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Nucleotide excision repair : a multi-step mechanism required to maintain genome integrity

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DYNAMIC IN VIVO INTERACTION OF DDB2
E3 UBIQUITIN LIGASE WITH UV-DAMAGED
DNA IS INDEPENDENT OF DAMAGE-
RECOGNITION PROTEIN XPC

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SUMMARY

Damage DNA binding protein 2 (DDB2) has a high affinity for UV-damaged DNA and has been implicated in the initial steps of global genome nucleotide excision repair (NER) in mammals. DDB2 binds to CUL4A and forms an E3 ubiquitin ligase. In this study, we have analyzed the properties of DDB2 and CUL4A in vivo. The majority of DDB2 and CUL4A diffuse in the nucleus with a diffusion rate consistent with a high molecular mass complex. Essentially all DDB2 binds to UV-induced DNA damage, where each molecule resides for 2 minutes. After the induction of DNA damage, DDB2 is proteolytically degraded with a half-life that is two orders of magnitude larger than its residence time on a DNA lesion. This indicates that binding to damaged DNA is not the primary trigger for DDB2 breakdown. The bulk of DDB2 binds to and dissociates from DNA lesions independently of damage-recognition protein XPC. Moreover, the DDB2-containing E3 ubiquitin ligase is bound to many more damaged sites than XPC, suggesting that there is little physical interaction between the two proteins. We propose a scenario in which DDB2 prepares UV-damaged chromatin for assembly of the NER complex.

INTRODUCTION

Genome integrity is continuously challenged by various sources that potentially damage DNA. Short-wavelength UV light introduces DNA injuries, such as 6-4 photoproducts (6-4 PPs) and cyclobutane pyrimidine dimers (CPDs), both of which can interfere with transcription and replication. The mammalian cell utilizes nucleotide excision repair (NER) to remove UV-induced DNA damage and various other bulky DNA lesions from the genome (de Laat et al., 1999; Hoeijmakers, 2001). Two different modes of NER exist: transcription-coupled NER (TC-NER) and global genome NER (GG-NER). TC-NER removes lesions from the transcribed strand of active genes, whereas GG-NER repairs damage at any other position in the genome (de Laat et al., 1999). More than 20 gene products are involved in mammalian NER (Aboussekhra et al., 1995) that employs a dual incision mechanism to remove lesions. A crucial step in the initiation of GG-NER is the detection of DNA lesions. Although the mechanism of damage detection in chromatin is not well understood, various studies have identified the XPC-RAD23B-CEN2 complex as the principal initiator of GG-NER (Araki et al., 2001; Sugawara et al., 1998; Sugawara et al., 2001; Volker et al., 2001). Detection of CPDs by damage-recognition protein XPC is inefficient and it has been suggested that the UV-damaged DNA binding (UV-DDB) complex plays a crucial role in CPD detection and repair. UV-DDB consists of DDB1 (p127) and DDB2 (p48) (Dulan et al., 1995; Takao et al., 1993), and mutations in the DDB2 subunit are responsible for the XP-E phenotype (Nichols et al., 2000; Rapic-Otrin et al., 2003). DDB2-deficient cells such as rodent and human XP-E cells, have a deficiency in the removal of CPDs and delayed removal of 6-4 PPs, particularly at low UV doses (Hwang et al., 1999; Moser et al., 2005; Tang et al., 2000). Transfection of DDB2 cDNA in hamster cells restores the binding activity of UV-DDB to damaged DNA, showing that DDB2 rather than DDB1 is responsible for binding to damaged DNA (Hwang et al., 1998; Li et al., 2006; Tang et al., 2000). The complete inactivation of DDB2 expression in mice results in a significant increase in the formation of malignant tumors (Yoon et al., 2005), whereas overexpression of DDB2 protects mice from the carcinogenic effects of UV-B irradiation (Alekseev et al., 2005), illustrating the tumor-preventing property of DDB2.

Recently, UV-DDB was implicated in 6-4 PPs repair *in vivo* (Alekseev et al., 2005; Moser et al., 2005), which supports results of several *in vitro* assays that demonstrated a higher affinity of UV-DDB for 6-4 PPs compared with CPDs (Fujiwara et al., 1999; Reardon et al., 1993; Treiber et al., 1992). UV-DDB has a 500,000-fold preference for damaged over non-damaged DNA (Hwang and Chu, 1993), which is significantly higher than the selectivity of XPC for damaged DNA (3,000-fold preference) (Sugawara et al., 1998). UV-DDB appears to bind to DNA lesions in the absence of functional XPC protein (Fitch et al., 2003; Wakasugi et al., 2001), in contrast to the NER components XPA, TFIIH, XPG and ERCC1-XPF, which all require XPC for binding to damaged DNA (Volker et al., 2001). Although much less efficiently, XPC binds to 6-4 PPs in XP-E cells, indicating that UV-DDB is not an absolute prerequisite for XPC binding to this particular type of lesion (Moser et al., 2005). DDB2 is crucial for the recognition and repair of CPDs and overexpression of DDB2 leads to enhanced recruitment of XPC to CPDs (Fitch et al., 2003). These observations strengthen

the hypothesis that UV-DDB facilitates and/or stabilizes the binding of XPC to UV-induced photoproducts.

Biochemical evidence suggests that UV-DDB is part of an E3 ub ligase complex that contains cullin 4A (CUL4A) (Shiyanov et al., 1999b) and the COP9 signalosome, which is a negative regulator of the ub ligase activity of the E3 DDB2 complex (Groisman et al., 2003). The ub ligase activity of the E3 DDB2 complex is transiently activated by UV irradiation and several substrates for ubiquitylation have been identified, including DDB2 itself, XPC and histones H2A, H3 and H4 (Groisman et al., 2003; Kapetanaki et al., 2006; Sugasawa et al., 2005; Wang et al., 2006). It is currently believed that DDB2 recruits XPC to lesions, after which both DDB2 and XPC are ubiquitylated (Sugasawa et al., 2005). Ubiquitylated DDB2 is proteolytically degraded (Chen et al., 2001; Nag et al., 2001; Rapic-Otrin et al., 2002) whereas the reversible ubiquitylation of XPC increases its affinity for DNA (Sugasawa et al., 2005). To investigate the interplay between DDB2 and XPC *in vivo*, we have analyzed the properties of fluorescently labeled DDB2 in wild-type and XPC-deficient cells. Our results indicate that the bulk of DDB2 interacts with lesions independently of XPC and that there is little interaction between the two recognition proteins on damaged DNA.

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RESULTS

Generation of cell-lines expressing YFP-tagged DDB2 and GFP-tagged CUL4A

To study the cellular distribution and dynamics of DDB2 we tagged the protein with enhanced yellow fluorescent protein (EYFP), which was fused to the C terminus of murine DDB2 (Fig. 1B) resulting in a DDB2-EYFP fusion protein that was stably expressed in human fibroblasts (MRC5-SV) (Moser et al., 2005). EYFP was also fused to the N terminus of DDB2 and stably expressed in the same cells. Both fusion proteins were found predominantly in the nucleus (Fig. 1A). Western blot analysis using anti-DDB2 antibodies showed that DDB2-EYFP migrates in SDS-PAGE with a mobility corresponding to the expected size of the full-length fusion protein (75 kDa; Fig. 1C) (Dualan et al., 1995). The fluorescently tagged DDB2 protein binds to UV-damaged DNA (Moser et al., 2005) and is proteolytically degraded after UV irradiation as shown by western blot analysis of whole cell extracts (Fig. 1C). This indicates that the fusion protein exhibits wild-type behavior. To quantify the expression level of DDB2-EYFP, we compared the fluorescence of a solution of EYFP protein of a known concentration with the fluorescence of cells expressing DDB2-EYFP. The average concentration of DDB2-EYFP in living cells was $0.7 \pm 0.1 \mu\text{M}$ (data not shown), which corresponds to 1.2×10^5 molecules in a $300 \mu\text{m}^3$ nucleus. The number of endogenous DDB2 molecules per cell is 1×10^5 , indicating that DDB2-EYFP is expressed at levels comparable to endogenous DDB2 (Keeney et al., 1993). Moreover, western blot analysis using a polyclonal antibody against DDB2 (Wang et al., 2005b) demonstrated that DDB2-EYFP is expressed at about the same level as endogenous DDB2 in the same cells (Fig. 1C). DDB2-EYFP was also expressed in XPC-deficient and XPA-deficient human fibroblasts (XP20MA-SV and XP12RO-SV, respectively) and displayed a similar cellular distribution as observed in wild-type cells (data not shown). In addition to DDB2, CUL4A

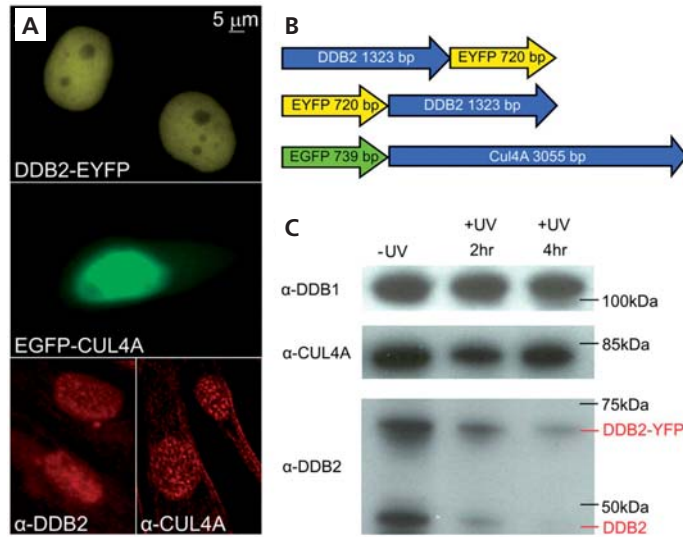


Fig. 1. Expression of DDB2-EYFP and EGFP-CUL4A. (A) Localization of DDB2 and CUL4A. The upper panel shows the distribution of DDB2-EYFP in an MRC5 cell, the middle image shows the distribution of EGFP-CUL4A in a HeLa cell and the lower images show the localization of endogenous DDB2 and CUL4A detected with specific antibodies in primary human cells (VH10). (B) Representation of the DNA constructs encoding fluorescently tagged DDB2 and CUL4A. (C) Western blot analysis of MRC5-SV40 cells expressing DDB2-EYFP. Whole cell extracts (10 μg) of non-UV-irradiated cells and UV-irradiated cells (2 and 4 hours after irradiation with 20 J/m²), were probed with antibodies against DDB2, DDB1 and CUL4A.

was fused to EGFP and expressed in HeLa cells, where it was found predominantly in the nucleus (Fig. 1A, B). The cellular distribution of endogenous CUL4A and DDB2 was mainly nuclear (Fig. 1A), showing that the localization of the fluorescent fusion proteins reflects that of the endogenous proteins.

DDB2 and CUL4A have the same mobility in undamaged cells

Analysis of purified proteins from insect cells and whole cell lysates suggested that DDB2 exists in at least four different assembly states *in vitro*: monomeric DDB2, heterodimeric DDB1-DDB2 and as a protein complex containing either ROC1/CUL4A or ROC1/CUL4A with COP9 (Kulaksiz et al., 2005). However, DDB2 isolated from cells is co-purified with DDB1, CUL4A and all subunits of COP9 (Groisman et al., 2003). To investigate whether DDB2 is part of different protein complexes *in vivo* we employed fluorescence recovery after photobleaching (FRAP) (Houtsmuller and Vermeulen, 2001; Rabut and Ellenberg, 2005), in which a strip (2 μm width) spanning the nucleus was bleached and the fluorescence recovery in the strip was monitored (Fig. 2A).

Most DDB2-EYFP was mobile in non-UV-irradiated cells monitored at 37°C (Fig. 2B) and a similar mobility was measured at 27°C (Fig. 2C). Curves generated by Monte Carlo simulations (Farla et al., 2004) were fitted to experimental FRAP curves, yielding an effective diffusion constant (D_{eff}) for DDB2 in undamaged cells of $2.4 \pm 0.4 \mu\text{m}^2/\text{second}$

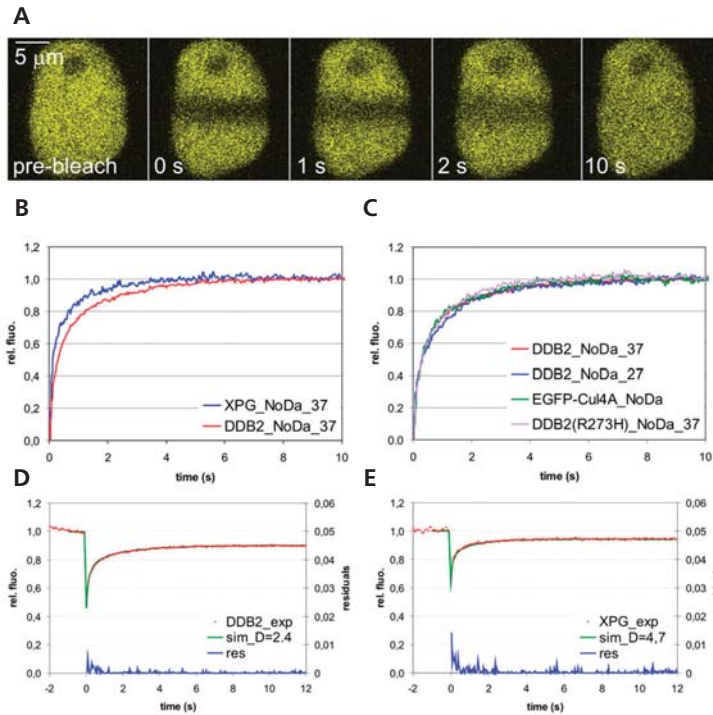


Fig. 2. FRAP analysis of DDB2 mobility in non-damaged cells (NoDa). (A) Example of a DDB2-EYFP cell in which a strip (2 μm) spanning the nucleus was bleached. (B) Recovery plots of DDB2-EYFP ($n=14$) and XPG-EGFP ($n=23$), normalized between 0 and post-bleach intensity after full equilibration. (C) Normalized recovery plots of DDB2-EYFP at 37°C and 27°C ($n=12$), DDB2(R273H)-EYFP ($n=13$) at 37°C and EGFP-CUL4A ($n=11$) at 37°C. (D) Recovery of DDB2-EYFP normalized to pre-bleach intensity (red line). Monte Carlo simulation assuming a diffusion constant of 2.4 $\mu\text{m}^2/\text{second}$ (green line) and the residuals (blue lines at the bottom) that are a measure for the quality of the simulation. (E) Recovery of XPG-EGFP normalized to pre-bleach intensity (red line), Monte Carlo simulation assuming a diffusion constant of 4.7 $\mu\text{m}^2/\text{second}$ (green line) and the residuals (blue lines).

(Fig. 2D). To compare the mobility of DDB2 to that of other NER proteins we performed FRAP on cells expressing NER endonuclease XPG-EGFP (Zotter et al., 2006). The rationale to investigate XPG is that the molecular mass of this protein (180 kDa) (O'Donovan et al., 1994) is about the same as the molecular mass of heterodimeric DDB1-DDB2 (175 kDa). The recovery of XPG-EGFP (Fig. 2B, E), $D_{\text{eff}}=4.7\pm1.0 \mu\text{m}^2/\text{second}$ was significantly faster than the recovery of DDB2-EYFP. In addition to DDB2, we have tagged the CUL4A protein with EGFP (Fig. 1B) and measured its mobility in non-UV-irradiated cells. The FRAP curves of EGFP-CUL4A and DDB2-EYFP at 37°C were similar, showing that both proteins move through the nucleus with the same mobility (Fig. 2C). To estimate the size of the protein complexes in which DDB2 resides, we compared the mobility of DDB2 and CUL4A with that of other GFP-tagged NER factors (TFIIH, ERCC1, XPG and XPA) and free EGFP (Hoogstraten et al., 2002; Houtsmuller et al., 1999; Rademakers et al., 2003; Zotter et al., 2006). We assumed that the shape of the different NER factors is the same and that the

mobility of these proteins is determined predominantly by their molecular mass. Based on these assumptions, we estimate that the majority of DDB2 and CUL4A has a diffusion rate consistent with high molecular mass complexes of 500-700 kDa (Fig. S1). CUL4A was recently reported to be part of several protein complexes, varying in size between 300 and 700 kDa (He et al., 2006), which is in agreement with our estimate. Immunoprecipitation studies have demonstrated that the naturally occurring DDB2 protein with an R273H substitution in the WD40 motif is unable to interact with the E3 ubiquitin ligase core proteins DDB1 and CUL4A (Chen et al., 2001; Rasic-Otrin et al., 2003; Shiyanov et al., 1999a). In contrast to these studies, purified R273H mutant DDB2 forms a complex with DDB1 in vitro (Wittschieben et al., 2005). We generated a YFP-tagged DDB2 (R273H) protein, which was expressed in human HeLa cells. The mobility of DDB2 (R273H) and wild-type DDB2 was similar (Fig. 2C), suggesting that mutant DDB2 is part of the DDB-CUL4A-ROC complex in vivo. Although DDB2 can exist in different assembly states in vitro (Kulaksiz et al., 2005), our results indicate that the majority of DDB2 in vivo resides in high molecular mass complexes of at least 500 kDa. Such a molecular mass is consistent with the size of the DDB-CUL4A-ROC (280 kDa) complex with the COP9 signalosome (450 kDa).

6

DDB2 recruitment to lesions is independent of XPC

To investigate how DDB2 associates with damaged DNA in vivo, we analyzed its binding to UV-induced lesions in human fibroblasts that expressed DDB2-EYFP and that were subjected to local UV irradiation (100 J/m² through 5 μ m pores) with UV-C light (Fig. 3A) (Moné et al., 2004). DDB2-EYFP accumulated at 37°C with a half-time ($t_{1/2}$) of 40 seconds (Fig. 3D), which is significantly faster than other GFP-tagged NER proteins tested so far. Similar results were obtained with DDB2 tagged with the red fluorescent protein mCherry (Shaner et al., 2004) (data not shown). To verify that the fluorescent tag does not affect the binding kinetics of DDB2 we measured accumulation of EYFP-DDB2 (i.e. N-terminally tagged DDB2; Fig. 1B), which displayed binding behavior similar to that of C-terminally tagged DDB2 (Fig. 3D). To monitor the binding of DDB2 and another NER protein simultaneously, we expressed DDB2-mCherry and XPG-EGFP in XPG-deficient rodent cells and measured the binding kinetics of both proteins upon local UV-C irradiation (Zotter et al., 2006). Results show that DDB2 binds considerably faster than XPG ($t_{1/2}$ of 40 seconds and 200 seconds, respectively) and that the binding kinetics of DDB2 in rodent and human cells are similar (data not shown). To investigate the kinetics of CUL4A recruitment to sites of DNA damage we transiently expressed EGFP-CUL4A in human HeLa cells (Fig. 3B) and exposed cells to local UV irradiation. Clear accumulation of GFP-CUL4A at UV-irradiated areas was detected. Since CUL4A alone does not bind to damaged DNA, this indicates that the GFP-CUL4A interacts with endogenous DDB2 (Li et al., 2006). The binding kinetics of EGFP-CUL4A ($t_{1/2}$ of 47 seconds) and DDB2-EYFP ($t_{1/2}$ of 40 seconds) were similar (Fig. 3D), suggesting that they reflect the assembly of the DDB-CUL4A-ROC 1 protein complex onto UV-damaged DNA. In agreement, DDB1-mCherry also binds to UV-damaged DNA with similar kinetics (S. Alekseev and W. Vermeulen, unpublished). To investigate whether

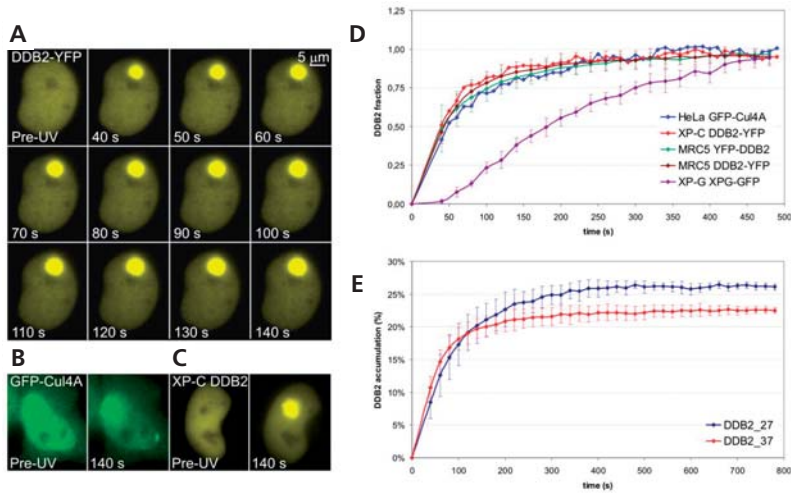


Fig. 3. Binding kinetics of DDB2 after local UV irradiation. (A) Example of a DDB2-EYFP cell that shows local accumulation after local UV-C irradiation with 100 J/m² through 5 μm pores at 37°C. (B) Example of a HeLa cell expressing EGFP-CUL4A and (C) an XP-C cell expressing DDB2-EYFP. Both fusion proteins accumulate after local UV-C irradiation through 5 μm pores at 37°C (B,C). (D) Quantification of accumulation kinetics of DDB2-EYFP in MRC5 cells (brown line, n=9), EYFP-DDB2 in MRC5 cells (green line, n=8), DDB2-EYFP in XP-C cells (red line, n=3) and EGFP-CUL4A in HeLa cells (blue line, n=3). Curves were normalized to the plateau value. Time point t=0 is defined as the start of UV irradiation. (E) Quantification of assembly kinetics of DDB2-EYFP at 37°C (red, n=9) and 27°C (blue, n=8). The local accumulation of DDB2-EYFP was measured and plotted as a percentage of the total EYFP fluorescence in the cell nucleus. The error bars represent the standard deviation.

the association of DDB2 with UV-damaged DNA involves a temperature-dependent step, for instance an enzymatic reaction, we analyzed its binding kinetics at 27°C. The initial slope of the assembly curves at 37°C and 27°C are similar (Fig. 3E), indicating that the association of DDB2 with damaged DNA is not temperature sensitive. Previous NER assembly approaches showed that exogenously expressed DDB2 binds to UV-induced DNA lesions in the absence of functional XPC (Fitch et al., 2003; Wakasugi et al., 2002). In this study, we show that endogenous DDB2 accumulates at sites of localized UV damage in cells lacking XPC, XPA, XPG and XPF (Fig. S2), indicating that the binding of DDB2 does not require any of these NER proteins. Although binding of DDB2 can be detected in several XP cell lines, it is possible that the kinetics of binding are different in NER-deficient cells. To investigate this, we expressed DDB2-EYFP in XP-C cells (XP20MA-SV40) (see Rademakers et al., 2003). Similar binding kinetics were measured in XP-C cells and wild-type cells (Fig. 3C, D). These findings show that DDB2 rapidly binds to damaged DNA independently of XPC.

Most DDB2 is bound to chromatin after global DNA damage induction

The distribution of DDB2-EYFP in non-UV-irradiated living fibroblasts is homogeneous although the protein is partially excluded from the nucleoli, possibly due to their very high

macromolecular concentration (Fig. 4A-C). To investigate if UV irradiation results in nuclear redistribution of DDB2 we globally irradiated cells (16 J/m^2), inducing a uniform distribution of damage throughout the nucleus. Confocal microscopy showed that the distribution of DDB2 changed upon UV irradiation, which is not observed for other NER factors except TFIIH (Volker et al., 2001). Irradiated cells show regions of dense and less dense DDB2 fluorescence (Fig. 4F-H), similar to the spatial distribution of chromatin (Kimura and Cook, 2001). In agreement, we observed co-localization between Cerulean-tagged histone H2A and DDB2-EYFP after global UV irradiation (Fig. 4H-J and line-scan Fig. 4L), which was not observed in non-UV-irradiated cells (Fig. 4C-E and line-scan Fig. 4K). This indicates that DDB2 binds to UV-damaged chromatin in interphase nuclei (Rapic-Otrin et al., 1997). Previous studies have shown that XPC readily associates with interphase and mitotic chromatin in the absence of DNA damage (D. Hoogstraten, PhD thesis, Erasmus University Rotterdam, 2003) (van der Spek et al., 1996). In contrast to XPC, the distribution of DDB2 in metaphase cells was homogenous and no association with mitotic chromatin could be observed (Fig. 4M). Global irradiation (16 J/m^2) of cells in metaphase resulted in rapid association of DDB2 with the condensed mitotic chromosomes after UV exposure (Fig.

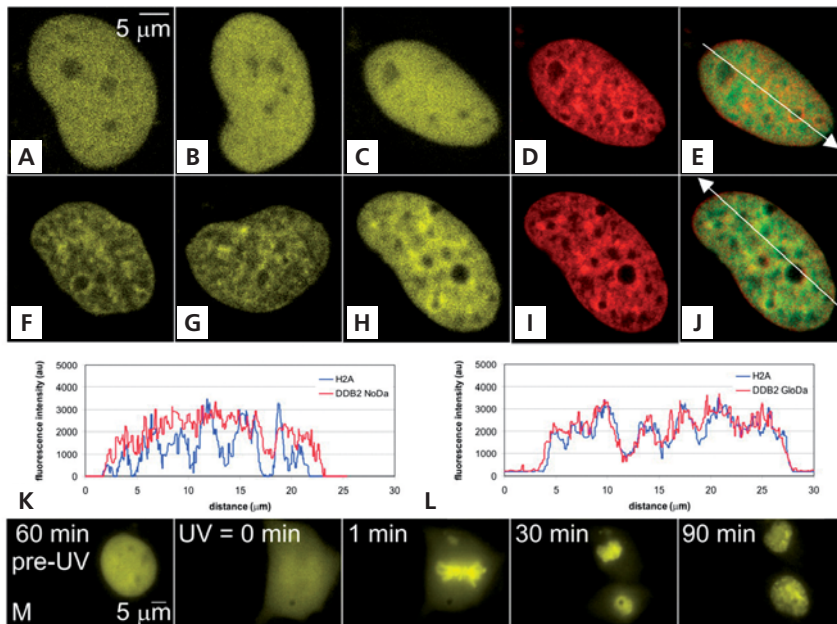


Fig. 4. Nuclear distribution of DDB2 after global UV exposure. (A,B) Representative confocal images of DDB2-EYFP cells (C-E) Example of a DDB2-EYFP cell (C) that was transfected with cerulean-histone H2A (D); the merged images of the two signals is shown in E. (K) Line scan along the arrow in E. (F,G) Representative confocal images of DDB2-EYFP cells that were exposed to global UV-C irradiation (16 J/m^2). (H-J) Globally UV-irradiated DDB2-EYFP expressing cell (H) that was transfected with cerulean-histone H2A (I); the merged images of the two signals is shown in J. (L) Line scan along the arrow in J. (M) Example of a cell expressing DDB2-EYFP, that was globally UV-C irradiated (16 J/m^2) in metaphase and monitored until cell division was completed.

4M). After mitosis, daughter cells displayed the characteristic chromatin localization as observed in irradiated interphase nuclei (Fig. 4M). Binding of GFP-tagged XPG to mitotic chromatin was not observed after global UV-C irradiation (data not shown). It is interesting to note that DDB2 is the only NER protein studied so far that displays this distribution pattern in interphase nuclei upon UV irradiation. Our findings show that DDB2 has the ability to bind to UV-damaged interphase and metaphase chromatin.

The amount of DDB2 that is proteolytically degraded is UV dose dependent

To investigate whether YFP-tagged DDB2 is degraded upon UV irradiation we analyzed whole cell extracts by western blotting using antibodies against DDB2 (Fig. 1C). Our analysis shows that DDB2-EYFP is degraded within 4 hours (Fig. 1C) and that expression of DDB2 is restored after 20 hours (data not shown). The amount of DDB1 and CUL4A remains unchanged after UV irradiation (Fig. 1C). Next, we used live-cell imaging to measure the degradation kinetics of DDB2-EYFP in living UV-irradiated cells up to 6 hours. We observed that locally UV-irradiated cells (100 J/m^2) showed a significant decrease in total nuclear fluorescence (within 6 hours) in contrast to cells that were not hit by local UV irradiation (Fig. 5A). Cells that were globally UV irradiated (16 J/m^2) showed a complete loss of nuclear fluorescence with a $t_{1/2}$ of 2 hours (Fig. 5B-D). To unambiguously show that the decrease in nuclear fluorescence was due to proteolytic degradation of DDB2-EYFP we treated cells with proteasome inhibitor MG-132 and observed that DDB2-EYFP levels remained unchanged up to 6 hours after irradiation at 16 J/m^2 (Fig. 5C). Irradiation with UV doses of 8 and 4 J/m^2 resulted in degradation of 75% and 50% of the DDB2 pool, respectively (Fig. 5D). Non-UV-irradiated DDB2-EYFP cells (Fig. 5D) and globally irradiated HeLa cells transfected with EYFP-NLS (8 J/m^2) showed no change in nuclear fluorescence between 0 and 6 hours. Previous experiments showed that DDB2 is degraded in several NER mutant cell lines, including XP-C (Rapic-Otrin et al., 2002). To determine if the rate of degradation is affected by the ongoing NER process, we measured degradation of DDB2-EYFP in XP-C and XP-A cells. The initial breakdown of DDB2 was faster in both XP lines than in wild-type cells irradiated with the same dose of UV (Fig. 5D). The kinetics of degradation in repair-proficient cells resembles the repair kinetics of 6-4 PPs in the UV dose range employed (van Hoffen et al., 1995). We suspect that breakdown of DDB2-EYFP in cells irradiated with UV doses of 4 and 8 J/m^2 is not complete because a significant fraction of 6-4 PPs is removed before all DDB2 is degraded. For example, NER proficient cells remove 50% of their 6-4 PPs in the first hour after a dose of 8 J/m^2 (Moser et al., 2005). It is possible that the breakdown of DDB2 is faster in NER-deficient cells due to the persistence of DNA damage in the genome of these cells. It is unclear why DDB2 is not completely degraded in XP-C and XP-A cells (Fig. 5D). Although a single high dose of UV-C can result in almost complete break-down of DDB2, such a situation, and thus complete break-down of DDB2, is not likely to occur in cells of an organism that is exposed to solar UV irradiation. Together, our data indicate that the amount of DDB2 that is proteolytically degraded is UV-dose dependent and that the rate of DDB2 breakdown depends on ongoing NER.

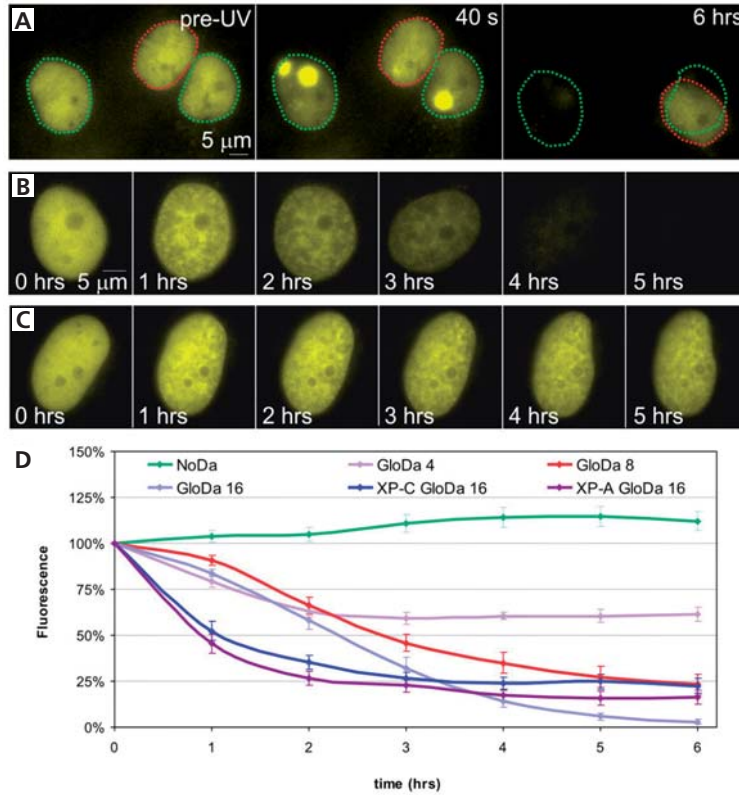


Fig. 5. UV-induced degradation of DDB2. (A) Two DDB2-YFP cells were locally damaged by UV irradiation (nuclei indicated by the green dotted outline), while a third cell was not irradiated (indicated by the red dotted outline). The same three cells are shown shortly after (40 seconds) and 6 hours after UV irradiation. (B) Example of a DDB2-EYFP cell that has been globally UV irradiated (16 J/m^2) and monitored for 5 hours. (C) Example of a globally UV irradiated (16 J/m^2) DDB2-EYFP cell that had been treated with $50 \mu\text{M}$ MG-132. (D) Quantification of the total nuclear fluorescence in the absence of UV-C irradiation (green line, NoDa, $n=8$) and after global UV irradiation (GloDa) of MRC5 human cells with 4 (light-purple line, $n=10$), 8 (red line, $n=12$) and 16 J/m^2 (light-blue line, $n=10$), and in XP-C (dark-blue line, $n=15$) and XP-A (dark-purple line, $n=7$) cells with 16 J/m^2 . All values were corrected for background fluorescence and the initial total nuclear fluorescence intensity was set to 100%. The error bars represent the standard error of the mean.

All DDB2 can bind to lesions in UV-irradiated cells

To examine the properties of bound and unbound DDB2 upon UV irradiation, we applied strip-FRAP on globally irradiated cells (Fig. 6A). The FRAP curves showed incomplete recovery (1 hour after 16 J/m^2 at 37°C) indicative of a significant immobile fraction (Fig. 6C). We observed fast but incomplete recovery (within 2 seconds) of DDB2 in irradiated cells. This initial very fast recovery ($t_{1/2}=0.1$ seconds) is probably due to fluorescence blinking, given that the average lifetime of GFP in a dark non-emissive state is around 2 seconds (Garcia-Parajo et al., 2000). Blinking of GFP is the result of switching between an emissive (anionic) on-state and a non-emissive (neutral) off-state (Dickson et al., 1997).

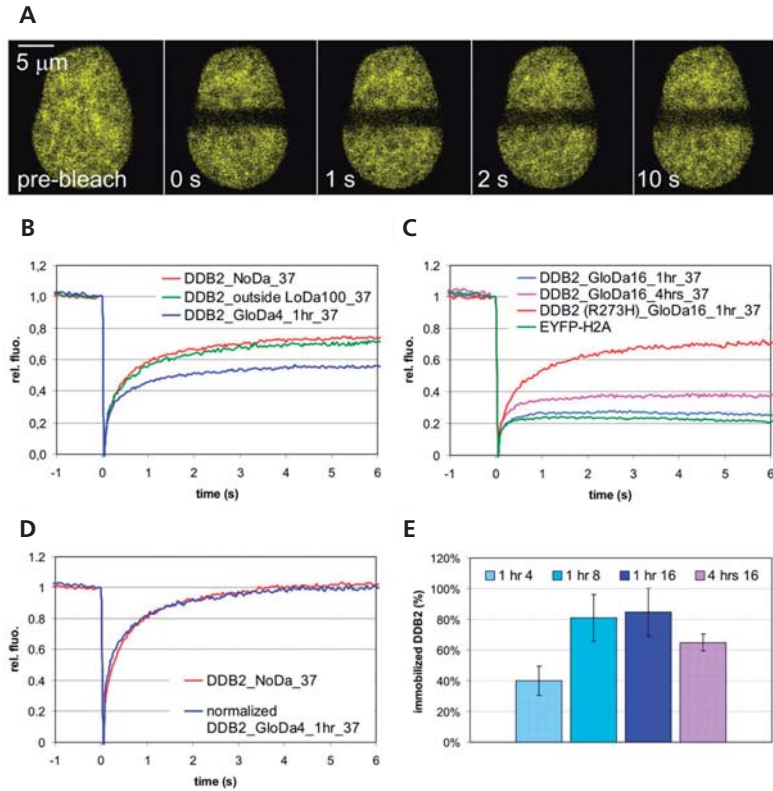


Fig. 6. FRAP analysis of DDB2 mobility in UV-irradiated cells. (A) Example of a globally UV-irradiated DDB2-EYFP cell in which a narrow strip (2 μm) across the nucleus was bleached. (B) Recovery kinetics of DDB2-EYFP in undamaged cells (red line, NoDa, $n=14$), in locally damaged cells (green line, outside LoDa, $n=14$) and in globally UV-irradiated cells (4 J/m^2 ; blue line, GloDa, $n=15$). The recovery plots are normalized to 0 and to the pre-bleach intensity. (C) The recovery plots of DDB2-EYFP in globally damaged cells 1 hour after (purple line, GloDa, $n=16$) and 4 hours after (blue line, GloDa, $n=15$) irradiation with 16 J/m^2 . The recovery plots of mutant R273H DDB2 (red line, $n=12$) and EYFP-histone H2A (green line, $n=15$) 1 hour after irradiation (16 J/m^2) are also shown. The recovery plots are normalized to 0 and to the pre-bleach intensity. (D) Normalized recovery of DDB2-EYFP in cells irradiated with 4 J/m^2 (blue line, GloDa). The recovery plot is normalized to post-bleach intensity and compared to DDB2-EYFP in undamaged cells (red line, NoDa). (E) UV-immobilized fraction of DDB2-EYFP in MRC5 cells 1 hour after global irradiation with 4, 8 and 16 J/m^2 and 4 hours after 16 J/m^2 . The immobilized fractions depicted in E were determined by Monte Carlo simulations. The bound fractions obtained from the ten best simulations that fitted the experimental data were averaged. The error bars represent the standard deviation.

The off-time is independent of excitation intensity whereas the on-time is shortened at high excitation intensities (such as the FRAP bleach pulse), which makes blinking a light-induced process (Garcia-Parajo et al., 2000). Comparable fast but incomplete recovery was measured with EYFP- and EGFP-tagged histone H2A (Fig. 6C and data not shown), which are immobile chromatin proteins on this time-scale (Phair and Misteli, 2000). In contrast to wild-type DDB2, the R273H mutant DDB2 protein was not immobilized after

UV-C irradiation (Fig. 6C). This confirms the inability of DDB2 R273H to bind to damaged DNA (Hwang et al., 1998) and demonstrates that the R273 residue is essential for binding to damaged DNA. Monte Carlo simulations of the experimental FRAP curves, which were corrected for blinking (Farla et al., 2004), yielded an immobile fraction of $85 \pm 15\%$ for DDB2, measured 1 hour after UV irradiation at a dose of 8 or 16 J/m² (Fig. 6E). Such a UV-C dose produces $3 \cdot 10^5$ 6-4 PPs and $1 \cdot 10^6$ CPDs (Perdiz et al., 2000), indicating that there is sufficient damage to bind all DDB2. Even 4 hours after UV irradiation (16 J/m²) we still observed a significant immobilization of DDB2 ($65 \pm 5\%$, %, Fig. 6C,E).

It is likely that this immobilization reflects binding to CPDs because the majority of 6-4PPs is removed within 4-6 hours (Moser et al., 2005; van Hoffen et al., 1995). For comparison, the fraction of immobilized XPC-EGFP decreases to background levels 2 hours after global irradiation at a UV dose of 8 J/m² (D. Hoogstraten, PhD thesis, Erasmus University Rotterdam, 2003), indicating that NER complexes assembled on slowly repaired CPDs cannot be detected. There is significant degradation of DDB2 between 1 (15% degradation; Fig. 5D) and 4 hours after UV (85% degradation; Fig. 5D). Considering this degradation, we estimate that the number of DDB2 molecules bound to damaged DNA between 1 and 4 hours after UV decreases with a factor of 8. A lower UV dose (4 J/m²) induced a less pronounced immobilization of DDB2 ($40 \pm 9\%$; Fig. 6B, E). Unbound DDB2 in these UV-irradiated cells has the same mobility as DDB2 in unchallenged cells (Fig. 6D). Accordingly, the mobility of DDB2 outside local UV-induced DNA damages (100 J/m² through 5 μ m pores) was similar to the mobility in undamaged cells (Fig. 6B), indicating that DDB2 molecules that are not bound to a lesion have the same mobility and thus a similar complex composition as DDB2 in non-damaged cells. These findings indicate that DDB2 binds to lesions in a UV-dose-dependent manner up to complete binding of all DDB2 molecules. Moreover, DDB2 that dissociates from lesions has a similar diffusion rate as DDB2 in undamaged cells, suggesting that the composition of the DDB2 complex that dissociates from lesions is similar to that in undamaged cells.

Release of DDB2 from lesions is XPC independent

To determine the dissociation kinetics of DDB2 from UV-damaged DNA, we applied fluorescence loss in photobleaching (FLIP). A region distant from the local UV-induced DNA damage was continuously bleached (Fig. 7A) and the decrease of fluorescence in the locally damaged area was measured (Fig. 7B). The half-time ($t_{1/2}$) of the FLIP curve is indicative for the k_{off} of DDB2 at sites of DNA damage. Directly after bleaching we observed a decrease of fluorescence intensity, which corresponded to the non-bound DDB2 pool. The overall decrease in the locally UV-irradiated area was much slower due to the binding of DDB2 to UV-induced damage. The $t_{1/2}$ of release of DDB2 at sites of UV damage (at 37°C) was 110 seconds (Fig. 7B). Surprisingly, the dissociation kinetics of DDB2 from UV-induced lesions was similar in cells lacking XPC (Fig. 7B), indicating that XPC has no influence on the release of DDB2. The residence time of DDB2 was significantly longer at 27°C ($t_{1/2}$ 220 seconds; Fig. 7B) compared with 37°C ($t_{1/2}$ 110 seconds), indicating that the dissociation of DDB2 from UV-damaged DNA is temperature sensitive.

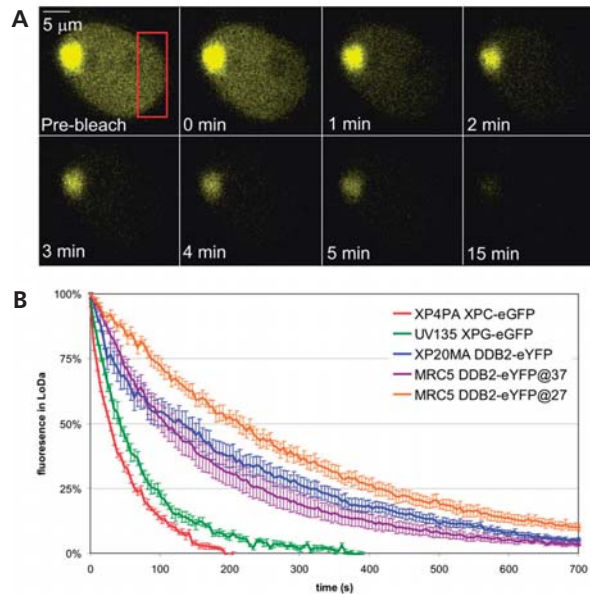


Fig. 7. Dissociation kinetics of DDB2. (A) Example of a DDB2-EYFP cell after local UV irradiation with 100 J/m^2 . A strip of about one third of the cell nucleus opposite the local damage was continuously bleached and the fluorescence decrease in the local damage was monitored. (B) Quantification of the decrease in fluorescence signal in the damaged area of the nucleus. The half-time ($t_{1/2}$) of a FLIP curve corresponds to the residence time of a protein molecule in the locally damaged area. FLIP was performed on DDB2-EYFP in MRC5 human fibroblasts at 37°C (purple line, $n=10$) and at 27°C (orange line, $n=10$), DDB2-EYFP in XP20MA human XP-C fibroblasts (blue line, $n=11$), XPF-EGFP in UV135 Chinese hamster ovary XP-G cells (green line, $n=8$) and XPC-EGFP in XP4PA human XP-C fibroblasts (red line, $n=9$). The error bars represent the standard error of the mean.

To compare the release kinetics of DDB2 with that of other NER proteins, we have previously applied FLIP on cell lines expressing XPC-EGFP and XPG-EGFP (D. Hoogstraten, PhD thesis, Erasmus University Rotterdam, 2003) (Zotter et al., 2006). Residence times of 28 and 45 seconds were measured for XPC-EGFP and XPG-EGFP, respectively ($t_{1/2}$ of the FLIP curves shown in Fig. 7B). Comparison of the residence time of DDB2 with the residence times of XPC-EGFP and XPG-EGFP shows that DDB2 resides significantly longer on UV-damaged DNA than XPC (about four times longer, since $t_{1/2}$ values are 110 seconds and 28 seconds, respectively) and about two times longer than the NER endonuclease XPG.

DISCUSSION

Mammalian nucleotide excision repair is initiated by the XPC complex, which has high affinity for UV-damaged DNA (Sugasawa et al., 1998; Volker et al., 2001). Besides XPC, mammalian cells express a second UV-damage-recognition protein with high affinity for UV-induced lesions: DDB2, which is part of an E3 ubiquitin (ub) ligase containing CUL4A,

ROC1 and DDB1 (Groisman et al., 2003). Currently, it is not clear how these two protein complexes cooperate in the DNA-damage-recognition step of mammalian NER in the living cell. To investigate the interplay between DDB2 and XPC *in vivo* we used quantitative live cell imaging of fluorescently tagged proteins.

DDB2 resides in a high molecular weight protein complex

We measured the mobility of EYFP-tagged DDB2 and of EGFP-tagged CUL4A in nuclei of living human fibroblasts (Fig. 2) and found that the mobility of both fusion proteins is similar and remarkably low. The apparent diffusion constants of DDB2 and CUL4A ($2.5 \mu\text{m}^2/\text{second}$) correspond to that of globular protein complexes with a molecular mass of between 500 and 700 kDa (see Fig. S1). This suggests that the majority of DDB2 resides in the DDB-CUL4A-ROC1 (280 kDa) E3 ub ligase complex, possibly associated with COP9 (450 kDa). The naturally occurring R273H mutant DDB2 protein displays a similar slow behavior as wild-type DDB2 (Fig. 2), suggesting that this protein is assembled into the DDB-CUL4A-ROC1 complex. In agreement with these results, DDB2 R273H was recently demonstrated to form a complex with DDB1 *in vitro* (Wittschieben et al., 2005). Although CUL4A and DDB2 have the same mobility in living cells, it should be noted that CUL4A is also part of a number of other protein complexes not containing DDB2 and therefore its mobility is not necessarily directly related to that of DDB2 (He et al., 2006). About 15 proteins containing WD40 repeats were shown to associate with DDB1-CUL4A (He et al., 2006). All of these DDB1-interacting proteins have a molecular mass of 50 kDa, comparable to DDB2. This might explain why the mobility of CUL4A is similar to the mobility of DDB2. Our results show that transiently transfected EGFP-CUL4A, which can be part of all of these DDB1-CUL4A complexes, accumulates at UV-damaged sites in significant quantities. CUL4A does not accumulate at UV-damaged sites unless associated with DDB2 (Li et al., 2006). Therefore, the accumulation of EGFP-CUL4A indicates that a significant fraction of CUL4A complexes contains DDB2. Recently, DDB2 was shown to bind to UV-damaged DNA after knock-down of DDB1 and CUL4A (El-Mahdy et al., 2006; Li et al., 2006). In the absence of DDB1, the proteins CUL4A and DDB2 do not interact (He et al., 2006), showing that DDB2 alone binds to UV-damaged DNA (Li et al., 2006). Our experiments suggest that the majority of DDB2 in living DDB1-containing cells is part of the DDB-CUL4A-ROC1 complex, rather than free DDB2. UV-induced DNA damage triggers the binding of DDB2 and CUL4A to UV-damaged chromatin (Figs 3, 4). Mutant DDB2 with a R273H substitution does not become immobilized after UV-C irradiation (Fig. 6), demonstrating that the R273 residue is essential for binding to damaged DNA. DDB2 molecules that dissociate from lesions have the same mobility and thus complex composition as DDB2 in undamaged cells (Fig. 6). This suggests that DDB2 that is released from damaged DNA is bound to COP9, which is the negative regulator of the DDB2 E3 complex. Our findings are consistent with a model in which activation of the DDB2 E3 ub ligase complex (by dissociation of COP9) only occurs on damaged chromatin and in which the bulk of DDB2 that dissociates from lesions is inactivated by binding to COP9. In this way the ub ligase activity is specifically directed to chromatin at DNA damaged sites.

DDB2 binds to DNA lesions independently of XPC

To investigate if the dynamic behavior of DDB2 is dependent on functional NER, we measured the accumulation of endogenous and YFP-tagged DDB2 in NER-deficient cells. Binding of endogenous DDB2 to UV-damaged DNA can be detected in the absence of functional XPC, XPA, XPG or XPF protein (see Fig.). Moreover, the association kinetics of DDB2-EYFP with UV-damaged DNA is not influenced by XPC (Fig. 3). Up to 85% of the cellular DDB2 pool becomes immobilized after UV irradiation (Fig. 6), which is considerably higher than the percentage of the cellular XPC pool (25%) that is immobilized after a similar UV dose (W.V., unpublished data). The number of endogenous DDB2 molecules per cell is 1×10^5 (Keeney et al., 1993), whereas there are 3×10^4 XPC molecules per cell (Araujo et al., 2001). Given that the expression level and the damage-bound fraction of XPC is lower than that of DDB2 (Araujo et al., 2001; Keeney et al., 1993), we estimate that shortly after UV irradiation the number of damage-bound DDB2 molecules is 10 times higher than the number of damage-bound XPC molecules (85% of 1×10^5 versus 25% of 3×10^4 for DDB2 and XPC, respectively). This comparison is possible since the fluorescently tagged proteins are present in the same amounts as their endogenous counterpart (see Materials and Methods, and the Results section). Immunoprecipitation experiments have indicated that XPC and DDB2 directly interact even in the absence of UV-induced lesions (Sugasawa et al., 2005). It has been suggested that DDB2 recruits XPC to UV-induced lesions resulting in DDB2 displacement (Sugasawa et al., 2005). If each DDB2 protein would recruit an XPC molecule, resulting in the dissociation of DDB2 due to inactivation by ubiquitylation (Sugasawa et al., 2005), the kinetics of DDB2 dissociation should be XPC dependent. In vivo measurements reveal that DDB2 resides about four times longer on UV-damaged DNA than XPC and that the release of DDB2 from UV-induced lesions is not affected by XPC (Fig. 7). These findings, in combination with the difference in the number of damage-bound XPC and DDB2 molecules, suggest that DDB2 does not physically interact with XPC on UV-damaged DNA. It is possible that there is an interaction between the two recognition factors on some (possibly poorly recognized) lesions resulting in the DDB2-mediated ubiquitylation of XPC (Sugasawa et al., 2005), but our results indicate that the bulk of DDB2 binds to lesions independently of XPC.

DDB2 primes chromatin around lesions to be targeted by NER

Based on our findings we propose the following model for DDB2 function in the living cell. DDB2 is part of an inactive E3 ub ligase complex that contains DDB1, CUL4A and the COP9 signalosome (Groisman et al., 2003). Essentially all DDB2 binds to UV-damaged DNA at high UV doses independently of XPC. It is likely that the E3 ub ligase binds to damages as a holocomplex, since DDB2 and CUL4A have the same rate of binding to lesions. A DDB2 molecule that binds to DNA damage is not inactivated immediately, but can dissociate and rebound several times to UV-damaged DNA, since the residence time of DDB2 is 2 minutes, whereas the half-time of breakdown is two orders of magnitude larger (2 hours). This indicates that a DDB2 molecule can bind about 75 times to UV-damaged DNA before it is degraded. The binding-deficient DDB2 mutant K244E is resistant to degradation. It has

been suggested that binding to damaged DNA is the trigger for DDB2 breakdown (Nichols et al., 1996; Raptic-Otrin et al., 2002). Our findings show that binding of DDB2 to UV-damaged DNA is not the primary trigger for its proteolytic degradation. Other factors are likely to be involved in the regulation of DDB2 breakdown, such as the checkpoint protein Claspin (Praetorius-Ibba et al., 2007). However, the exact trigger for DDB2 breakdown remains to be elucidated.

Our findings show that DDB2 interacts with UV-damaged DNA independently of XPC. It is attractive to speculate that DDB2 marks lesions to be targeted by NER. Several studies have shown that DDB2 interacts with chromatin remodeling complexes, such as p300 histone acetyltransferase (Datta et al., 2001; Raptic-Otrin et al., 2002). The DDB2 E3 ubiquitin ligase was recently shown to ubiquitylate histone H2A, H3 and H4 (Kapetanaki et al., 2006; Wang et al., 2006), linking DDB2 to chromatin remodeling. We propose a scenario in which the binding of DDB2 to a lesion and subsequent remodeling creates a local chromatin environment that facilitates the assembly of a NER complex. The ubiquitylation of histone H3 and H4 results in a weakened interaction with DNA (Wang et al., 2006). Thus, DDB2-dependent histone ubiquitylation could result in the dissociation of histones from the DNA. Evidence for this is that histone H3 was recently shown to be incorporated into newly repaired chromatin in a CAF-1-dependent manner (Polo et al., 2006). Alternatively, the DDB2-dependent ubiquitylation of histones might facilitate binding of XPC through the ubiquitin-associated (UBA) domains of RAD23B (Bertolaet et al., 2001; Kapetanaki et al., 2006). After the initial massive binding of DDB2 to UV-induced lesions, thereby marking these sites for NER, the effective DDB2 concentration is gradually lowered by its proteolytic degradation. Breakdown may be important because otherwise potential binding sites for XPC are occupied by DDB2. Evidence for this comes from *in vitro* studies using purified components, showing that NER is inhibited by the addition of UV-DDB in the absence of ubiquitylation factors (e.g. E1, E2 and ub itself). Inhibition is relieved when ubiquitylation factors are added (Sugasawa, 2006; Sugawara et al., 2005). The importance of DDB2 degradation *in vivo* is illustrated by the compromised removal of CPDs upon interference with DDB2 proteolysis (El-Mahdy et al., 2006; Groisman et al., 2003; Wang et al., 2005a).

Together, our results give insight into the early steps of DNA damage detection. DDB2 diffuses in the undamaged nucleus as part of an E3 ubiquitin ligase. In UV-damaged cells most DDB2 binds to DNA lesions. We provide evidence that DDB2 functions independently of the damage-recognition protein XPC. Although DDB2 is not part of the pre-incision complex, it has a central role in NER, probably by preparing chromatin around DNA lesions for assembly of the pre-incision complex.

MATERIALS AND METHODS

Cell lines. The cell lines used in this study were human fibroblasts MRC5-SV expressing DDB2-EYFP and EYFP-DDB2, XPC-deficient XP20MA-SV expressing DDB2-EYFP, XPA-deficient XP12RO-SV, XPC-deficient XP4PA-SV expressing XPC-EGFP (D. Hoogstraten, PhD thesis, Erasmus University Rotterdam, 2003), XPA-deficient XP2OS-SV expressing EGFP-XPA (Rademakers et al., 2003), XPG-deficient XPCS1RO-SV expressing XPG-EGFP (Zotter et al., 2006), XPB-deficient XPCS2BA-SV expressing

XPB-EGFP (Hoogstraten et al., 2002), HeLa cells, Chinese hamster ovary cells: UV135 expressing XPG-EGFP (Zotter et al., 2006) and 43-3B expressing ERCC1-GFP (Houtsmuller et al., 1999). The expression level of all GFP-tagged repair proteins is comparable to the level of endogenous proteins as shown by western blot analysis (D. Hoogstraten, PhD thesis, Erasmus University Rotterdam, 2003) (Hoogstraten et al., 2002; Houtsmuller et al., 1999; Rademakers et al., 2003; Zotter et al., 2006). MRC5-SV cells were cultured in Ham's F12 medium supplemented with 400 µg/ml G418, XP20MA-SV cells were cultured in DMEM supplemented with 600 µg/ml G418 and HeLa cells were cultured in DMEM. The cell lines expressing GFP-tagged NER proteins were cultured in a 1:1 mixture of DMEM:Ham's F10 medium. All media contained glutamine (Gibco, Breda, The Netherlands) supplemented with antibiotics and 10% FCS and all cells were cultured at 37°C in an atmosphere of 5% CO₂.

DNA constructs. Murine DDB2 cDNA was ligated in-frame into pEYFP-N1 and pEYFP-C1 (Moser et al., 2005) (Clontech Laboratories, CA) and stably expressed in MRC5-SV cells and XP-C cells (XP20MA-SV). DDB2 was also ligated with EGFP and mCherry. CUL4A cDNA was ligated in frame with EGFP and transiently transfected into HeLa cells using Lipofectamine 2000 (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. DDB2-EYFP was transiently transfected in XP12RO-SV cells and monitored 5 days after transfection. PCR-based overlap extension (Ho et al., 1989) was used to generate a DDB2 mutant protein with an R273H substitution. Briefly, two products were amplified from the DDB2 gene using two different primer sets, each containing the same nucleotide substitution (details available on request). The first PCR product covers the first 837 bp of the DDB2 gene, whereas the second PCR product encompasses the last 512 bp of the DDB2 gene. Both products contain the region with the G818A mutation that results in an R273H amino acid substitution. In a subsequent 'fusion' reaction, both overlapping products were added together and amplified using PCR. The generated product was subsequently inserted into pEYFP-N1 with *EcoRI* and *BamHI* and transiently transfected into HeLa cells.

Quantification of DDB2-EYFP expression level. To quantify the expression level of DDB2-EYFP, the cellular fluorescence of living cells was compared to the fluorescence of purified EYFP protein from *E. coli*. The concentration of purified EYFP protein was determined by the extinction coefficient of 72,000 M⁻¹.cm⁻¹ (Kremers et al., 2006). Confocal slices of EYFP solutions (1 and 4 µM) and living cells (*n*=44) were quantified using Metamorph software (Molecular Devices Corporation, Sunnyvale, CA).

Immunoblot analysis. Western blot analysis was performed as described previously (Fousteri et al., 2006). DDB2 was detected with mouse polyclonal anti-DDB2 antibodies (1:2000, a gift from A. A. Wani, The Ohio State University, OH), DDB1 was detected with goat polyclonal anti-DDB1 antibodies (1:600, Abcam, Cambridge, MA) and CUL4A was detected with rabbit polyclonal CUL4A antibodies (1:300, Santa Cruz, CA). Protein bands were visualized via chemiluminescence (ECL-Plus, Amersham Biosciences, Uppsala, Sweden) using horseradish peroxidase (HP)-conjugated secondary antibodies and exposure to ECL-Hyperfilms (Amersham Biosciences, Uppsala, Sweden).

Immunofluorescence. Immunolabeling was carried out as described previously (Moser et al., 2005). Primary antibodies used were: mouse monoclonal antibody against XPB (a gift from J. M. Egly, IGMC, Illkirch, France), rabbit polyclonal antibody against DDB2 (Acris, Hiddenhausen, Germany), rabbit polyclonal against CUL4A (1:100, Santa Cruz, CA) and goat polyclonal against DDB1 (1:300, Abcam, Cambridge, MA). Secondary antibody used were: Alexa Fluor 488-conjugated goat anti-mouse antiserum (Molecular probes/Invitrogen, CA) and Cy3-conjugated goat anti-rabbit antiserum (Jackson ImmunoResearch Laboratories, West Grove, PA). All antibodies were diluted in PBS containing 0.15% glycine and 0.5% bovine serum albumin.

Microscopic analysis. Assembly and degradation kinetics were measured on a Zeiss Axiovert 200M wide field fluorescence microscope, equipped with a 100x Plan-Apochromat (1.4 NA) oil immersion lens (Zeiss, Oberkochen, Germany) and a Cairn Xenon Arc lamp with monochromator (Cairn research, Kent, UK). Images were recorded with a cooled CCD camera (Coolsnap HQ, Roper Scientific). FRAP and FLIP experiments were performed on a Zeiss LSM 510 confocal microscope, equipped with a 63x Plan-A (1.4 NA) oil immersion lens (Zeiss, Oberkochen, Germany) and a 60 mW argon laser (488 and 514 nm). Both microscopes were equipped with an objective heater and cells were examined in microscopy medium (137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 20 mM D-glucose and 20 mM Hepes) at 37°C or 27°C.

UV irradiation. For all experiments, cells were irradiated with a UV source containing four UV lamps (Philips TUV 9W PL-S) above the microscope stage. The UV dose rate was measured to be 3 W/m² at

254 nm. For induction of global UV damage, cells were rinsed with microscopy medium and irradiated for 1.5 seconds (4 J/m²), 3 seconds (8 J/m²), 6 seconds (16 J/m²), or 12 seconds (32 J/m²). For induction of local UV damage, cells were UV irradiated through a polycarbonate mask (Millipore Billerica, MA) with pores of 5 µm (Moné et al., 2001) and subsequently irradiated for 39 seconds (100 J/m²).

Fluorescence recovery after photobleaching (FRAP). FRAP analysis was used to measure the mobility of DDB2-EYFP and EGFP-CUL4A as described by Houtsmuller and co-workers (Hoogstraten et al., 2002; Houtsmuller et al., 1999; Rademakers et al., 2003; Zotter et al., 2006). The data were normalized to pre-bleach intensity or to post-bleach intensity after full equilibration using the second data point for normalization to 0. Normalization to post-bleach intensity was used to compare the shape of different FRAP curves.

Fluorescence loss in photobleaching (FLIP). FLIP analysis was performed by continuously bleaching a third of a locally UV-irradiated nucleus as previously described (Essers et al., 2002; Hoogstraten et al., 2002). Fluorescence in the locally damaged area was monitored with low laser intensity. All values were background corrected.

Monte Carlo simulations. For analysis of FRAP data, experimental FRAP curves were normalized to pre-bleach values and corrected for monitor bleaching. FRAP curves were generated by Monte Carlo simulations in which three parameters were varied: diffusion constant (ranging from 0.04 to 36 µm²/second), immobile fraction (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 99.9%) and time spent in an immobile state (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, seconds) (Farla et al., 2004; Zotter et al., 2006). Fluoresce blinking was also simulated based on FRAP measurements on cells expressing EGFP-H2A and EYFP-H2A (Rabut and Ellenberg, 2005). Simulated curves that fitted the experimental curves were selected based on the method of least squares. The reported error is the standard deviation calculated from ten independent simulations. Monte Carlo simulations were based on a model of random diffusion in an ellipsoid volume and simple binding kinetics representing binding to immobile elements. Diffusion was simulated by deriving novel positions $M(x+dx, y+dy, z+dz)$ for all mobile molecules $M(x, y, z)$ after each time step, where $dx=G(r_1)$, $dy=G(r_2)$ and $dz=G(r_3)$, r_i is a random number (0 r_i 1) chosen from a uniform distribution, and $G(r)$ is an inversed cumulative Gaussian distribution with $\mu=0$ and $\sigma^2=6Dt$, where D is the diffusion coefficient and t is time measured in unit time steps. Immobilization was based on simple binding kinetics described by: $k_{on}/k_{off}=F_{imm}/(1-F_{imm})$, where F_{imm} is the relative number of immobile molecules. The chance for each particle to become immobilized was defined as $P_{immobilise}=k_{on}/k_{off} \cdot F_{imm}/(1-F_{imm})$, where $k_{off}=1/t_{imm}$, and t_{imm} is the average time a molecule is immobile, measured in unit time steps. The chance for each particle to be released from an immobile state was defined as: $P_{mobilise}=k_{off}=1/t_{imm}$. The FRAP procedure was simulated on the basis of an experimentally derived three-dimensional laser intensity profile providing a chance for each molecule to get bleached during simulation of the bleach pulse based on the three-dimensional position of the molecule.

Assembly kinetics. Cells were grown in glass bottom dishes (MatTek, Ashland, MA) and locally UV-irradiated as described by van Driel and co-workers (Moné et al., 2004; Zotter et al., 2006). Accumulation after local irradiation was quantified with Object-Image software. Time courses were normalized with respect to the plateau level. Start of the UV irradiation was defined as $t=0$.

Degradation kinetics. To measure the degradation of DDB2-EYFP, cells were globally irradiated with 4, 8, 16 and 32 J/m². For the treatment of cells with proteasome inhibitors, MG-132 (Sigma, Zwijndrecht, The Netherlands) was dissolved in DMSO at 10 mM. Three hours prior to UV irradiation, medium was replaced with microscopy medium containing 50 µM MG132 (Pajonk et al., 2005). Cells were monitored for 6 hours and the fluorescence intensities at every time point were quantified.

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SUPPLEMENTARY FIGURES

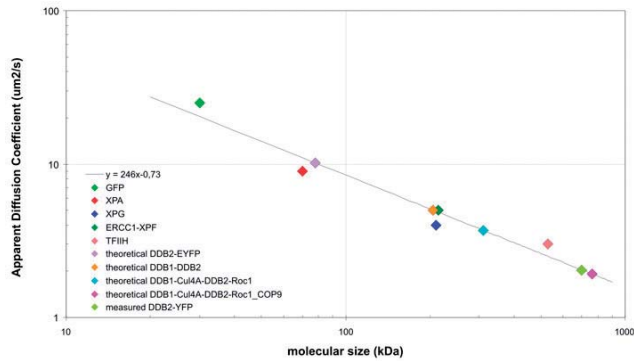


Fig. S1. Relationship between the apparent diffusion constant and the molecular mass of a protein. The mobility of EGFP alone (30 kDa) and of the EGFP-tagged NER proteins XPA (70 kDa), XPG (210 kDa), ERCC1-XPF (215 kDa) and TFIIH (500 kDa) was determined by FRAP and subsequent Monte Carlo simulations, yielding apparent diffusion constants for all fusion proteins and EGFP alone. The data points were fitted with the exponential equation $y=246x^{-0.73}$, where x is the molecular mass in kDa and y the apparent diffusion constant in $\mu\text{m}^2/\text{second}$. Using this equation, diffusion constants were derived for monomeric DDB2 (78 kDa), dimeric UV-DDB (205 kDa), multi-protein complex DDB-CUL4A-ROC1 (310 kDa) and DDB-CUL4A-ROC1 associated with COP9 (760 kDa). The calculated diffusion constants are plotted in the same graph as the measured diffusion constant for DDB2-EYFP that fits well to the expected diffusion constant of DDB-CUL4A-ROC1 associated with COP9. All values include an additional 30 kDa because of the presence of yellow or green fluorescent protein.

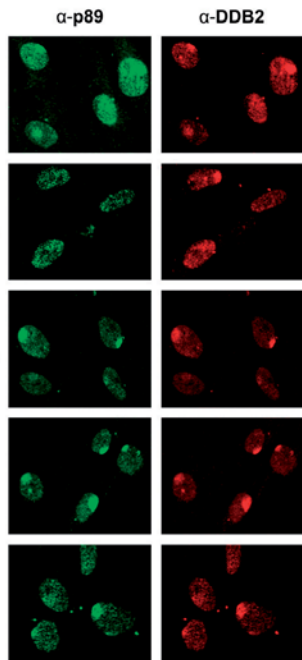


Fig. S2. Accumulation of endogenous DDB2 and TFIIH (p89 subunit) after local UV damage (30 J/m²) in wild-type human cells (VH10) and several NER mutant cell-lines (XP-C, XP-A, XP-G and XP-F). The left column show the immunofluorescent labeling with anti-XPB antibody (green) and the right column shows labeling with anti-DDB2 antibody (red).



