The deubiquitinating protein USP24 interacts with DDB2 and regulates DDB2 stability

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Damage-specific DNA-binding protein 2 (DDB2) was first isolated as a subunit of the UV-DDB heterodimeric complex that is involved in DNA damage recognition in the nucleotide excision repair pathway (NER). DDB2 is required for efficient repair of CPDs in chromatin and is a component of the CRL4DBB2 E3 ligase that targets XPC, histones and DDB2 itself for ubiquitination. In this study, a yeast two-hybrid screening of a human cDNA library was performed to identify potential DDB2 cellular partners. We identified a deubiquitinating enzyme, USP24, as a likely DDB2-interacting partner. Interaction between DDB2 and USP24 was confirmed by co-precipitation. Importantly, knockdown of USP24 in two human cell lines decreased the steady-state levels of DDB2, indicating that USP24-mediated DDB2 deubiquitination prevents DDB2 degradation. In addition, we demonstrated that USP24 can cleave an ubiquitinated form of DDB2 in vitro. Taken together, our results suggest that the ubiquitin-specific protease USP24 is a novel regulator of DDB2 stability.

Introduction

Nucleotide excision repair (NER) is the principal pathway for repair of UV-induced cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts in human cells. UV-DDB (UV-damaged DNA-binding protein) is a heterodimeric protein complex consisting of 127 kDa DDB1 and 48 kDa DDB2,1,4 which participates in damage recognition in global genome (GG)-NER. Mutations in DDB2 result in defective NER, as reflected in the heritable sun-sensitive skin condition, xeroderma pigmentosum group E (XP-E).1,4,5 Cells derived from XP-E patients are deficient in CPD repair and show reduced 6–4PP repair.2,6-9 Deletion of the DDB2 gene in mice significantly impairs the repair of UV lesions and leads to hypersensitivity to UV-induced skin cancers.10

DDB2 is in complex with the CUL4-RING E3 ubiquitin ligase complex (CRL4DBB2), consisting of DDB1, CUL4 and RBX1.11-15 DDB2 is thought to be the substrate receptor targeting the E3 ligase complex to DNA damage sites to facilitate GG-NER. Of note, DDB1 and CUL4 have been shown to be in complex with other proteins, including CSA, a transcription-coupled, NER-specific protein.11,15 Consistent with its classification as an E3 ligase, XPC, histone H2A, H3, H4 and DDB2 itself have been identified as ubiquitination targets of the CRL4DBB2 E3 ligase complex.16,17,22 The E3 ligase CRL4DBB2 is found in complex with the COP9 signalosome (CSN).19-21 In the absence of UV damage, CSN is associated with CRL4DBB2 and regulates its E3 ligase activity by de neddylation. After UV irradiation, CSN disassociates from CRL4DBB2, allowing DDB2 binding to the damage sites and subsequent DDB2 ubiquitination by CRL4DBB2.24,26

Several lines of evidence suggest that DDB2 plays a key role in the repair of UV damage only in the context of chromatin. Although UV-DDB binds strongly to UV-damaged DNA,27-31 it stimulates NER of naked DNA only slightly in vitro.32-34 XP-E cell extracts display proficient NER of naked DNA in vitro, suggesting that UV-DDB has a role in the repair of DNA in chromatin.35 DDB2 binds the lesion independent of XPC,36 and XPC recruitment to UV damage is significantly decreased in the absence of functional DDB2.10,37,38 DDB2 can co-localize with both CPDs and 6-4PPs in vivo, while XPC seems to bind 6-4PPs efficiently, but not CPDs. This suggests the necessity of DDB2 in GG-NER is specific for CPD repair.38 Importantly, it has been suggested that the observed high affinity of DDB2 for 6-4PPs aids in the targeting of XPC to 6-4PPs when low levels of damage are present.39 DDB2 autoubiquitination leads to the loss of DNA damage binding and rapid DDB2 degradation.16,19,40-42 XPC ubiquitination, in contrast, retains the complex at the site of UV damage without immediate proteasomal degradation. The differential response of XPC and DDB2 upon ubiquitination has been linked to an ubiquitin-dependent damage handover from DDB2 to XPC.16,43,44 Recent findings show that UV-DDB associates preferentially with lesions in internucleosomal sites. In addition, UV-DDB and DDB2 ubiquitination are required to retain XPC at the linker regions. However, while UV-DDB facilitates XPC binding to nucleosomal DNA lesions, this does not appear to require DDB2 ubiquitination.45 Luijsterburg et al. demonstrated that chromatin regions containing UV lesions undergo ATP-dependent chromatin decondensation that is strictly dependent on the presence of
p21.50-52 DDB2 is involved in SOD2 transcription and stimulates the DNA damage response as well, via regulation of remodeling factors.47,48 Furthermore, DDB2 has been shown to associate with the histone acetyltransferases CBP/p300. 41,49

Besides its role in NER, DDB2 is involved in cellular processes. DDB2 ubiquitination is mediated by the CRL4DDB2 E3 ligase, and its ubiquitination and subsequent degradation are essential for its functions in NER. USP24 belongs to the ubiquitin-specific protease family of deubiquitinating enzymes (DUBs) and contains the characteristic Cys and His motifs at the core enzymatic domain.58 It has the ability to remove ubiquitin from an ubiquitinated substrate, thereby regulating the stability and activity of this substrate. Since DDB2 is also ubiquitinated in human cells in the absence of exogenous DNA damage,60 our identified interaction between DDB2 and USP24 immediately suggests a novel mechanism by which the ubiquitin moiety attached to DDB2 can be removed to prevent DDB2 degradation. To determine the functional relevance of DDB2-USP24 interactions, we tested whether USP24 affects the state-dependent levels of DDB2. As indicated in Figure 3A–C, knockdown of USP24 decreased the steady-state levels of DDB2 in both HeLa and 293T cells. Importantly, knockdown of USP24 using three different USP24-specific siRNAs all led to decreased levels of DDB2 in HeLa and 293T cells, when compared with the control siRNA-transfected cells (Fig. 3B and C). In contrast, USP24 knockdown had no detectable effect on the levels of XPC in the two cell lines, another DNA lesion recognition protein which is also ubiquitinated by CL4DDB2, suggesting a specific role of USP24 in the control of DDB2 stability (Fig. 3B and C). We note that, although UV irradiation induces the levels of ubiquitination of DDB2 and XPC,16,22 both proteins are ubiquitinated in human cells in the absence of exogenous DNA damage.60

Results

Identify new DDB2-interacting proteins using yeast-two hybrid screening. Besides its role in NER, DDB2 is involved in proteolysis51 and transcriptional regulation.57,58 In order to gain a comprehensive picture of DDB2 functions, we took the yeast two-hybrid screen approach to establish a DDB2 protein interaction network. We screened a normalized universal human cDNA library using the full-length DDB2 as the bait. In silico search of the NCBI database allowed the identification and classification of potential DDB2-interacting proteins into 11 diverse processes (Fig. 1). These processes include transcription and RNA metabolic process, protein degradation, metabolism, cell cycle, cell differentiation, apoptosis, DNA repair and signal transduction, cell adhesion and motility, cellular component. These data suggest that DDB2 may play previously unknown roles in many cellular processes and provide a framework for the understanding of DDB2 functions.

DDB2 interacts with USP24. Among the potential DDB2-interacting proteins identified by the yeast-two hybrid screen, there are two ubiquitin-specific proteases (USPs), USP24 and USP53. USPs recognizes and removes the ubiquitin moiety from proteins.59 The substrate of USP24 was previously unknown. To investigate whether DDB2 is a direct target of USP24, we set out to evaluate interactions in vivo by co-immunoprecipitation. By using the USP24-specific antibody, we examined the interaction between the endogenous DDB2 and USP24 proteins. Western blot analysis showed that USP24 was present in the anti-DDB2 immunoprecipitates from HeLa cell extracts, but not in the control immunoprecipitates (Fig. 2A). Reciprocal co-immunoprecipitation experiment showed that DDB2 was detected in the anti-USP24 immunoprecipitates (Fig. 2B). To avoid detecting the 55 KDa IgG heavy chains during western blotting, we expressed a His-tagged DDB2 in HeLa cells and precipitated His-DDB2 using Ni-NTA beads. Western blot showed again that USP24 was present in the His-DDB2 precipitates (Fig. 2C). Importantly, USP24 was not present in the precipitates from extracts prepared from HeLa cells without His-DDB2 expression (Fig. 2C). These observations suggest that USP24 interacts with DDB2 in vivo, as suggested by our yeast two-hybrid screen.

USP24 regulates the stability of DDB2 in vivo. Ubiquitination is crucial in the physiological regulation of many cellular processes. DDB2 ubiquitination is mediated by the CRL4DDB2 E3 ligase, and its ubiquitination and subsequent degradation are essential for its functions in NER. USP24 belongs to the ubiquitin-specific protease family of deubiquitinating enzymes (DUBs) and contains the characteristic Cys and His motifs at the core enzymatic domain.58 It has the ability to remove ubiquitin from an ubiquitinated substrate, thereby regulating the stability and activity of this substrate. Since DDB2 is also ubiquitinated in human cells in the absence of exogenous DNA damage,60 our identified interaction between DDB2 and USP24 immediately suggests a novel mechanism by which the ubiquitin moiety attached to DDB2 can be removed to prevent DDB2 degradation. To determine the functional relevance of DDB2-USP24 interactions, we tested whether USP24 affects the state-dependent levels of DDB2. As indicated in Figure 3A–C, knockdown of USP24 decreased the steady-state levels of DDB2 in both HeLa and 293T cells. Importantly, knockdown of USP24 using three different USP24-specific siRNAs all led to decreased levels of DDB2 in HeLa and 293T cells, when compared with the control siRNA-transfected cells (Fig. 3B and C). In contrast, USP24 knockdown had no detectable effect on the levels of XPC in the two cell lines, another DNA lesion recognition protein which is also ubiquitinated by CL4DDB2, suggesting a specific role of USP24 in the control of DDB2 stability (Fig. 3B and C). We note that, although UV irradiation induces the levels of ubiquitination of DDB2 and XPC,16,22 both proteins are ubiquitinated in human cells in the absence of exogenous DNA damage.60
USP53 interacts with DDB2, but knockdown of USP53 has no effect on DDB2 stability. USP53 is another deubiquitinating enzyme identified in the yeast two-hybrid screening. Interestingly, we also confirmed that USP53 interacts with DDB2 by co-immunoprecipitation (Fig. 4A). However, knockdown of USP53 expression in HeLa cells had no detectable effect on XPC and DDB2 expression (Fig. 4B). Thus, the physiological relevance of USP53-DDB2 interactions is unclear.

USP24 cleaves an ubiquitinated form of DDB2 in vitro. To examine if USP24 can target ubiquitinated DDB2 in vitro, we treated HeLa cells expressing His-tagged DDB2 with MG132, a proteasome inhibitor, for 1 h and exposed HeLa cells to UVC (10 J/m²) to stimulate DDB2 ubiquitination for another hour, Ni-NTA beads were then used to pull down modified His-DDB2. Using a His-tag-specific antibody, we detected a ~110 KDa band by western blot (Fig. 4C). Identical band could be picked up using an antibody specific for ubiquitin (data not shown), indicating that this is an ubiquitinated form of His-DDB2. Interestingly, addition of USP24 precipitated by an anti-USP24 antibody led to cleavage of this ubiquitinated form of His-DDB2, with the appearance of several bands corresponding to ubiquitin chains of different sizes (Fig. 4C). Thus, USP24 can cleave an ubiquitinated form of DDB2 in vitro.

USP24, a nuclear protein, does not bind to UV damage sites. Since DDB2 is known to bind to UV damage sites in human cells and UV-stimulated DDB2 ubiquitination is believed to occur at sites of DNA damage, we next examined if USP24 also binds to UV damage sites. Immunofluorescence staining showed that both USP24 and DDB2 were present in the nucleus, suggesting that both are nuclear proteins (Fig. 5A). However, unlike DDB2, which is recruited to CPD sites 15 min after UV irradiation (Fig. 5B), USP24 did not accumulate at the CPD sites (Fig. 5B). We also performed a time-course experiment but failed to detect USP24 accumulation at UV damage sites up to 3 h after UV irradiation (data not shown). These results suggest that USP24-mediated deubiquitination of modified DDB2 does not occur at the sites of DNA damage.

Discussion

In this study, we identified a novel regulator of DDB2 stability using a yeast two-hybrid approach. We demonstrated biochemically that USP24 binds to DDB2 and regulates DDB2 stability in human cells. Our data suggest that USP24 stabilizes DDB2 by removing the ubiquitin moiety from modified DDB2, thereby preventing DDB2 degradation. As shown in Figure 1, identified potential DDB2-interacting proteins largely fall into 11 classes, with some overlaps, on the basis of the cellular processes in which they are involved. These protein interaction data indicate that DDB2 may play other novel roles in human cells.

Key players in essential pathways are often subject to ubiquitin regulation mediated by one or several ubiquitin ligases or deubiquitinating enzymes (DUBs). Ubiquitin-specific proteases are a family of DUBs that participate in the removal of ubiquitin and ubiquitin-like modifications from an ubiquitinated substrate, thereby regulating the localization and activity of this substrate. Likewise, these proteases remove polyubiquitin from target proteins, rescuing them from degradation by the proteasome. The human genome encodes about 95 functional DUBs, and only a few of those have been assigned functions or substrates. For example, USP7 has been shown to deubiquitinate p53, with overexpression of USP7 resulting in p53 stabilization. In recent years, several USPs have been found to participate in the DNA damage response, including USP1, USP7, USP10, USP13, USP28, and USP29. The function and substrate of
ubiquitin moiety from ubiquitinated DDB2, thereby preventing DDB2 degradation by the proteosomes (Fig. 6). Interestingly, we identified COPS2/CSN2 from the yeast two-hybrid screen that may interact with DDB2 (data not shown). CSN2 is a subunit of CSN, an eight-subunit isopeptidase complex. CSN, via the proteolytic activity of its CSN5 subunit, removes the ubiquitin-like NEDD8 from cullins. Removal of NEDD8 consequently abrogates the stimulating effect of neddylation on cullin-based ubiquitin ligases. Recruitment of ubiquitinated DDB2 in vitro by USP24. Ubiquitinated DDB2 was purified from UV-treated HeLa cells expressing Hig-tagged DDB2 in the presence of MG132. USP24 was partially purified from HeLa cells by immunoprecipitation using an USP24 antibody and protein G beads, followed by extensive washing to remove its binding partners. Cleavage reactions were performed at 37°C for 1 h.

Figure 3. USP24 depletion in human cells destabilizes DDB2. (A) siRNA-mediated USP24 depletion in 293T cells decreases the steady-state levels of DDB2. (B) USP24 knockdown in 293T cells by three siRNAs destabilizes DDB2, but not XPC, in 293T cells. (C) Knockdown of USP24 in HeLa cells by three siRNAs and its effect on the steady-state levels of DDB2 and XPC.

Figure 4. (A) Co-immunoprecipitation of USP53 and DDB2: IP was performed using an anti-USP53 antibody, following by western blot (WB) using both USP53 and DDB2 antibodies. (B) Knockdown of USP53 in HeLa cells has no detectable effect on the steady-state levels of DDB2 and XPC. (C) Cleavage of ubiquitinated DDB2 in vitro by USP24. Ubiquitinated DDB2 was purified from UV-treated HeLa cells expressing Hig-tagged DDB2 and its effect on the steady-state levels of DDB2 and XPC. USP24 depletion in human cells destabilizes DDB2. 293T cells. USP24 1 2 3 USP24 DDB2 Tubulin 293T cells USP24 DDB2 Tubulin 293T cells USP24 DDB2 Tubulin HeLa cells USP24 DDB2 Tubulin HeLa cells

USP24 were previously unknown. DDB2 represents the first example of a specific substrate that USP24 can directly deubiquitinate and stabilize in human cells.

Since DDB2 is a component of the CRL$^\text{4DDB2}$ E3 ligase and one physiological substrate of this E3 ubiquitin ligase complex is DDB2 itself, it will be interesting to explore the regulation of DDB2 with or without DNA damage. Our results suggest that DDB2 modified by ubiquitination can be recycled by USP24 (Figs. 3 and 4). We are investigating the involvement of USP24 in the control of DDB2 stability after UV irradiation. We speculate that there is a basal level of DDB2 ubiquitination in human cells, presumably mediated by the same CRL$^\text{4DDB2}$ E3 ligase. Indeed, mass spectrometric analysis showed that DDB2 is also ubiquitinated in the cells in the absence of exogenous DNA damage. Thus, USP24 plays an important role in the maintenance of steady-state levels of DDB2 by constantly removing the ubiquitin moiety from ubiquitinated DDB2, thereby preventing DDB2 degradation by the proteosomes (Fig. 6).

Interestingly, we identified COPS2/CSN2 from the yeast two-hybrid screen that may interact with DDB2 (data not shown). CSN2 is a subunit of CSN, an eight-subunit isopeptidase complex. CSN, via the proteolytic activity of its CSN5 subunit, removes the ubiquitin-like NEDD8 from cullins. Removal of NEDD8 consequently abrogates the stimulating effect of neddylation on cullin-based ubiquitin ligases. Recruitment of CRL$^\text{4DDB2}$ to sites of DNA damage in chromatin appears to correlate with CSN release from the E3 ligase. Consistent with our data, CSN was co-immunoprecipitated with the DDB2 E3 complex from undamaged cells. Our data further suggest that CSN2 may mediate the interaction between CSN complex and DDB2 E3 ligase to inhibit the basal level DDB2 ubiquitination (Fig. 6). Therefore, both CSN and USP24 are important for the
control of the steady-state levels of DDB2: (1) CSN-binding to DDB2 E3 ligase presumably inhibits DDB2 ubiquitination by silencing the stimulating effect of neddylation on the E3 ligase; and (2) ubiquitinated DDB2 can be recycled by USP24 to prevent degradation (Fig. 6).

Materials and Methods

Yeast two-hybrid screen. Matchmaker Gold yeast two-hybrid screening was performed according to manufacturer’s instruction. Briefly, the full-length of the coding region of DDB2 was inserted in frame into the multiple cloning sites of the DNA-BD vector, pGBKt7 (Clontech), to generate the bait plasmid pGBKt7-DDB2, which was subsequently confirmed by sequencing. The pGBKt7-DDB2 was transformed into the bait strain Y2HGold. DDB2 bait strain Y2HGold was mated with the pre-transformed Y187/pACT2 normalized universal human Mate & Plate cDNA library according to the Clontech protocol. Diploid yeast cells were plated on a nutrient deficiency medium SD plate without Trp and Leu (DDO) and analyzed for their ability to grow in the presence of highly toxic drug Aureobasidin A (125 ng/ml, Clontech) and regulate α-galactosidase expression, which hydrolyzes 5-bromo-4-chloro-3-indolyl-a-d-galactopyranoside (X-α-gal, 40 μg/ml; Clontech) to produce a blue-end product. The selected colonies were restreaked on SD plate without Trp, Leu, His and Ade (QDO) containing Aureobasidin A and X-α-gal for further selection.

Sequencing and sequencing data analysis. Plasmid DNA from yeast was isolated and transformed into Escherichia coli DH5α for propagation. Plasmid DNA from E. coli was then sequenced (www.genewiz.com/). Nucleotide and deduced protein sequences were identified using BLAST (www.blast.ncbi.nlm.nih.gov/) and online services of EMBL-EBI (www.ebi.ac.uk/QuickGO/).

Cell culture. HeLa and 293T cells were obtained from ATCC. All cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) at 37°C and 5% CO₂.

Co-immunoprecipitation. Cells from two 10-cm culture dishes were obtained and lysed with co-IP buffer (20 mM HEPES, 0.2 mM EDTA, 5% glycerol, 150 mM NaCl, 1% NP40, Roche complete EDTA-free protease inhibitor cocktail). Lysates were cleared by centrifugation (13,000 rpm, 10 min); the supernatant was incubated with USP24 antibody or DDB2 antibody overnight at 4°C and pulled down with protein G agarose beads (Millipore). Beads were washed three times with co-IP buffer and one time with TE buffer and eluted with one bead volume of 2X Laemmli SDS sample buffer at 95°C.

RNA interference. USP24 siRNA oligonucleotides and nonspecific siRNA were obtained from Sigma in a purified and annealed duplex form. The sequences, targeting three different

Figure 5. USP24, localized primarily in the nucleus, does not bind to UV damage sites. (A) Immunofluorescence staining of USP24 and DDB2. DAPI nucleus staining was shown. (B) Micropore UV irradiation followed by immunofluorescence staining using antibodies specific for USP24, UV lesion CPDs and DDB2.

Figure 6. A model depicting the involvement of USP24 in DDB2 recycling. A low level of DDB2 is ubiquitinated continuously by the DDB1-CRL4E3 E3 ligase. We propose that USP24 deubiquitinates modified DDB2 and prevent its degradation by the proteasome. CSN-binding to DDB2 E3 ligase presumably inhibits DDB2 ubiquitination by silencing the stimulating effect of neddylation on the E3 ligase. Our yeast two-hybrid screen data suggest that interaction between the CRL4DDB2 E3 ligase and the CSN complex is bridged by the CSN2 subunit of CSN and DDB2.
regions of USP24 gene, were as follows: duplex-1, 5'-GAA ACU CAG GGU UAC UAC U-3'; duplex-2, 5'-CUU CUA CUG UGG CUU GCU U-3'; duplex-3, 5'-GCA CAA UAC UGU GAC CGA U-3'. siRNA transfections were performed with RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Antibodies, microprobe UV irradiation and immunofluorescence staining. Protein concentrations of the cell lysates were quantified and separated by SDS-PAGE. Immunoblot analysis was performed using chemiluminescent detection. The antibodies were obtained from following sources: USP24 (1:1,000, Novus, Cat No: NB100–40830), DDB2 (1:500, Santa Cruz biotechnology, Cat No: sc-81246). Microprobe UV irradiation and immunofluorescence staining were performed as described previously.47

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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