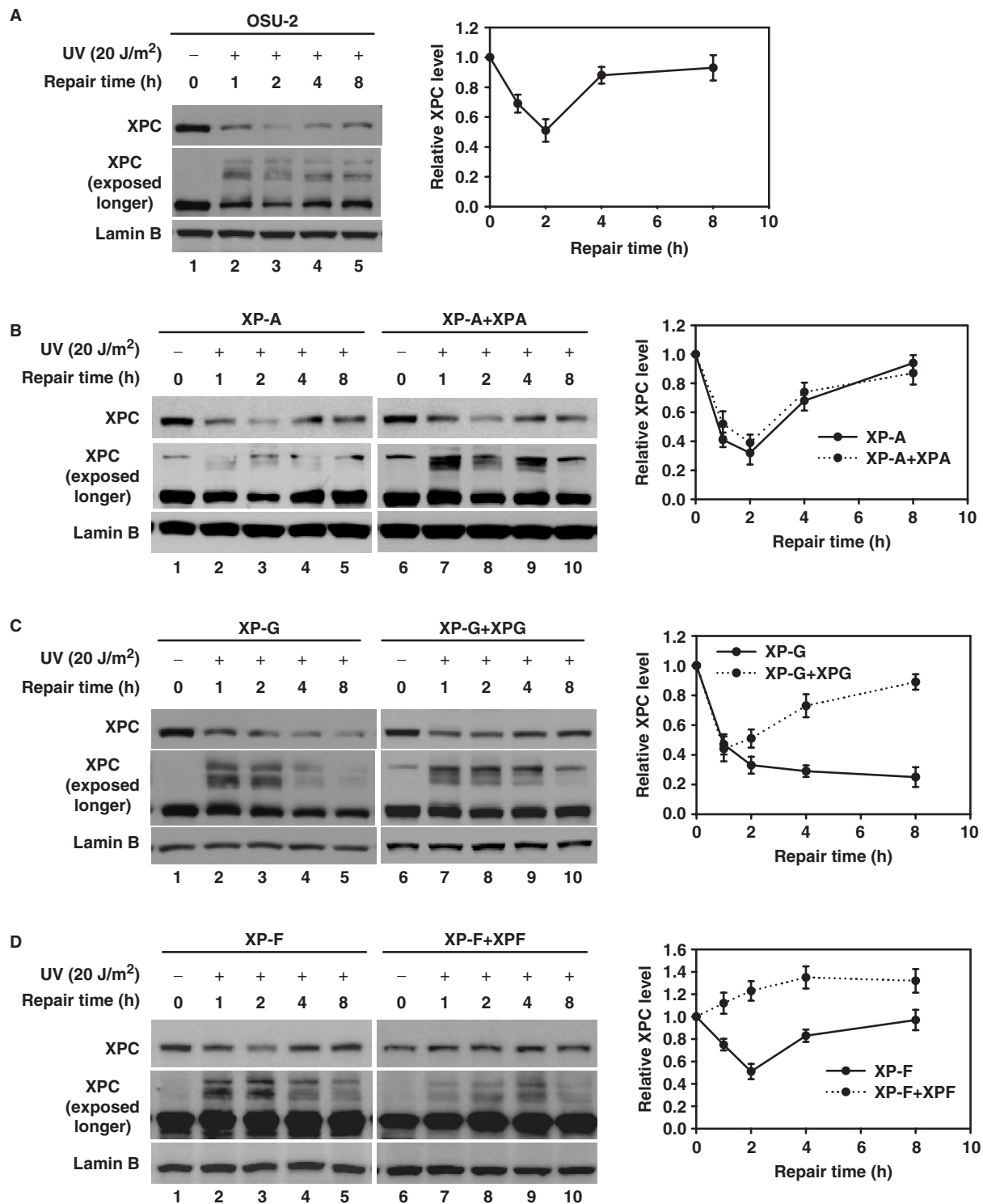


Figure 2. The dynamic of UV-induced XPC degradation. NER-proficient OSU-2 cells (A), various NER-deficient XP cells, like XP-A (B), XP-F (C), XP-G (D) as well as their corresponding repair factor-complemented cell lines were UV irradiated at 20 J/m² and then incubated for the indicated times. Whole cell lysates were subjected to immunoblotting as described in Figure 1A. The levels of total XPC in each lane were quantified and normalized by the initial amount of XPC and Lamin B and plotted on the right.

in XP-G cells was able to restore the normal XPC dynamics (Figure 2C, lanes 6–10), indicating that XPG is required for the recovery of XPC protein following repair of UV damage in fully repair-competent cells. Interestingly,

the ectopic expression of XPF in XP-F cells prevented the expected XPC decrease observed upon UV treatment (Figure 2D, lanes 6–10). Since the UV-induced XPC degradation is easily seen in CHX-treated XPF-corrected

repair-proficient XP-F cells (Supplementary Figure 2), we conclude that the XPF protein does not interfere with the XPC degradation. Nonetheless, XPF protein could additionally be stimulating the new synthesis of XPC which is also inducible upon UV irradiation.

DDB2 is essential for promoting UV-induced XPC degradation

The requirement of DDB2 protein for the UV-induced XPC modifications (ubiquitylation and sumoylation) has previously been reported by our laboratory and others (26,27). Here, we extend this work by investigating the role of DDB2 in UV-induced XPC degradation. We approached this question by first following the post-irradiation fate of XPC protein in experiments with DDB2-deficient XP-E cells. The results clearly show that UV-induced XPC degradation fails to occur in cells lacking DDB2 (Figure 3A). Since XP-E cells posed difficulty in transfecting cDNA constructs, we used another DDB2-deficient Chinese Hamster V79 cell line to further observe the effect of restoring DDB2 into these cells on UV-induced XPC degradation. As expected, DDB2 expression restored the XPC degradation following UV irradiation (Figure 3B). This DDB2-mediated response was more clearly demonstrable in another cell line, 041, that lacks the DDB2 because of the absence of p53 inducer. As shown in Figure 3C, XPC remains fully intact upon UV irradiation of these cells. However, transient transfection of DDB2 cDNA into these cells restored the normal UV-induced XPC degradation with a distinct dose-response relationship, i.e. greater XPC degradation with higher DDB2 expression. Finally, we tested whether the expression of mutated DDB2 can functionally substitute for the wild-type DDB2. Two XP-E mutations are single amino acid substitutions (K244E and R273H) corresponding to XP-E patients XP82TO and the related individuals XP2RO and XP3RO, respectively (40). Extracts from cells of these lines are defective in the ability to bind UV-irradiated DNA fragments (9). These two naturally occurring mutants of DDB2, R273H and K244E, along with wild-type DDB2, were separately and stably transfected into 041 cells and evaluated for the fate of XPC. As expected, only the wild-type DDB2 promotes UV-induced XPC degradation (Figure 3D), which unambiguously indicates that the damaged DNA binding activity of DDB2 is a strict requirement for it to participate in the XPC degradation.

DDB1 and Cul4A protect XPC from degradation upon UV irradiation

DDB-Cul4A E3 ligase is believed to be functionally essential for the XPC ubiquitylation upon UV irradiation (26). Furthermore, our results indicate that DDB2, as one of the subunits of this same E3 ligase, is also required for UV-induced XPC degradation. However, the role of other subunits of this E3 ligase in this important cellular process is not known. In order to address this question, we utilized a siRNA-based gene silencing strategy to squelch the activity of individual complex components within cells. As shown in Figure 4A and B,

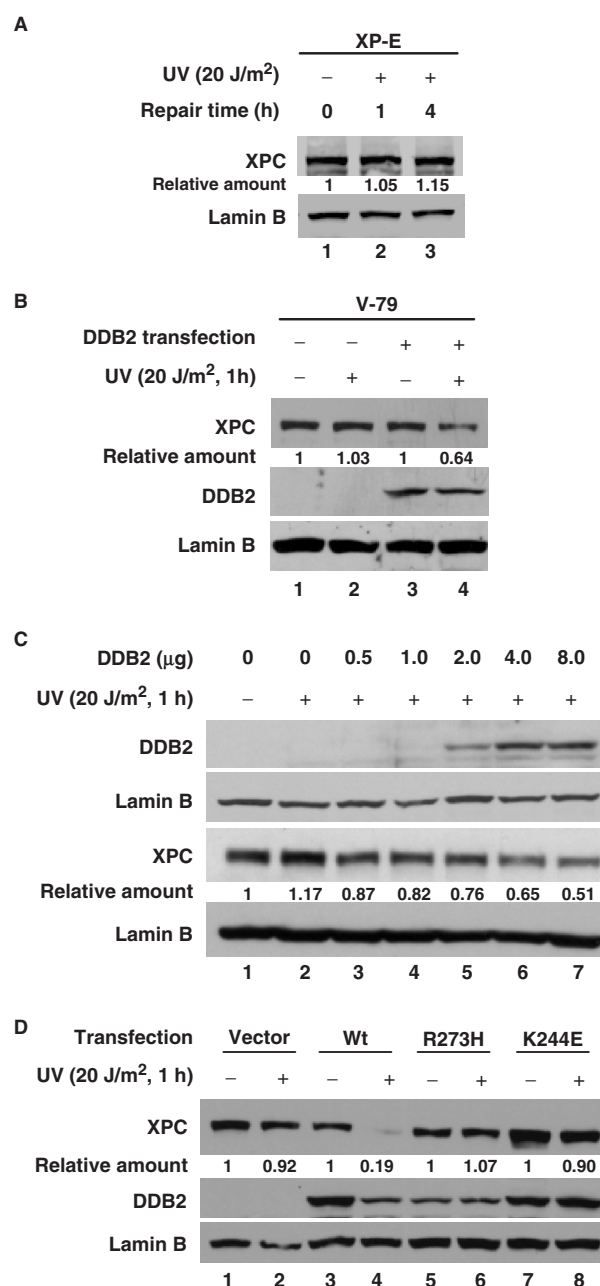
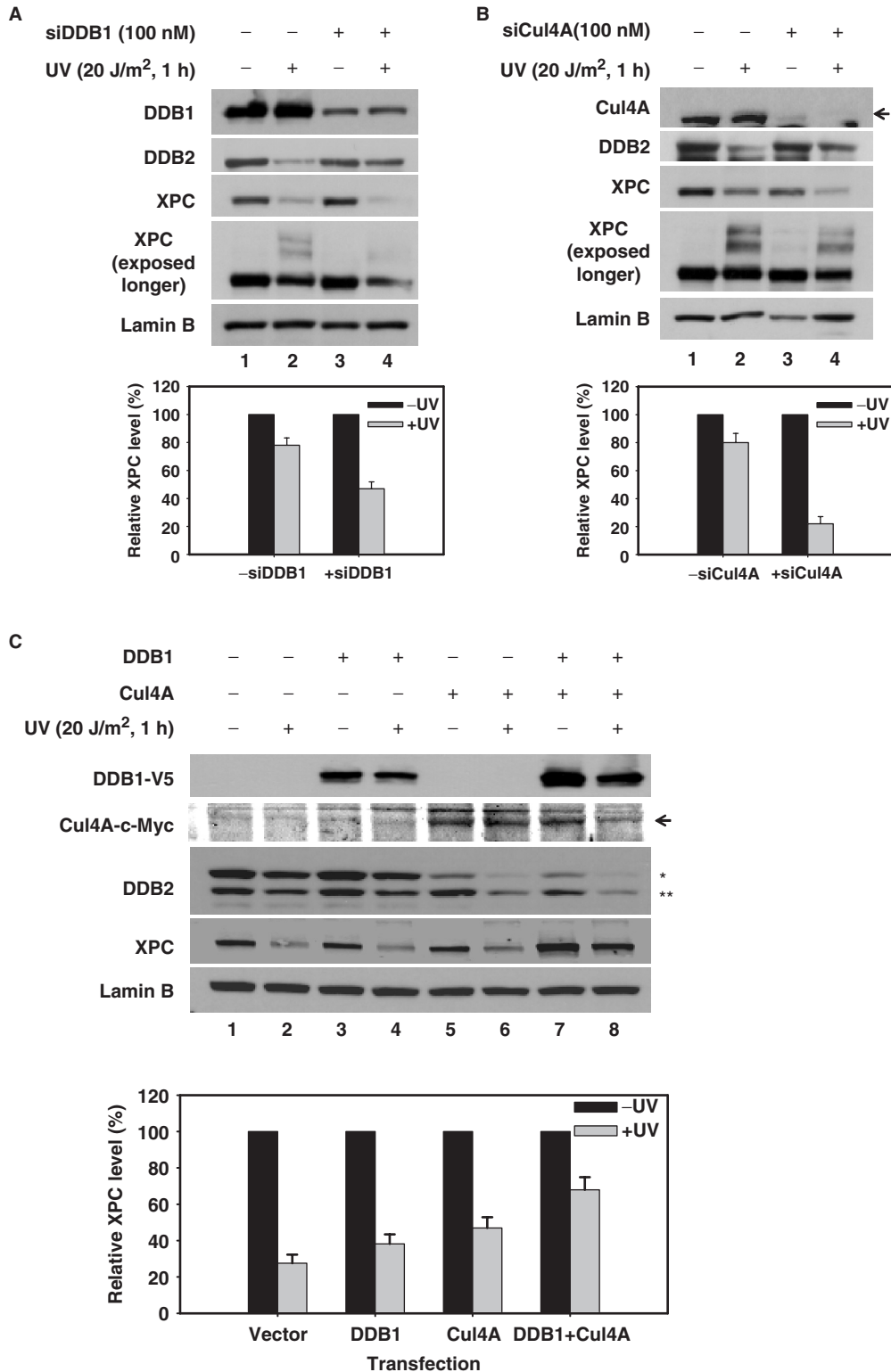


Figure 3. DDB2 is required and promotes UV-induced XPC degradation. (A) XP-E cells were UV irradiated at 20J/m² and incubated for the indicated times. The whole cell lysates were subjected to immunoblotting using anti-XPC antibody. (B) V79 cells were transiently transfected with DDB2-FLAG and UV irradiated at 20J/m². After incubation for another 1h, the whole cell lysates were prepared and subjected to immunoblotting using anti-XPC antibody. (C) 041 cells were transiently transfected with various amounts of DDB2-FLAG. Twelve hours post-transfection, cells were split into two 60 mm plates and grown for another 24h. The cells were mock or UV irradiated at 20J/m² and incubated for another 1h. The whole cell lysates from mock-irradiated cells were subjected to immunoblotting using anti-DDB2 antibody, and those from UV-treated cells were subjected to immunoblotting using anti-XPC antibody. (D) 041 cells stably expressing wild-type or mutant (R273H and K244E) DDB2-FLAG were UV irradiated at 20J/m² and incubated for another 1h. The whole cell lysates were subjected to immunoblotting using anti-DDB2 and anti-XPC antibodies. Relative amount of total XPC at various times post-UV were quantified relative to the respective unirradiated levels and normalized by Lamin B.



knocking down the expression of DDB1 or Cul4A in normal human fibroblasts caused an expected inhibition of the UV-induced DDB2 degradation. However, the absence of DDB1 or Cul4A clearly enhanced the XPC degradation. Interestingly, there was a simultaneous reduction in the UV-induced XPC modifications. These results indicate that DDB1 and Cul4A are required for the stabilization of XPC through an influence on its protein modifications. To further confirm this finding, we tested if over-expression of DDB1 and Cul4A can protect XPC from degradation in another cell line, i.e. HeLa-DDB2 cells. It is worthy to note that HeLa cells have both DDB1 and DDB2 (41) and UV-induced XPC modification and degradation in HeLa cells are similar to those of OSU-2 cells [(27) and unpublished data]. We over-expressed either V5-tagged DDB1, or c-Myc-tagged Cul4A or both DDB1 and Cul4A in HeLa-DDB2 cells. All transfections involving the over-expression of Cul4A promoted the degradation of DDB2. Consistent with a previous report (42), this indicates the normal function of ectopically expressed Cul4A. Interestingly, over-expression of either DDB1 or Cul4A or of both DDB1 and Cul4A components in cells dramatically inhibit UV-induced XPC degradation (Figure 4C). Taken together, these data indicate that both DDB1 and Cul4A can protect XPC from being degraded upon UV irradiation and this effect is mainly through allowing the modifications of XPC protein.

Sumoylation and degradation involve the same site in XPC protein

Our previously published studies indicated that UV-induced sumoylation of XPC inhibits its degradation following UV irradiation (27), suggesting that sumoylation site in XPC may be involved in XPC degradation. In order to address this question, we needed to determine and manipulate the potential sumoylation sites in XPC protein. The linkage between SUMO and its target proteins occurs through an isopeptide bond between the C-terminal carboxyl group of SUMO and the ϵ -amino group of a lysine residue in the substrate. The majority of the sumoylation sites follow a consensus motif with ψ -K-X-E (43,44) or ψ -K-X-E/D (45), where ψ is a large hydrophobic amino acid, generally isoleucine, leucine or valine; K is the lysine residue that is modified; X is any residue and D or E is an acidic residue. This motif is bound directly by Ubc9, the sole SUMO-conjugating enzyme. We used SUMOplot (<http://www.abgent.com/doc/sumoplot>) to predict the putative sumoylation sites in XPC protein. SUMOplot provides the probability of the SUMO consensus sequence (SUMO-CS) potentially engaged in SUMO attachment. The SUMOplot analysis revealed six putative sumoylation sites in XPC protein, e.g. K81, K89, K113, K183, K655 and K917 (Figure 5A). In order to assess the valid sumoylation site in XPC, we either mutated the putative lysine to alanine (K655A and K917A), or used an existing 1–117 amino acids deletion XPC construct (pXPC3, Δ 1–117) (46) to experimentally test the possible

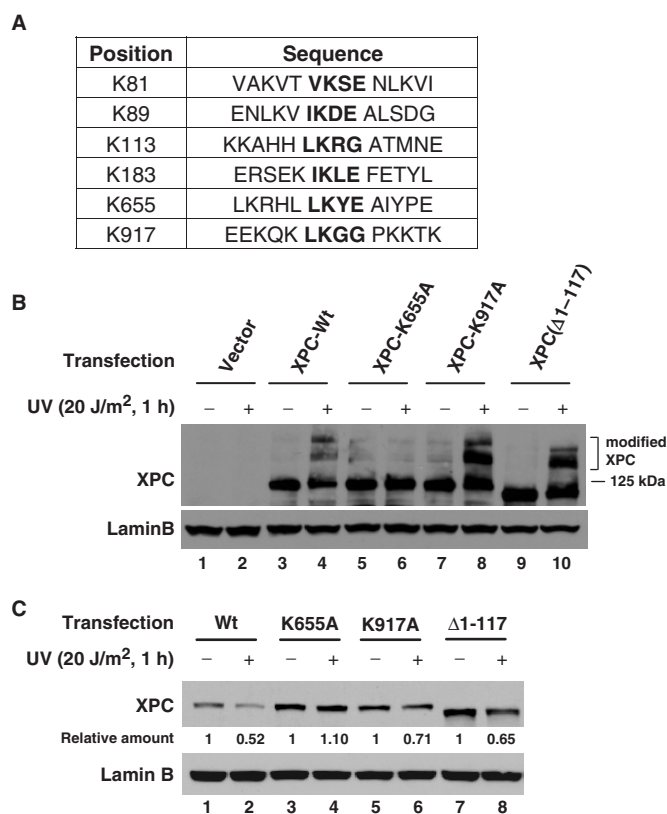


Figure 5. K655 is the critical site for UV-induced XPC modification and degradation. (A) Six putative SUMO sites in XPC protein predicted by SUMOplot are depicted. (B and C) Wild-type XPC and three mutant XPC (K655A, K917A and Δ 1-117) were generated and transiently co-transfected with DDB2-FLAG into XP-C cells. Twenty-four hours post-transfection, the cells were split into two plates and grown for another 24 h, one plate was mock-irradiated and another was UV-irradiated at 20 J/m² and allowed to repair for 1 h. The whole cell lysates were subjected to immunoblotting using anti-XPC antibody. The blots in 'B' were exposed longer to show the modified protein forms. Relative amounts of total XPC following UV irradiation were quantified relative to the respective unirradiated levels and normalized by Lamin B controls.

sumoylation-specific lysine. Since immortalized XP-C (GM15983) cells exhibited reduced DDB2 level, possibly due to disrupted p53 via SV40 large T antigen (data not shown), the XPC constructs were transiently co-transfected with DDB2 into these XP-C cells and the cells were UV irradiated at 20 J/m² followed by 1 h for repair. The modified forms of XPC protein were detected by western blot analysis. As shown in Figure 5B, mutation of K655 to alanine (K655A) produced a XPC that was unable to undergo modifications *in vivo* (lanes 5, 6), whereas mutation of K917 to alanine (K917A) and deletion of 1–117 amino acids had no effect on the protein's modification competence (lanes 7–10). This result indicates that K655 is the site responsible for sumoylation and other modification of XPC protein. Furthermore, we also tested the UV-induced degradation prowess of various XPC forms. Upon UV irradiation, ectopically expressed wild-type XPC could be degraded to the same extent as endogenous XPC, whereas K655A XPC does not undergo any

degradation (Figure 5C, lanes 1 and 2 versus 3 and 4). In contrast, other mutations such as K917A and Δ 1-117 did not affect UV-induced XPC degradation (lanes 5–8), suggesting once again that K655 is also an essential residue for the XPC degradation.

Blocking UV-induced XPC degradation compromises NER via inhibition of XPG recruitment to damage sites

Since K655A mutation abrogates UV-induced XPC degradation, we used this construct to study the function of XPC degradation in NER following UV irradiation. XPC-Wt or XPC-K655A constructs were transiently co-transfected with DDB2 into XP-C cells, and the characteristics of XPC, e.g. its binding to hHR23B and its recruitment to damaged DNA sites were evaluated. The result indicates that K655A mutation does not affect the complex forming ability of XPC and hHR23B (data not shown). In addition, both XPC-Wt and XPC-K655A could be recruited to CPD sites upon UV irradiation (Figure 6A). The recruitment of other NER factors, which are placed into the repair complex subsequent to XPC, was also analyzed. TFIIH (XPB) and XPA exhibit the normal recruitment to the UV-damage sites in both XPC-Wt and XPC-K655A expressing cells (Figure 6B and C). On the other hand, XPG protein, while recruited as normal to the damage sites in XPC-Wt expressing cells, was severely impaired in its damage site recruitment in XPC-K655A transfected cells (Figure 6D). These results indicate that K655A mutation-induced abrogation of XPC degradation hampers the recruitment of XPG to the damage sites. We also evaluated the effect of XPC-K655A mutation on the efficiency of NER. XP-C cells with transiently expressed XPC-Wt or XPC-K655A were UV irradiated at 10J/m² and allowed to repair for a 24h period. The CPD remaining in DNA were quantified and the repair rates compared among different cell types. Figure 6E shows that the expression of XPC-Wt and XPC-K655 is comparable in two transfected cell lines. As expected, CPD were not repaired in XP-C cells transfected with vector alone (Figure 6F). Moreover, the transfection of XPC-K655A was unable to restore the DNA repair ability of XPC cells like that achieved with the XPC-Wt construct. These data suggests that inhibition of XPC degradation by K655A mutation severely affects the function of XPC in NER.

DISCUSSION

Modification, degradation and induction of XPC occur in tandem within irradiated cells

The alterations of XPC levels in cells irradiated with UV have been reported either as no change (26), or as an increase (29,30,33). Our previous work, however, detected a decrease in XPC level immediately upon UV irradiation (27). Similarly, a study in *Saccharomyces cerevisiae* also demonstrated that Rad4 is degraded upon UV irradiation (34). In the present study, we carried out an in-depth mechanistic investigation of the fate of XPC and confirm that the observed decrease of XPC following

UV irradiation is a result of active XPC degradation. UV-induced XPC degradation occurs very early and can be seen for more than 2 h. In the meantime, as reported by other groups, XPC expression is also induced so that the new synthesis of XPC becomes an overwhelming event after 4 h and masks the decrease of XPC level invoked earlier. At this point, the cumulative measurement of the dual opposing effects is reflected as a net increase. Importantly, we show that UV-induced XPC degradation is not triggered by the typical protein ubiquitylation process. The mechanistic studies reveal that 26S proteasome can directly bind XPC to affect its degradation.

Ubiquitylation and sumoylation of XPC following UV irradiation of cells is already established (26,27), albeit the nature of the two independent modifications has not been fully resolved. The function of XPC ubiquitylation, which has also been studied extensively *in vitro*, is not for the purpose of its degradation, but to augment DNA binding of XPC. However, the function of XPC sumoylation has so far remained unclear. Since we have found that inhibition of XPC sumoylation increases UV-induced XPC degradation (27), it can be surmised that at least one function of XPC sumoylation is to protect XPC from being destroyed. Therefore, XPC undergoes degradation and modifications simultaneously following UV irradiation and in essence the degradation of XPC is intimately regulated by modifications, i.e. more modifications resulting in lesser degradation.

With regards irradiation-related XPC protein induction, our data argues that XPG is required for this process because, in the absence of XPG, the level of XPC does not increase following UV irradiation. In addition, the transfection of XPG into XP-G cells restores the XPC increase after 4 h of UV irradiation. Because XP-A and XP-F cells exhibit normal XPC degradation and induction kinetics, we can rule out the possibility that blocking of XPC induction is due to transcription inhibition from un-repaired lesions located in the transcribed strand of the *XPC* gene. Therefore, XPG may be an important factor in DNA damage-induced XPC expression, and it would be enlightening to unravel the role of XPG in XPC production.

DDB–Cul4A components differentially regulate XPC degradation

The DDB–Cul4A complex is a new class of cullin-containing ubiquitin E3 ligases (47). Previous studies have indicated that the DDB–Cul4A E3 ligase regulates the autoubiquitylation and proteolysis of DDB2 in response to DNA damage (42,48). In addition, DDB–Cul4A complex is also required for UV-induced ubiquitylation of XPC, but this modification does not serve as the signal for proteolysis. Nevertheless, our present study demonstrates that the subunits of this E3 complex, DDB2, DDB1 and Cul4A, also regulate UV-induced XPC degradation. These regulatory events, however, serve different functions. DDB2 is required and promotes XPC degradation upon UV irradiation, whereas DDB1 and Cul4A protect XPC from being degraded. DDB2 has been shown to be a critical factor in the

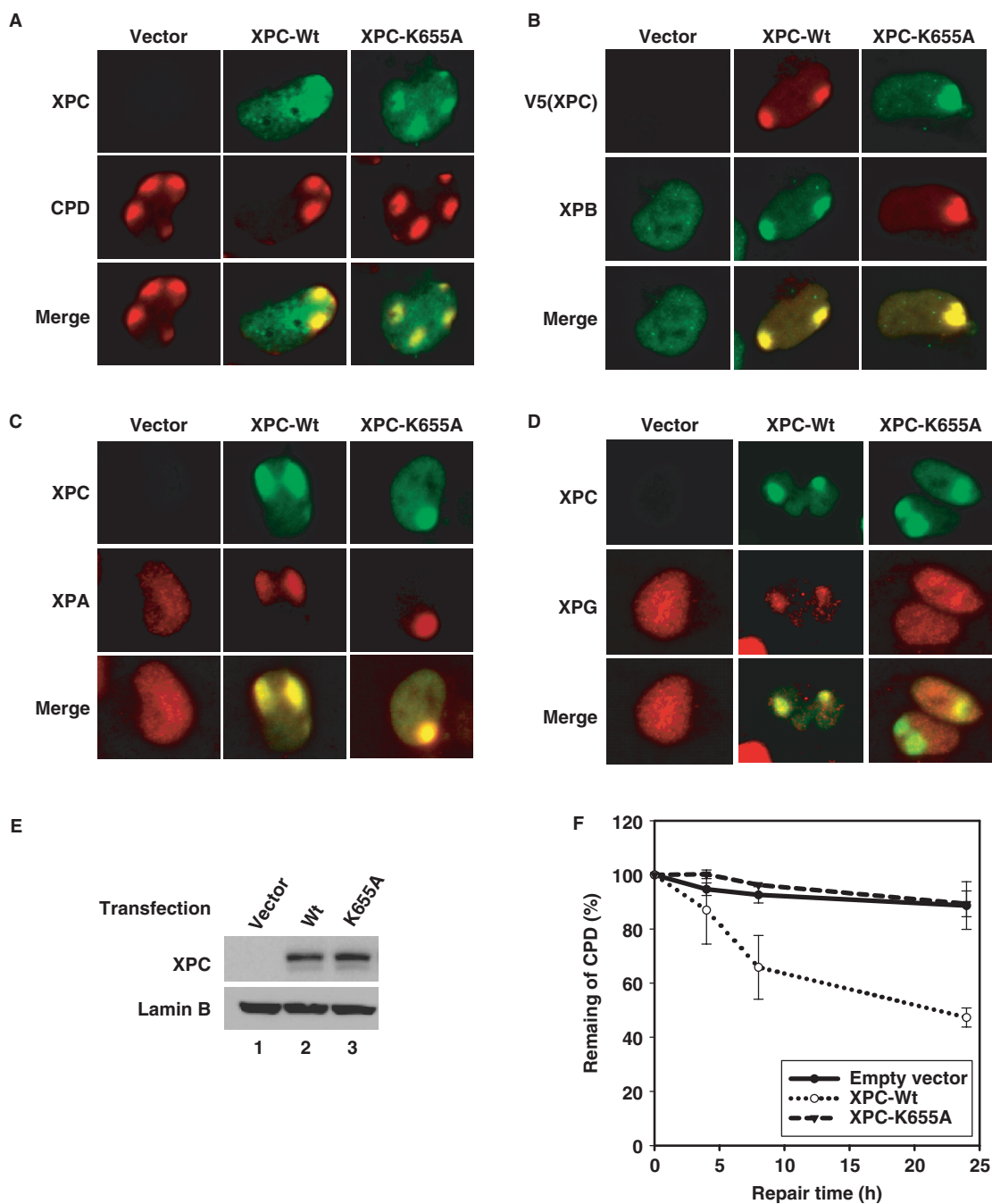


Figure 6. K655A mutation compromises NER of UV-induced CPD through inhibiting XPG recruitment. (A–D) XP-C cells grown on coverslips were co-transfected with DDB2-FLAG and either wild-type or K655A mutant XPC for 48 h, then UV irradiated through a 5 μm micropore filter at 100 J/m². After incubation for another 30 min, the cells were fixed, permeabilized and then subjected to dual immunofluorescent staining with rabbit anti-XPC and mouse anti-CPD antibodies (A), or mouse anti-V5 (for XPC) and rabbit anti-XPB antibodies (B), or rabbit anti-XPC and mouse anti-XPA antibodies (C), or rabbit anti-XPC and mouse anti-XPG antibodies (D). (E and F) XP-C cells were co-transfected with DDB2-FLAG and either wild type or K655A mutant XPC for 24 h, then the cells were split into five 60 mm plates and incubated for another 24 h. Whole cell lysates prepared from one plate of each transfection were subjected to immunoblotting using anti-XPC antibody to confirm the expression of XPC (E). Cells in other plates were UV irradiated at 10 J/m² and allowed to repair for the indicated times. Genomic DNA was isolated and the identical amount of DNA was subjected to immuno-slot blotting using anti-CPD antibody to detect the CPD remaining in each samples (F).

removal of CPD, most likely by allowing the recruitment of XPC to the damage sites (17,18). For instance, in DDB2-deficient XP-E cells, XPC cannot be recruited to the damage sites and consequently XPC cannot be degraded. In addition, only the wild-type DDB2, but not

its mutant forms, has the ability to trigger UV-induced XPC degradation. Since mutated DDB2 cannot bind to UV-damaged DNA, we propose that XPC degradation occurs at the damage sites, and the role of DDB2 in this event is to help promptly recruit XPC to UV lesions.

DDB1 and Cul4A have been reported to be involved in the proteolysis of several proteins, such as DDB2 (42), p27^{Kip1} (49) and CDT1 (50). However, in this study, we demonstrate that DDB1 and Cul4A did not promote XPC degradation, but instead protect XPC from destruction by the proteasome. In addition, knocking down the expression of either DDB1 or Cul4A impairs UV-induced XPC modifications. In light of the earlier observation that Ubc9 knockdown impaired UV-induced XPC modification while promoting its degradation (27), we conclude that both XPC ubiquitylation and sumoylation can prevent XPC degradation upon UV irradiation. The fact that XPC modifications as well as degradation involve the same lysine residue of the XPC protein reinforces this conclusion.

Based on this and other studies, we propose that DDB2 has two distinct functions in UV-induced XPC degradation. On the one hand, DDB2 helps the recruitment of XPC to the UV lesions and XPC has to undergo degradation to execute repair. On the other hand, DDB2 brings DDB-Cul4A E3 ligase to the damage sites to allow protective ubiquitylation of XPC so as to prevent its degradation before repair is complete. Thus, the prevention of inappropriate degradation of XPC by the DDB-Cul4A E3 activity enables XPC to execute its function in genomic repair.

XPC degradation has an important role in NER

In this study, we mutated the XPC K655 to alanine to understand the function of UV-induced XPC degradation in NER. Mutation at this site blocked both UV-induced XPC modifications as well as its degradation. As described above, XPC sumoylation is believed to inhibit XPC degradation while XPC ubiquitylation is shown to enhance the binding of XPC to damaged DNA as well as inhibit XPC degradation. It may be noted that ubiquitylation of XPC was not found to promote the dual incision in a reconstituted NER reaction with purified proteins (26). Similarly, inhibition of XPC sumoylation, by knockdown of Ubc9 expression, did not affect the efficiency of NER (27). Therefore, it can be reasoned that the observed effect of K655A mutation on DNA repair is a consequence of eliminating its ability to degrade XPC.

It has already been reported that during assembly of NER factors, XPC-hHR23B and XPG cannot simultaneously exist in the repair complex and that the entry of XPG into the complex coincides with XPC-hHR23B leaving the complex (51). In contrast, XPC-hHR23B and XPA-RPA complexes can simultaneously bind to distorting DNA lesions (52). Here, we have provided *in vivo* evidence showing that XPC degradation is a prerequisite for XPG recruitment to the damage sites. If XPC cannot be degraded (as in the case of K655A mutation), the recruitment of XPG to the damage sites is obviously compromised and as a result impairs the efficiency of CPD repair. However, in XP-E cells lacking UV-DDB activity, NER of 6-4PP is almost normal (53), even though UV-induced XPC degradation does not occur. This means that XPG can still be recruited

to 6-4PP in the absence of XPC degradation. Structural analysis of DNA lesions has revealed that 6-4PP induces significant helix distortion (54), including disruption of base pairing and this structural distortion accommodates all needed proteins to allow the required assembly of the repair machinery. Thus, it seems that XPC degradation is not necessary here to make space for incoming XPG. In contrast, the distortion induced by CPD is much less pronounced (54,55). The DNA helix distortion induced by CPD could be too subtle to render sufficient space for all the NER factors to simultaneously congregate at the damage site. In this case, XPC-hHR23B complex will have to leave the damage site and, therefore, after serving the damage recognition function XPC is degraded to make the space needed for XPG recruitment.

In summary, this study demonstrates that XPC can be degraded independent of ubiquitylation upon UV irradiation. The level of XPC is very important for cells to execute GGR, even though XPC degradation is necessary for efficient removal of CPD. Moreover, the UV-induced XPC degradation is controlled by XPC modifications to avoid excessive depletion of XPC from the cells. Meanwhile, XPC expression is also induced following UV irradiation so that the new synthesis replenishes the depleted XPC to ensure the presence of sufficient XPC for the upcoming rounds of damage removal.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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