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The Ubiquitin Ligase Activity in the DDB2 and CSA Complexes Is Differentially Regulated by the COP9 Signalosome in Response to DNA Damage

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Summary

Nucleotide excision repair (NER) is a major cellular defense against the carcinogenic effects of ultraviolet light from the sun. Mutational inactivation of NER proteins, like DDB and CSA, leads to hereditary diseases such as xeroderma pigmentosum (XP) and Cockayne syndrome (CS). Here, we show that DDB2 and CSA are each integrated into nearly identical complexes via interaction with DDB1. Both complexes contain cullin 4A and Roc1 and display ubiquitin ligase activity. They also contain the COP9 signalosome (CSN), a known regulator of cullin-based ubiquitin ligases. Strikingly, CSN differentially regulates ubiquitin ligase activity of the DDB2 and CSA complexes in response to UV irradiation. Knockdown of CSN with RNA interference leads to defects in NER. These results suggest that the distinct UV response of the DDB2 and CSA complexes is involved in diverse mechanisms of NER.

Introduction

Nucleotide excision repair (NER) acts on a wide variety of helix-distorting DNA lesions, including the cyclobutane pyrimidine dimers (CPDs) and (6–4) pyrimidine-pyrimidone photoproducts induced by UV light (Friedberg et al., 1995; Svejstrup, 2002). The core reaction of NER involves removal of an approximately 27–30 nt oligonucleotide fragment containing the photoproduct. This excision reaction occurs by positioned hydrolysis of the phosphodiester bonds at precise distances 5′ and 3′ to the lesion, exclusively on the damaged DNA strand. Once the damage-containing fragment is excised, the gap generated in the DNA duplex is repaired by DNA synthesis using the opposite, normal DNA strand as a template (Friedberg et al., 1995; Svejstrup, 2002). It has been shown that NER can operate via two pathways: global genome repair (GGR) and transcription-coupled repair (TCR). GGR repairs the DNA damage over the entire genome, while TCR removes DNA lesions significantly faster from the transcribed strand of active genes than from the nontranscribed strand or the bulk of DNA (Friedberg et al., 1995; Svejstrup, 2002).

Various types of NER-defects are found in individuals with the inherited syndromes xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) (Bootsma et al., 1998). XP patients are sun sensitive and generally show a greatly increased incidence of UV-induced skin cancers. Cell fusion studies have identified seven XP complementation groups (XP-A through XP-G) and a variant form (XP-V). In general, patients with XP-A, -B, -D, -F, and -G are defective in both TCR and GGR, while those with XP-C are defective only in GGR (Bootsma et al., 1998). XP-V defines patients with the clinical symptoms of XP, which shows normal NER but defective *trans*-lesion DNA synthesis (Friedberg et al., 2002).

XP-E cells are biochemically heterogeneous and some, but not all, XP-E cell lines lack an activity that binds to damaged DNA (Friedberg et al., 1995; Bootsma et al., 1998). This activity is associated with a UV-damaged DNA binding protein (DDB) composed of the DDB1 (or p127) and DDB2 (or p48) subunits (Keeney et al., 1993). Mutations in the *DDB2* gene, which encodes a WD40 repeat-containing protein, have been found in XP-E cell lines that are lacking the damaged DNA binding activity. The absence of this activity has been linked to the deficiency in GGR of CPDs in these XP-E cells (Friedberg et al., 1995; Bootsma et al., 1998). Although the exact mechanisms whereby DDB contributes to NER are not clear, DDB has been shown to stimulate binding of XPA and replication protein A (RPA) to damaged DNA (Wakasugi et al., 2001). This result suggests that DDB stimulates NER by recruiting core NER factors to damaged DNA via interaction with XPA and RPA.

Like XP patients, CS patients show hypersensitivity to sunlight but have no predisposition to skin cancer. However, CS patients show a distinctive array of severe developmental and neurological abnormalities as well as premature aging (Friedberg et al., 1995; Bootsma et al., 1998). Classical CS is caused by mutations in either the *CSA* or *CSB* genes. *CSA*- and *CSB*-deficient cells (CS-A and CS-B, respectively) are proficient in GGR but show a defect in TCR. While mutations in the *CSA* and *CSB* genes account for over 90% of CS patients, mutations in the *XPB*, *XPD*, and *XPG* genes also underlie a small number of CS cases (XP-B/CS, XP-D/CS, and XP-G/CS, respectively) (Friedberg et al., 1995; Bootsma et al., 1998). Of these gene products, CSB, XPB, XPD, and XPG appear to play a role in general transcription (reviewed in Selby and Sancar, 1997; Lee et al., 2002), suggesting that transcriptional defects are the underlying cause of growth and developmental defects in CS. Moreover, CSB, XPB, XPD, and XPG appear to be involved in TCR of oxidative lesions such as thymine glycol and 8-oxoguanine. TCR of oxidative damage in a transcribed sequence has been shown to be defective in CS-B, XP-B/

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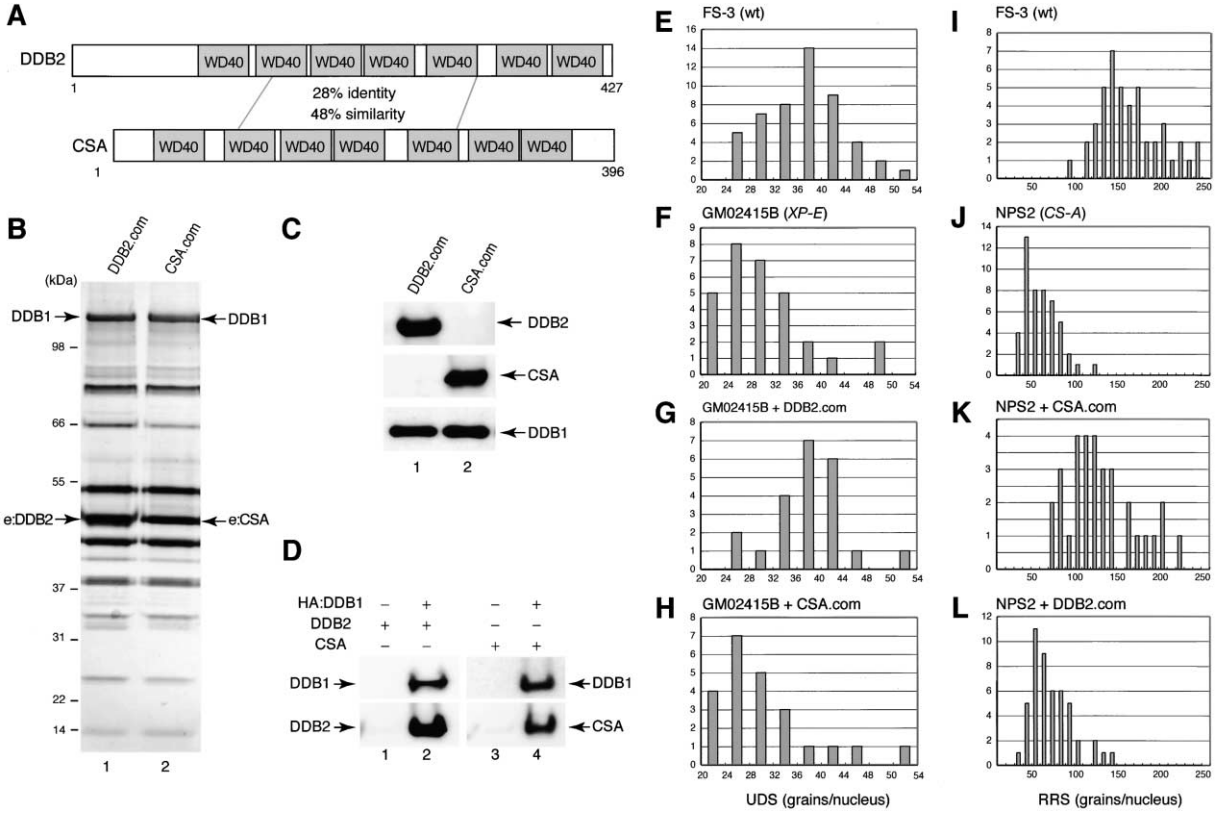

Figure 1. DDB2 and CSA Are Integrated into Nearly Identical Complexes via Interaction with DDB1

(A) Schematic representation of the comparative analyses of DDB2 and CSA. DDB2 and CSA share sequence similarity in the restricted region. Positions of predicted WD40 repeats are shown.

(B) Silver staining of the DDB2 and CSA complexes. The DDB2 and CSA complexes were immunopurified from nuclear extracts prepared from HeLa cells expressing FLAG-HA-epitope-tagged DDB2 and CSA, respectively. The complexes were separated on a 10%–30% glycerol gradient by ultracentrifugation, and the peak fractions of the DDB2 (lane 1) and CSA (lane 2) complexes were resolved by SDS-PAGE and visualized by silver staining.

(C) Both of the DDB2 and CSA complexes contain DDB1. The DDB2 (lane 1) and CSA (lane 2) complexes were immunoblotted against anti-DDB2 (top), anti-CSA (middle), and anti-DDB1 (bottom) antibodies.

(D) DDB1 directly binds not only to DDB2, but also to CSA. HA-tagged DDB1 was coexpressed with DDB2 (lane 2) or CSA (lane 4) in SF9 cells via the baculovirus expression system. As controls, DDB2 (lane 1) and CSA (lane 3) were expressed by themselves. After immunoprecipitating with anti-HA antibody, the precipitated materials were analyzed by immunoblotting with anti-HA (top), anti-DDB2 (bottom, lanes 1 and 2), or anti-CSA (lanes 3 and 4) antibodies.

(E–L) The DDB2 and CSA complexes contribute to GGR and TCR, respectively. Histograms show the UV-induced unscheduled DNA synthesis (UDS) (E–H) and the recovery of RNA synthesis after UV irradiation (RRS) (I–L) in normal FS-3 (E and I), GM02415 XP-E (F, G, and H), and NPS2 CS-A (J, K, and L) fibroblasts. The DDB2 (G and L) or CSA (H and K) complex was microinjected into respective fibroblasts. Ordinate indicates frequency of nuclei with grain numbers shown on the abscissa.

CS, XP-D/CS, and XP-G/CS cells (Cooper et al., 1997; Le Page et al., 2000). Therefore, defective TCR of oxidative lesions, perhaps in addition to defective transcription, may be the underlying cause of CS.

Here, we report purification of DDB2 and CSA, which play roles in GGR and TCR, respectively. Strikingly, DDB2 and CSA associate with an identical set of polypeptides, making the only distinction between the two complexes DDB2 and CSA themselves. Like DDB2, CSA binds directly to DDB1; thus DDB2 and CSA appear to be integrated into nearly identical complexes. Nevertheless, biochemically, these complexes are quite different, serving distinct functions in GGR and TCR. The results provide new insights into mechanisms whereby DDB2 and CSA, respectively, contribute to GGR and TCR.

Results

Purification of Functional Complexes Containing DDB2 and CSA

DDB2 and CSA, which respectively contribute to GGR and TCR, have WD40 repeats and share 48% sequence similarity in the middle region containing the second to fifth WD40 repeats (Figure 1A). To explore the molecular roles of these factors, we purified them as multimeric complexes. DDB2 and CSA were stably expressed as FLAG-HA-epitope fusions (e-DDB2 and e-CSA) in HeLa cells by retroviral transduction. e-DDB2 and e-CSA were each purified from the corresponding nuclear extracts by immunoprecipitation with anti-FLAG antibody followed with anti-HA antibody. The purified complexes

were further separated on a 10%–30% glycerol gradient by ultracentrifugation. Silver staining of the peak fraction of each complex is shown in Figure 1B. As a control, we performed mock purification from nontransduced HeLa cells; we detected no polypeptides by silver staining (data not shown; see Ikura et al., 2000; Ogawa et al., 2002), indicating that all detectable polypeptides in these complexes are specific to e-DDB2 or e-CSA. Surprisingly, all the polypeptides associated with e-DDB2 and e-CSA are indistinguishable on the SDS-PAGE gel. Nevertheless, immunoblotting shows no detectable CSA in the DDB2 complex or DDB2 in the CSA complex (Figure 1C), indicating that these complexes are distinct and independent.

DDB2 and CSA Both Interact with DDB1

Using mass spectrometric analyses, DDB1 was detected in the 127 kDa band of both DDB2 and CSA complexes (Figure 1B). Consistently, immunoblotting with anti-DDB1 antibody identified DDB1 in both complexes (Figure 1C). While DDB2 has been shown to be a heterodimeric partner for DDB1 (Keeney et al., 1993), to our knowledge a relationship between CSA and DDB1 has not previously been reported. Our data suggest that CSA, which possesses sequence similarity to DDB2 (Figure 1A), also interacts with DDB1. To test this possibility, HA-epitope-tagged DDB1 was coexpressed with DDB2 or CSA in Sf9 cells (Figure 1D). Like DDB2, CSA was coimmunoprecipitated with HA-DDB1. Thus, we conclude that CSA and DDB2 are each integrated into similar complexes via interaction with DDB1.

The DDB2 and CSA Complexes Play Roles in GGR and TCR, Respectively

Because of the striking similarities between the DDB2 and CSA complexes, we tested whether these complexes can specifically complement NER defects when they are microinjected into the corresponding mutant cells. First, NER activity in DDB-deficient XP-E fibroblasts (GM02415B) was determined by UV-induced unscheduled DNA synthesis (UDS) that mainly reflects GGR (Carreau and Hunting, 1992). Consistent with the fact that XP-E patients exhibit the mildest clinical signs of UV sensitivity and skin cancer among XP groups, GM02415B fibroblasts retained 82% of NER activity compared with FS-3 normal fibroblasts (Figures 1E and 1F). Importantly, microinjection of the DDB2 complex into GM02415B fibroblasts restored UDS to 102% of the level in the normal fibroblasts (Figure 1G), whereas microinjection of the CSA complex showed negligible effects (Figure 1H).

Next, we tested the effects of the DDB2 and CSA complexes on the TCR of CS-A fibroblasts. Since lack of recovery of RNA synthesis after UV irradiation (RRS) is ascribed to a defect in TCR (Troelstra et al., 1992), we estimated RRS as a measure of TCR. In Nps2 CS-A fibroblasts, RRS declined to 41% of the level in the normal fibroblasts (Figures 1I and 1J). As expected, microinjection of the CSA complex into Nps2 fibroblasts restored RRS to 84% of that level (Figure 1K), whereas the DDB2 complex had almost no effect (Figure 1L). Thus, we conclude that the DDB2 and CSA complexes we purified are biologically functional and play roles in GGR and TCR, respectively.

The DDB2 Complex Binds Tightly to Chromatin in a UV-Dependent Manner

To better understand how DDB2 contributes to GGR, we attempted to purify it following UV irradiation. Our preliminary experiments revealed that most of the DDB2 protein is present in the nuclear pellet fraction after UV irradiation. Empirically, we found that DDB2 can be efficiently solubilized together with mononucleosomes by digesting the pellet fraction with excess amounts of micrococcal nuclease. This allowed us to prepare a solubilized chromatin fraction from the nuclear pellet fraction. As shown in Figure 2A, the majority of DDB2 was found in the nuclear extract fraction prior to UV irradiation (lane 1). However, after UV irradiation, DDB2 was primarily detected in the solubilized chromatin fraction (lane 4). Importantly, histones H2A, H2B, H3, and H4 copurified with DDB2 from the solubilized chromatin fraction after UV irradiation (lane 4), suggesting that DDB2 is tightly associated with the UV-damaged chromatin. Moreover, only a subset of the subunits in the DDB2 complex was stoichiometrically purified from the solubilized chromatin fraction (compare lanes 1 and 4) (discussed later).

Unlike the DDB2 complex, the CSA complex did not copurify with nucleosomes after UV irradiation (data not shown). We confirmed these binding specificities in vitro by performing gel-shift experiments (Figure 2B). As reported for the DDB1/DDB2 heterodimer (Keeney et al., 1993), the DDB2 complex bound to DNA in a UV-damage-dependent manner (lanes 5 and 6). In contrast, the CSA complex did not bind to DNA regardless of UV damage (lanes 3 and 4). These results suggest that DDB2, rather than DDB1, plays a role in recognition of UV-damaged DNA.

The CSA Complex Binds to RNA Polymerase II

We next purified the CSA complex from cells that either had or had not been UV irradiated. In contrast to the DDB2 complex (Figure 2A), a significant amount of the CSA complex was purified from the solubilized chromatin fraction regardless of UV irradiation (see Figure 5B). However, a 240 kDa band, which associates substoichiometrically with the CSA complex, was predominantly found in the complex purified from UV-irradiated cells (Figure 2C). Mass spectrometric analyses identified the largest subunit of RNA polymerase II in this band. Consistent with these data, immunoblot analysis revealed that the hyperphosphorylated form of the largest subunit of RNA polymerase II (the IIo form), which is predominantly present in transcription elongation complexes (Reinberg et al., 1998), in the CSA complex drastically increased after UV irradiation (Figure 2D, lane 4). In contrast, similar amounts of the hypophosphorylated form of the largest subunit of RNA polymerase II (the IIa form) were found in the CSA complex regardless of UV irradiation (lanes 3 and 4). In contrast to the CSA complex, only a trace amount of the largest subunit of RNA polymerase II was found in the DDB2 complex (lanes 1 and 2). Taken together, these results indicate

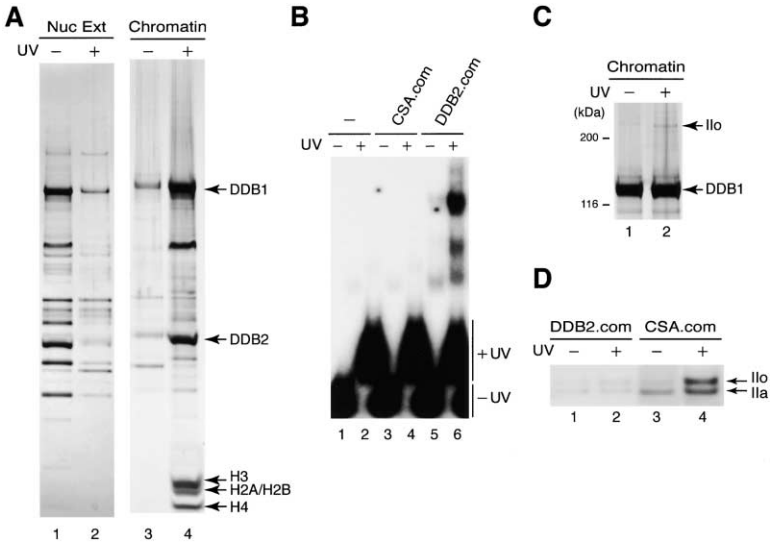

Figure 2. The DDB2 and CSA Complexes Bind to Distinct Targets

(A) The DDB2 complex tightly binds to chromatin in vivo after UV irradiation. The DDB2 complex was purified from the nuclear extract (lanes 1 and 2), and solubilized chromatin (lanes 3 and 4) fractions were prepared from cells subjected to UV irradiation (lanes 2 and 4) or from control cells (lanes 1 and 3). The purified complexes were resolved by SDS-PAGE and visualized by silver staining. The positions of DDB1, DDB2, and core histones are shown.

(B) The DDB2 complex, but not the CSA complex, binds to UV-damaged DNA in vitro. ^{32}P-labeled DNA probe from cells without (lanes 1, 3, and 5) and with (lanes 2, 4, and 6) UV irradiation were incubated without (lanes 1 and 2) and with the DDB2 (lanes 3 and 4) or CSA (lanes 5 and 6) complexes. The products were resolved by native PAGE and visualized by autoradiography. The positions of free probes without and with UV irradiation are shown.

(C) The CSA complex binds to RNA polymerase IIo in an UV-irradiation-dependent manner. The CSA complex was purified from the solubilized chromatin fraction prepared from cells subjected to UV irradiation (lane 2) or from control cells (lane 1). The positions of DDB1 and RNA polymerase IIo are shown. (D) The CSA complex, but not the DDB2 complex, binds to RNA polymerase II in vivo. The DDB2 (lanes 1 and 2) and CSA (lanes 3 and 4) complexes purified from the solubilized chromatin fraction prepared from UV-irradiated cells (lanes 2 and 4) and nonirradiated cells (lanes 1 and 3). The purified complexes were analyzed by immunoblotting with antibody against the largest subunit of RNA polymerase II. The positions of RNA polymerase IIo and IIa are shown.

that although the CSA complex binds to RNA polymerase IIa regardless of DNA damage, it accumulates on RNA polymerase IIo stalled at DNA lesions after UV irradiation.

Ubiquitin Ligase Activity Associated with the DDB2 and CSA Complexes

Mass spectrometric analyses identified an identical set of polypeptides from the DDB2 and CSA complexes: cullin 4A (Cul4A), Roc1 (Rbx1), and all the subunits of COP9 signalosome (CSN), CSN1 to CSN8 (Figure 3A, lanes 1 and 3). Consistently, these factors were detected in both complexes by immunoblot analysis (Figure 3B, lanes 1 and 3), but not in the mock-purified control (data not shown). Cul4A is a member of the cullin family of ubiquitin ligase subunits (also known as E3s) that promote ubiquitin transfer to the substrates, the last step in a three-enzyme cascade involving the ubiquitin-activation (E1) and ubiquitin-conjugating (E2) enzymes (Hershko and Ciechanover, 1998). It has been proposed that modification of cullin family members with the NEDD8 ubiquitin-like protein stimulates ubiquitin ligase activity (Liakopoulos et al., 1998; Podust et al., 2000). Conversely, CSN appears to act as a negative regulator for cullin-based ubiquitin ligases by promoting deconjugation of the NEDD8 from cullin family members (Lyapina et al., 2001; Yang et al., 2002).

Since these data suggest that the DDB2 and CSA complexes play roles in regulating ubiquitination pathways, we first measured ubiquitin ligase activity in these complexes. Because the identity of relevant substrates is uncertain, we measured ubiquitin-polymerizing activity using Uba1 E1 and UbcH5 E2 enzymes, which function with other cullin family members (Jackson et al., 2000). Although the complete complexes of DDB2 and CSA displayed no activity (Figure 4A, lanes 3 and 6), the partial complexes of DDB2 and CSA, which were obtained by glycerol gradient sedimentation and are devoid of CSN (Figure 3A, lanes 2 and 4), displayed robust activity (Figure 4A, lanes 2 and 5). Both partial complexes were able to catalyze ubiquitin polymerization (Figure 4A, top) as well as autoubiquitination of Cul4A (bottom). Since the partial complexes lack CSN (Figure 3B, lanes 2 and 4), these results raised the possibility that CSN is a negative regulator of ubiquitin ligase activity.

Deubiquitinating Activity Associated with CSN

S. pombe CSN5 (spCSN5) has been shown to possess the His-X-HIS-X_{10}-Asp metalloprotease motif that functions as a catalytic site for the NEDD8 isopeptidase activity (Cope et al., 2002). Given that ubiquitin and NEDD8 have closely related structures (Whitby et al., 1998), we examined whether CSN5 is a catalytic subunit for the ubiquitin isopeptidase as well as for NEDD8 isopeptidase activity. The residues in the metalloprotease motif of spCSN5, which are crucial for NEDD8-deconjugating activity (Cope et al., 2002), are conserved in human CSN5 (hCSN5). Therefore, we introduced a point mutation into the conserved Asp residue in the hCSN5 metalloprotease motif. Given that the recombinant CSN5 protein per se has no isopeptidase activity (Cope et al., 2002), we expressed the wild-type and mutant FLAG-HA epitope-tagged hCSN5 in HeLa cells to be integrated into the CSN complex. SDS-PAGE analysis of the purified materials revealed that wild-type and mutant CSN5 are integrated into very similar complexes (Figure 4B), indicating that the mutation in the CSN5 metalloprotease motif does not disrupt the CSN complex.

To measure isopeptidase activities in the purified

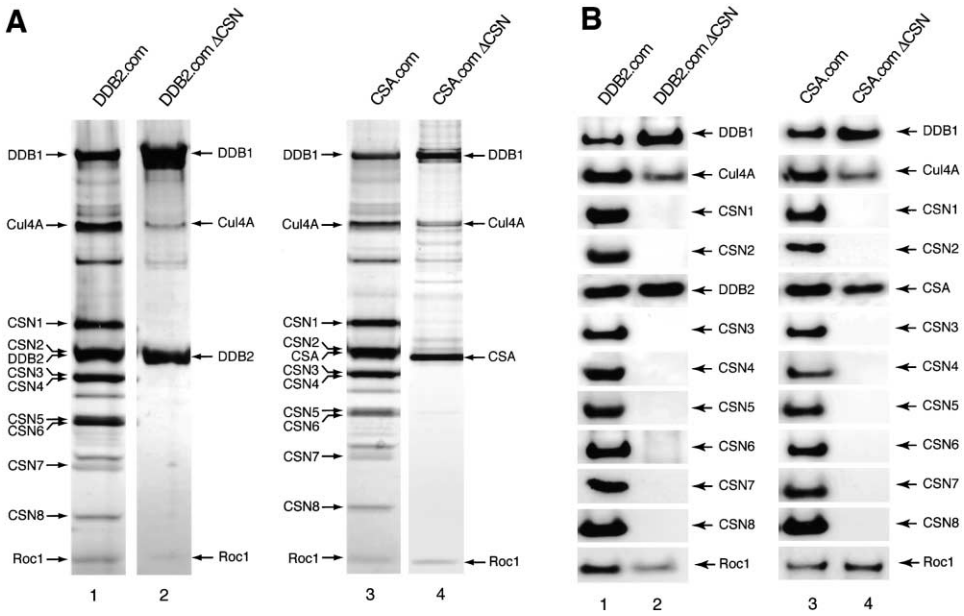

Figure 3. Cul4A, Roc1, and CSN Are Subunits of the DDB2 and CSA Complexes

(A) Subunit compositions in the DDB2 and CSA complexes. Mass-spectrometrically identified polypeptides in the DDB2 (lane 1) and CSA (lane 3) complexes are shown. The partial complexes of DDB2 (lane 2) and CSA (lane 4), a slowly sedimenting fraction from glycerol gradient sedimentation, are also shown.

(B) Immunoblot analyses of the DDB2 and CSA complexes. The complete and partial DDB2 (lanes 1 and 2) and CSA (lanes 3 and 4) complexes were analyzed by immunoblotting with the antibodies indicated.

complexes, the following substrates were employed. The NEDD8-conjugated Cul4A-containing DDB2 complex purified from cells 30 min after UV irradiation was used for a NEDD8 isopeptidase assay (see Figure 5C). Although ubiquitination of Cul4A cannot be detected in vivo by our system (data not shown), Cul4A in the DDB2 complex lacking CSN can be artificially polyubiquitinated in vitro (see Figure 4A, lane 2). Polyubiquitin-conjugated Cul4A, a substrate for the ubiquitin isopeptidase assay, was prepared by in vitro ubiquitination reactions.

The wild-type and mutant CSN complexes were incubated with these substrates, and isopeptidase activity was determined by analyzing Cul4A by immunoblotting. As expected, NEDD8-deconjugating activity was detected in the wild-type CSN5 complex, but not in the mutant CSN complex (Figure 4C). These results suggest that the metalloprotease motif of CSN5 is the catalytic site for the NEDD8-deconjugating activity. Unexpectedly, the mutant CSN complex showed ubiquitin-deconjugating activity that converts multiubiquitinated Cul4A to monoubiquitinated Cul4A (Figure 4D). This activity may be specific to the CSN complex given that the mock-purified control possessed no such activity. In contrast, the wild-type CSN5 completely deconjugated ubiquitin, producing nonubiquitinated Cul4A. These results suggest that at least two distinct ubiquitin isopeptidase activities associate with CSN: activities that deconjugate ubiquitin from Cul4A ubiquitin and activities that depolymerize polyubiquitin. The former activity could be catalyzed by the metalloprotease motif of CSN5, whereas the latter activity has not yet been characterized.

The Distinct UV Response of the DDB2 and CSA Complexes

We next tested how CSN in the DDB2 and CSA complexes regulates ubiquitin ligase activity following UV irradiation. As shown in Figures 2A and 5A, most of the DDB2 is transferred from the nuclear extract fraction to the solubilized chromatin fraction after UV irradiation. Importantly, CSN disappeared from the DDB2 complex purified from the solubilized chromatin fraction after UV irradiation (Figure 5A, lanes 1 and 4; see also Figure 2A). Moreover, conjugation of NEDD8 on Cul4A accompanied this disappearance, presumably leading to activation of ubiquitin ligase activity. However, it is intriguing that the CSA complex responded to UV treatment differently (Figure 5B). As described above, the CSA complex is found in the solubilized chromatin fraction regardless of UV irradiation. In contrast to the DDB2 complex, even more CSN was detected after UV irradiation in the CSA complex. Furthermore, regardless of UV irradiation, no NEDD8 conjugation of Cul4A was detected.

To further characterize the different UV responses of the DDB2 and CSA complexes, we purified these complexes from the solubilized chromatin fraction at different time points after UV irradiation. Immunoblot analysis of the purified complexes reveals that the amount of DDB2 reaches a maximum after 30 min UV irradiation and gradually decreases thereafter (Figure 5C). Thirty minutes after UV irradiation, a significant amount of Cul4A in the DDB2 complex is modified by NEDD8 and deconjugated at the later time points. Importantly, CSN, which dissociates from the DDB2 complex after UV irradiation, reassociates 2 hr after UV irradiation. Reassociated CSN could contribute to deconjugation of NEDD8

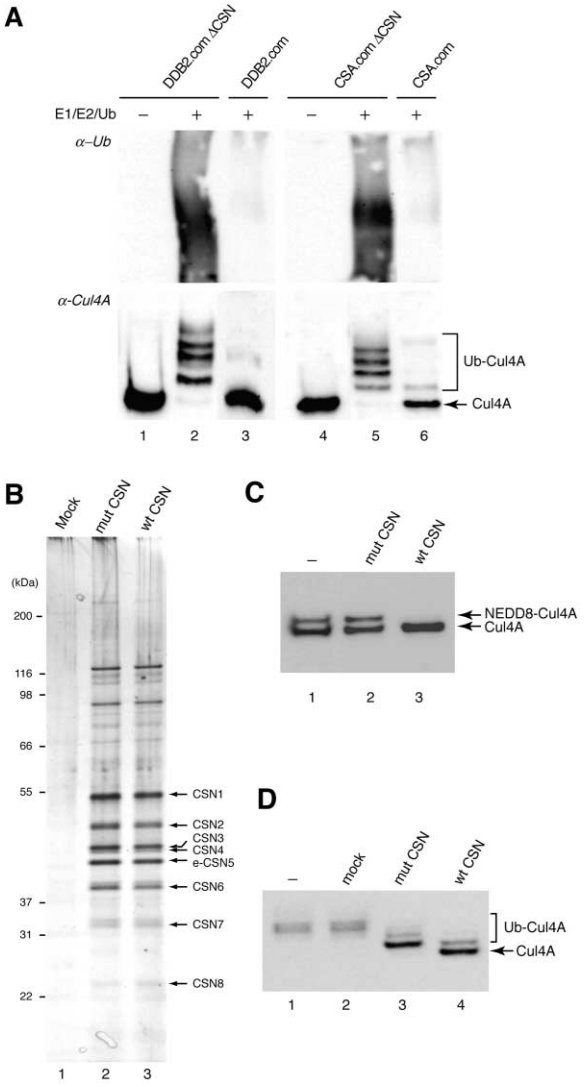

Figure 4. The DDB2 and CSA Complexes Possess Ubiquitin Ligase and Ubiquitin Isopeptidase Activities

(A) The partial DDB2 and CSA complexes lacking CSN have ubiquitin ligase activity. The partial DDB2 (lanes 1 and 2) and CSA (lanes 4 and 5) complexes and the complete DDB2 (lane 3) and CSA (lane 6) complexes were incubated with (lanes 2, 3, 5, and 6) or without (lanes 1 and 4) E1, E2, and ubiquitin. Polyubiquitin chains and ubiquitinated Cul4A were respectively determined by immunoblotting with anti-ubiquitin (top) and anti-Cul4A (bottom) antibodies.

(B) Purification of the mutant and wild-type CSN complexes. The mutant and wild-type CSN complexes were purified from nuclear extracts prepared from HeLa cells expressing FLAG-HA-epitope-tagged mutant and wild-type CSN5, respectively. The mutant (lane 2) and wild-type (lane 3) CSN complexes as well as mock-purified control (lane 1) were resolved by SDS-PAGE and visualized by silver staining. The positions of CSN1 to CSN8 are shown.

(C) NEDD8 deconjugating activity in CSN. NEDD8-conjugated Cul4A was incubated with buffer (lane 1), mutant CSN (lane 2), and wild-type CSN (lane 3). Modified and unmodified Cul4A were separated by DSD-PAGE and detected by immunoblotting with anti-Cul4A antibody.

(D) Ubiquitin deconjugating activity in CSN. Ubiquitinated Cul4A was incubated with buffer (lane 1), the mock-purified material (lane 2), mutant CSN (lane 3), and wild-type CSN (lane 4). Experiments were performed as described in (D).

from Cul4A and deubiquitination via ubiquitin isopeptidase activity, leading to inactivation of ubiquitin ligase activity of the DDB2 complex. These data suggest that shortly after UV irradiation, ubiquitin ligase activity in DDB2 complex is activated by dissociating CSN from the complex and conjugating NEDD8 to Cul4A. Subsequent inactivation is restored by reassociating CSN in the complex.

The amount of CSA in the solubilized chromatin fraction is almost unchanged by UV irradiation (Figure 5D). Without UV irradiation, RNA polymerase IIa is preferentially found in the CSA complex. Thirty minutes after UV irradiation, significant amounts of RNA polymerase IIo and IIa are found in the complex. Thereafter, the level and form of associated RNA polymerase II gradually returns to the original state. In contrast to the DDB2 complex, modification of Cul4A by NEDD8 could not be detected. Additionally, the level of CSN in the CSA complex increased 30 min after UV irradiation and then declined. These data suggest that ubiquitin ligase activity in the CSA complex, which is active without UV irradiation, is rapidly inactivated after UV irradiation by recruiting CSN and then restored at the later time points. Taken together, our data lead to the conclusion that CSN responds to UV treatment differently, depending on the complexes, providing diverse regulation of ubiquitin ligase activity.

CSN Is Required for GGR and TCR

Our results suggest that positive and negative regulation of ubiquitin-mediated protein degradation plays a key role in TCR and GGR. To address whether CSN is required for TCR and GGR, we attempted to knockdown CSN5 by retrovirus-derived siRNA. We employed two different siRNA constructs, designated CSN5 siRNA-1 and siRNA-2. BJ1 fibroblasts were infected with these retroviruses, and transduced subpopulations were selected on the basis of their sensitivity to puromycin. Immunoblot analysis revealed that both CSN5 siRNA-1 and siRNA-2 efficiently repressed expression of CSN5 without affecting that of CSN1 and CSN8 (Figure 6A), suggesting that knockdown of CSN5 does not cause disruption of the entire complex. Although the expression level of CSN5 is significantly reduced in these cell lines, no growth retardation was observed under normal growth conditions (data not shown).

We measured UDS and RRS, which respectively reflect GGR and TCR (Troelstra et al., 1992), in these cell lines as well as control BJ1 fibroblasts. Notably, in the BJ1 fibroblasts expressing CSN5 siRNA-1 and siRNA-2, UDS decreased to 55% and 48%, respectively, compared with the control fibroblasts (Figures 6B–6G). Similarly, in these cell lines, RRS also declined to 49% and 43%, respectively. From these results we conclude that loss of CSN5 leads to defects in GGR and TCR.

Discussion

The DDB2 and CSA Complexes Are Ubiquitin Ligases

Here, we show that DDB2 and CSA both link to a cullin-based ubiquitin ligase and that regulation of the ligase activity by CSN plays a crucial role in NER. Cullin-based

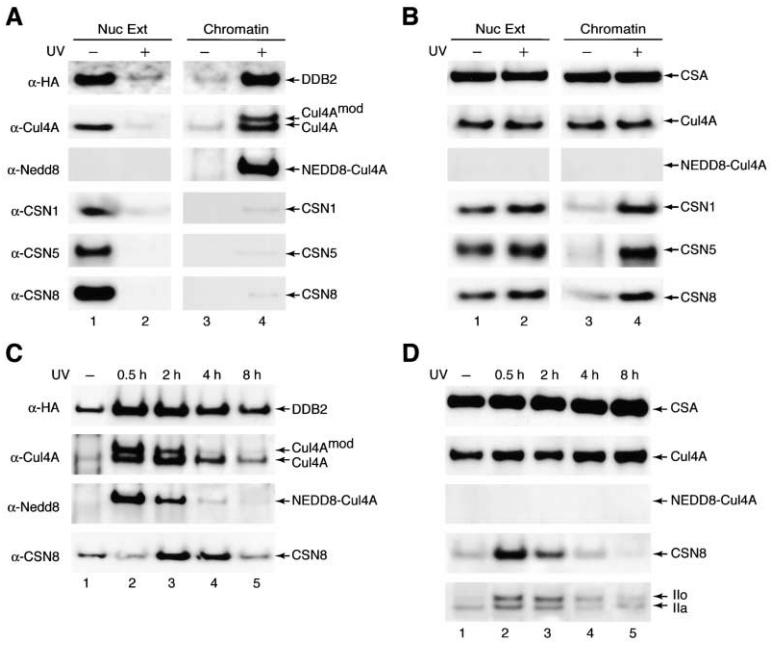

Figure 5. The Distinct UV Response of the DDB2 and CSA Complexes

(A) UV response of the DDB2 complex. The DDB2 complex was purified from nuclear extract (lanes 1 and 2) and solubilized chromatin (lanes 3 and 4) fractions prepared from cells before (lanes 1 and 3) and 30 min after (lanes 2 and 4) UV irradiation. The purified complexes were analyzed by immunoblotting with the antibodies indicated.

(B) UV response of the CSA complex. Experiments were performed as described in (A) except that CSA was purified.

(C) Dissociation of CSN from the DDB2 complex in response to UV irradiation. The DDB2 complex was purified from the solubilized chromatin fraction prepared from cells before (lane 1) and at different time points after (lanes 2 to 5) UV irradiation. The purified complexes were analyzed by immunoblotting with the antibodies indicated.

(D) Association of CSN with the CSA complex in response to UV irradiation. Experiments were performed as described in C except that CSA was purified.

ubiquitin ligases consist of cullin, a RING finger protein, adaptors, and a substrate recognition protein. The well-characterized SCF complex consists of Cul1, the Roc1 RING finger protein, the Skp1 adaptor, and an F box protein that binds directly to substrates (Weissman, 2001). Structural and biochemical data suggest that ubiquitin is transferred directly from E2-ubiquitin, which is tethered on SCF via interaction with Roc1, to substrates (Weissman, 2001; Zheng et al., 2002). Like SCF and other cullin-based ubiquitin ligases, the DDB2 and CSA complexes contain Cul4A and Roc1 (Figure 3). However, the relevant substrates of these complexes still remain to be identified. DDB2 has been reported to be ubiquitinated in a cell-cycle-dependent manner when DDB2 and Cul4A are transiently overexpressed (Nag et al., 2001). Conversely, DDB2 in the complexes purified from cells under various conditions is not ubiquitinated (data not shown).

RNA polymerase IIo has been shown to be very rapidly ubiquitinated after UV irradiation (Bregman et al., 1996; Ratner et al., 1998). Since such a modification cannot be seen in CS-A and CS-B cells, a role for ubiquitination of RNA polymerase IIo in TCR has been suggested (Bregman et al., 1996). However, the RNA polymerase IIo that copurifies with CSA from the solubilized chromatin fraction of the UV-irradiated cells is not ubiquitinated (data not shown). Moreover, ubiquitin ligase activity in the CSA complex appears to be suppressed by CSN upon UV irradiation (Figure 5). Thus, the CSA complex in the solubilized chromatin fraction may not contribute to ubiquitination of RNA polymerase IIo immediately after UV irradiation. However, it should be stressed that we have analyzed the CSA complex only in the nuclear extract and solubilized chromatin fractions. Since both the ubiquitination of RNA polymerase IIo and the translocation of CSA to the nuclear matrix are dependent on CSB (Kamiuchi et al., 2002), the CSA complex may ubiquitinate RNA polymerase IIo after translocation to the nuclear matrix, where it cannot be solubilized under the conditions we employed.

CSN Is Associated with a Ubiquitin Isopeptidase Activity

CSN is structurally and architecturally related to the lid subcomplex of the 26S proteasome, which mediates degradation of ubiquitin-conjugated proteins (Glickman et al., 1998). The 26S proteasome consists of a 20S proteolytic core cylinder and is capped on either side by a 19S regulatory particle that can be further subdivided into a lid and base (Glickman et al., 1998). Ubiquitin-conjugated proteins are recognized and unfolded by the 19S complexes (Elsasser et al., 2002) and funneled into the 20S proteolytic core cylinder for degradation (Kisselev and Goldberg, 2001). Recent reports show that the Rpn11 subunit of the proteasome lid subcomplex possesses ubiquitin isopeptidease activity, which is responsible for depolymerization of the ubiquitin chains from substrates prior to protein degradation (Verma et al., 2002; Yao and Cohen, 2002).

Likewise, the CSN5 subunit of CSN, which shares sequence similarity to Rpn11, has been shown to have isopeptidase activity that deconjugates NEDD8 from cullin-NEDD8 (Cope et al., 2002). This work supports the notion that CSN-mutations in *Arabidopsis* leads to deficient NEDD8-deconjugating activity and accumulation of NEDD8-conjugated cullins (Schwechheimer et al., 2001). NEDD8-conjugation of cullins has been shown to stimulate ubiquitin ligase activity both in vitro and in vivo (Liakopoulos et al., 1998; Read et al., 2000; Yang et al., 2002). Thus, CSN could serve as a negative regulator of ubiquitin ligase activity by deconjugating NEDD8 from cullin-NEDD8.

Here, we demonstrate that CSN also has ubiquitin isopeptidase activity in vitro (Figure 4). The DDB2 and CSA complexes containing CSN show no ubiquitin ligase activity. However, when these complexes lack

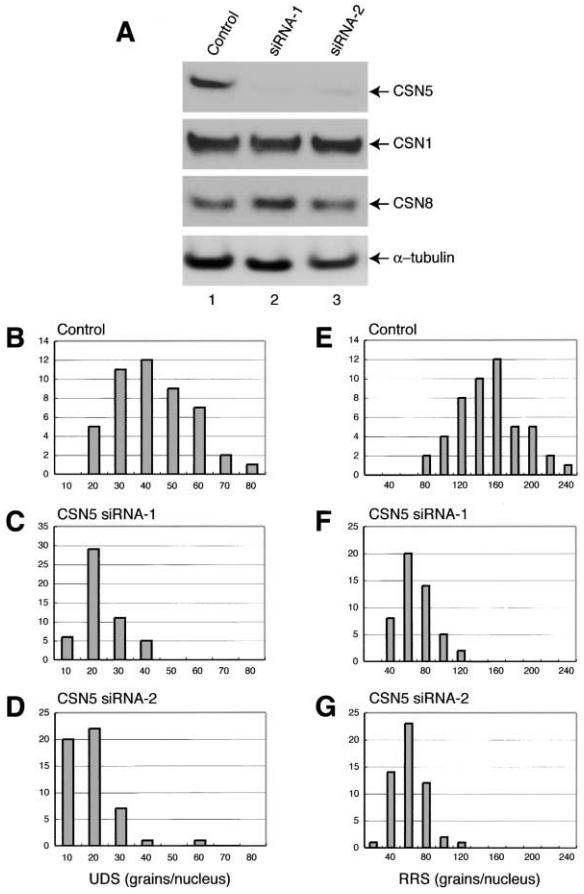

Figure 6. Knockdown of CSN5 Leads to Defects of GGR and TCR

(A) CSN5, CSN1, CSN8, and α-tubulin in control BJ1 fibroblasts (lane 1) and BJ1 fibroblasts stably expressing CSN5 siRNA-1 (lane 2) or siRNA-2 (lane 3) were determined by immunoblotting. (B–G) UDS (B–D) and RRS (E–G) in control BJ1 fibroblasts (B and E) and BJ1 fibroblasts stably expressing CSN5 siRNA-1 (C and F) or siRNA-2 (D and G). Experiments were performed as described in Figure 2.

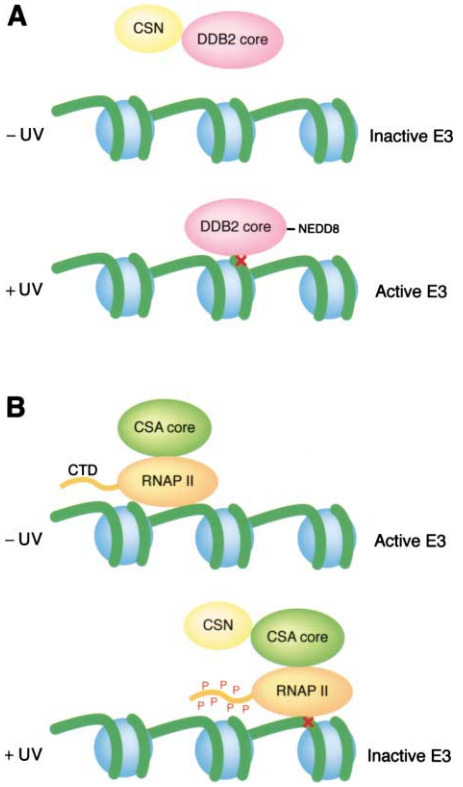

Figure 7. Model for the Distinct UV Response of the DDB2 and CSA Complexes

(A) In the absence of UV irradiation, the DDB2 core complex (DDB2, DDB1, Cul4A, and Roc1) exists in the soluble nuclear extract as a CSN bound form. Upon UV irradiation, the DDB2 complex tightly binds to damaged chromatin. Subsequently, CSN is dissociated from the DDB2 core complex, leading to activation of the ubiquitin ligase E3 activity. At the same time, Cul4A in the core complex is conjugated by NEDD8, further stimulating the E3 activity. (B) In the absence of UV irradiation the CSA core complex (CSA, DDB1, Cul4A, and Roc1) exists as a CSN-free form. Given that RNA polymerase II is found in the complex, the CSA complex may be incorporated into the transcription preinitiation complex and travel along with RNA polymerase II during elongation. Upon UV irradiation, the CSA complex accumulates on RNA polymerase IIo stalled at DNA lesions after UV irradiation. Subsequently, the CSA core complex recruits CSN, leading to inactivation of the ubiquitin ligase E3 activity. Such a dynamic regulation of the ubiquitin ligases would play a crucial role in nucleotide excision repair.

CSN, they exhibit robust ubiquitin ligase activity. Furthermore, experiments with the mutant CSN complex indicate that the CSN5 metalloprotease activity contributes to deconjugation of ubiquitin that directly attaches to substrates. Additionally, CSN associates with the isopeptidase activity that depolymerizes polyubiquitin. While the major components of the CSN complex are CSN1 to CSN8, it also contains many substoichiometric polypeptides that specifically associate with CSN5 (Figure 4B). Further analyses of the purified CSN complex could reveal the identity of additional ubiquitin isopeptidase in the CSN complex. Taken together, our data suggest that CSN has the capacity to negatively regulate the ubiquitin ligase activity of cullin-based ubiquitin ligase by two mechanisms: suppression of ubiquitin ligase activity by deconjugating NEDD8 from cullin-NEDD8 and depolymerization of ubiquitin chains by ubiquitin isopeptidase activity.

Distinct Regulation of Ubiquitin Ligase Activity in the DDB2 and CSA Complexes

The DDB2 and CSA complexes both contain CSN, a negative regulator of ubiquitin ligase activity. Strikingly, association of CSN with these complexes is differentially regulated by UV signals (Figure 7). In the DDB2 complex, CSN rapidly dissociates from the complex after UV irradiation. At the same time, Cul4A in the complex is modified by NEDD8, suggesting that the ubiquitin ligase activity is stimulated in response to UV irradiation in vivo. After DNA repair has occurred, CSN presumably reassociates with the DDB2 complex, leading to deconjugation of NEDD8 from Cul4A. In contrast, CSN rapidly associates with the CSA complex in the solubilized chromatin fraction after UV irradiation, presumably leading to suppression of the ubiquitin ligase activity. Such dynamic regulation of the ubiquitin ligase activities of the CSA and DDB complexes by CSN implies that CSN should play a central role in GGR and TCR. Our observation

that knockdown of CSN5 causes defects in GGR and TCR (Figure 6) supports this claim.

The difference between CSN behavior in the DDB2 and CSA complexes reflects the special feature of these two NER pathways. The GGR pathway removes lesions from both transcriptionally active and inactive genes. In contrast, the TCR pathway is confined to the transcribed strand of active genes and is dependent on RNA polymerase II activity (Friedberg et al., 1995; Svejstrup, 2002). Importantly, repair of the transcribed strand is approximately two to four times more rapid than in the nontranscribed strand. Given this general framework, a number of models could be put forth for GGR and TCR based on our results. The model we favor supports the differential activities of the CSA and DDB2 complexes regulated by CSN upon DNA damage. Specifically, in the absence of transcription, access to the DNA might be hindered by the presence of highly ordered chromatin. DNA damage sustained in transcriptionally inert regions must be repaired in order to preserve genomic integrity. The activity of the DDB2 protein has been shown to play a vital role in the repair of UV-induced lesions in transcriptionally inactive regions. How the repair machinery gains access to the DNA lesions buried within these regions remains unknown. Our finding that in vivo DDB2 is part of a cullin-containing E3 ligase complex provides, to our knowledge, the first insight into this question. Since the E3 ligase activity of DDB2 is only activated after DNA damage, it is possible that the necessity of this activity is linked to the need to remove physical barriers that otherwise prevent access to the DNA lesions. Ubiquitination and subsequent degradation of chromatin components may be part of how DNA damage binding factors gain access to lesions and nucleate the assembly of the excision repair machinery.

In TCR, a process that is dependent on RNA polymerase II, a stalled RNA polymerase molecule would serve as an activating signal for the transcription repair coupling factors. We find that even in the absence of damage, CSA associates with the hypophosphorylated forms of the polymerase. By assembling onto the RNA polymerase II preinitiation complex at the promoter, CSA, perhaps in conjunction with CSB, would confer upon the polymerase the ability to execute TCR at any time. Preserving the integrity of these factors and perhaps others at the site of a stalled RNA transcript is likely vital to the TCR process. Therefore, silencing of the CSA-associated E3 ligase activity by CSN may be necessary to permit the recruitment, assembly, and subsequent repair of the DNA lesion by the NER machinery during TCR. Further biochemical characterization of these reactions and the identification of the relevant substrates of the CSA and DDB2 E3 ligases will permit rigorous testing of this model both in vitro and in vivo.

Experimental Procedures

Purification of the DDB2 and CSA Complexes

HeLa cells stably expressing N-terminally FLAG-HA-epitope-tagged DDB2 or C-terminally FLAG-HA-epitope-tagged CSA were prepared as described previously (Ikura et al., 2000; Ogawa et al., 2002). e-DDB2 and e-CSA were purified from the nuclear extract and from solubilized chromatin fractions by immunoprecipitation with anti-FLAG antibody followed by anti-HA antibody according to our standard method (Ikura et al., 2000; Ogawa et al., 2002). Where indicated, purified complexes were further separated on a 10%–30% glycerol gradient by ultracentrifugation.

For UV irradiation, cells were grown on tissue culture dishes, washed with PBS, irradiated with UV at 25 J/m^2, and incubated in fresh media for the periods indicated. To prepare nuclear extracts, 2.5 $\times$ 10^8 cells were suspended in 4.5 ml of the hypotonic buffer (10 mM Tris-HCl [pH 7.3], 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.2 mM PMSF) and disrupted by Dounce homogenization. Nuclei were collected by centrifugation at 2000 $\times$ g for 15 min at 4°C and resuspended in 2 ml of the extraction buffer (15 mM Tris-HCl [pH 7.3], 1 mM EDTA, 0.4 M NaCl, 1 mM $MgCl_2$, 10% glycerol, 10 mM β-mercaptoethanol, and 0.2 mM PMSF). After incubating on ice for 30 min, the samples were centrifuged at 20,000 $\times$ g for 30 min at 4°C, and the supernatant was used as the nuclear extract fraction. The nuclear pellet fraction was washed and resuspended in the micrococcal nuclease buffer (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.3 M sucrose, 0.1% Triton X-100, and complete protease inhibitor cocktail, [Roche, Basel]). Micrococcal nuclease was added at 3 U/ml and the samples incubated for 10 min at room temperature, whereupon the reaction was terminated by adding 5 mM EGTA and 5 mM EDTA. The samples were centrifuged at 2000 $\times$ g for 5 min at 4°C, and the supernatant was used as the solubilized chromatin fraction.

Interaction of DDB1 with DDB2 and CSA

Recombinant HA-tagged DDB1 was coexpressed with recombinant FLAG-tagged DDB2 or CSA in Sf9 cells via the Bac-to-Bac baculovirus expression system (Invitrogen). HA-tagged DDB1 was purified from Sf9 cell extracts by immunoprecipitation with anti-HA antibody-conjugated agarose. Copurified FLAG-tagged DDB2 and CSA were detected by immunoblotting with anti-FLAG antibody.

DNA Repair Assay

After microinjection of the cells with either DDB2 or CSA complex, measurements of UV-induced unscheduled DNA synthesis (UDS) and recovery of RNA synthesis after UV irradiation (RRS), corresponding to global genome repair and transcription-coupled repair, respectively, were performed as described (Nakatsu et al., 2000). Briefly, 24 hr after microinjection, GM02415 XP-E cells were UV irradiated at 30 J/m^2 and subjected to 2 hr incubation in DMEM containing [^{3}H]thymidine (100 μCi/ml; specific activity: 30 Ci/mmol), washed with PBS, fixed with methanol, and processed for autoradiography to measure UDS. RRS was determined as follows. NPS2 CS-A cells were exposed to 15 J/m^2 of UV 24 hr after microinjection, incubated for an additional 24 hr under normal culture conditions, washed with PBS, and subsequently incubated for another 1 hr in DMEM containing [^{3}H]uridine (100 μCi/ml; specific activity: 27 Ci/mmol). Cells were then fixed and processed for autoradiography. Silver grains derived from [^{3}H]thymidine or [^{3}H]uridine incorporated into the cells were counted on the 20–50 nuclei.

UV-Damaged DNA Binding In Vitro

The 208 bp DNA EcoR1 fragment of 5S rRNA gene (Logie and Peterson, 1997) was labeled with ^{32}P-dATP by fill-in reaction. The labeled DNA was irradiated with UV at 10 kJ/m^2 and used as a probe. The DDB2 and CSA complexes were incubated with 0.1 ng of probe in 10 μl of the reaction buffer (20 mM Tris-HCl [pH 7.5], 6 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, and 0.1 mg/ml poly dGdC) at 4°C for 30 min and were subsequently resolved by 4% PAGE (Acrylamide/Bis-acryl 29:1) in TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). Electrophoresis was carried out at 4°C for 2 hr at 100V with circulating TE buffer.

In Vitro Ubiquitin Ligase Assay

To measure ubiquitin ligase activity in the DDB2 and CSA complexes, samples were incubated at 30°C for 60 min in 15 μl of assay buffer containing 0.1 μg Uba1 E1, 0.03 μg UbcH5b E2, 5 μg ubiquitin, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM DTT, and 4 mM ATP. To determine deubiquitinating activity in CSN after completion of ubiquitination reaction, 0.2 μg of purified CSN was added to the reaction mixture, which was further incubated at 30°C for 30 min.

Purification of CSN and Isopeptidase Assay

N-terminally FLAG-HA-epitope-tagged wild-type or mutant (D151N) CSN was stably expressed in HeLa cells and purified as described (Ikura et al., 2000; Ogawa et al., 2002). The substrates for NEDD8- and ubiquitin-deconjugating activities were prepared as follows. NEDD8-Cul 4A-containing DDB2 complex was immunopurified from the solubilized chromatin fraction of e-DDB2-expressing HeLa cells 30 min after UV irradiation as described above. Ubiquitinated Cul4A was obtained by in vitro ubiquitination of the partial DDB2 complex lacking CSN as described above. After the ubiquitination reaction, the complex was purified by immunoprecipitation with M2 anti-Flag antibody-conjugated agarose and used as a substrate. These substrates were incubated with 50 ng of the wild-type and mutant CSN in 10 μl of CSK buffer (10 mM Pipes-KOH [pH 6.8], 100 mM NaCl, 300 mM sucrose, and 3 mM $MgCl_2$) at 30°C for 30 min. After incubation, modification of Cul4A was analyzed by immunoblotting with Cul4A antibody.

CSN5 Knockdown

The following siRNA precursors were expressed into BJ1 fibroblasts (BD Biosciences Clontech) via pSUPER.retro (Brummelkamp et al., 2002). These transcripts are predicted to be folded into a 19 bp stem-loop structure and processed to functional siRNA. Note that the target 19 nt sequences are underlined. Transduced subpopulations were selected with 3 μg/ml puromycin. CSN5 siRNA-1: 5′GCU CAGAGUAUCGAUGAAAUUCAAGAGAUUUCAUCGAUACUCUGAG CUU 3′ CSN5 siRNA-2: 5′CAUGCAGGAAGCUCAGAGUUUCAAGA GAACUCUGAGCUUCCUGCAUGUU 3′.

Antibodies

Antibody employed are as follows: anti-CSA (Santa Cruz Biotechnology, Inc.), anti-CSN1 to CSN8 (Affiniti), anti-multiubiquitin (FK2) (MBL), anti-DDB1 and -DDB2 (Vesna Rapic Otrin and Arthur Levine, University of Pittsburgh School of Medicine, Pittsburg, PA), anti-Cullin 4A antibody (Pradip Raychaudhuri, University of Illinois at Chicago, Chicago, IL), and anti-RNA polymerase II (Danny Reinberg, Robert Wood Johnson Medical School, Piscataway, NJ).

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