Molecular Cell 22, 383–394, May 5, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.molcel.2006.03.035

Histone H3 and H4 Ubiquitylation by the CUL4-DDB-ROC1 Ubiquitin Ligase Facilitates Cellular Response to DNA Damage

Hengbin Wang,[1,2,4,*] Ling Zhai,[1,4] Jun Xu,[1] Heui-Yun Joo,[1] Sarah Jackson,[2] Hediye Erdjument-Bromage,[3] Paul Tempst,[3] Yue Xiong,[2] and Yi Zhang[2,*]

[1] Department of Biochemistry and Molecular Genetics
University of Alabama at Birmingham
Kaul Human Genetics Building Room 402A
720 South 20th Street
Birmingham, Alabama 35294

[2] Department of Biochemistry and Biophysics
Lineberger Comprehensive Cancer Center
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599

[3] Molecular Biology Program
Memorial Sloan Kettering Cancer Center
1275 York Avenue
New York, New York 10021

Summary

Posttranslational histone modifications play important roles in transcription and other chromatin-based processes. Compared to acetylation, methylation, and phosphorylation, very little is known about the function of histone ubiquitylation. Here, we report the purification and functional characterization of a histone H3 and H4 ubiquitin ligase complex, CUL4-DDB-ROC1. We demonstrate that CUL4-DDB-ROC1-mediated H3 and H4 ubiquitylation occurs both in vitro and in vivo. Importantly, CUL4-DDB-ROC1-mediated H3 and H4 ubiquitylation is regulated by UV irradiation. Reduction of histone H3 and H4 ubiquitylation by knockdown of CUL4A impairs recruitment of the repair protein XPC to the damaged foci and inhibits the repair process. Biochemical studies indicate that CUL4-DDB-ROC1-mediated histone ubiquitylation weakens the interaction between histones and DNA and facilitates the recruitment of repair proteins to damaged DNA. Thus, our studies uncover CUL4-DDB-ROC1 as a histone ubiquitin ligase and demonstrate that histone H3 and H4 ubiquitylation participates in the cellular response to DNA damage.

Introduction

Posttranslational modifications of histones play an important role in regulating many aspects of chromatin biology (Martin and Zhang, 2005; Peterson and Laniel, 2004). Compared to acetylation and methylation, histone ubiquitylation is less well characterized (Jason et al., 2002). Recent identifications of enzymes involved in histones H2B and H2A ubiquitylation have revealed critical functions for this modification in transcriptional regulation, recombination, and the damage checkpoint response (Giannattasio et al., 2005; Osley, 2004; Wang et al., 2004; Yamashita et al., 2004).

Although histone ubiquitylation has been known for more than three decades (Goldknopf et al., 1975), its functional significance remained elusive until very recently (Osley, 2004). Yeast histone H2B is ubiquitylated at lysine 123 (equivalent to lysine 120 in mammals), and uH2B accounts for about 10% of total cellular H2B (Robzyk et al., 2000). Rad6 and Bre1 have been identified as the ubiquitin conjugating and ligation enzymes responsible for this modification (Hwang et al., 2003; Robzyk et al., 2000; Wood et al., 2003). Subsequent studies have demonstrated a role for H2B ubiquitylation in transcriptional activation (Henry et al., 2003; Kao et al., 2004). Furthermore, deletion of H2B deubiquitinase Ubp10 impairs silencing at telomere and rDNA regions (Emre et al., 2005; Gardner et al., 2005). Interestingly, H2B ubiquitylation was found to be required for subsequent methylation of H3K4 and H3K79 (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002), suggesting a role for H2B ubiquitylation in the regulation of other histone modifications and chromatin function. Recently, it has been shown that RNF20/40, the mammalian counterpart of Bre1, possesses ubiquitin ligase activity for histone H2B and that H2B ubiquitylation could regulate H3K4 and H3K79 methylation (Kim et al., 2005; Zhu et al., 2005), indicating that the role of H2B ubiquitylation in transcription is evolutionally conserved. In addition to transcription, H2B ubiquitylation has recently been implicated in meiotic recombination and DNA damage checkpoint control (Giannattasio et al., 2005; Yamashita et al., 2004).

In contrast to budding yeast, histone H2A is the most abundant ubiquitylated protein in mammals (West and Bonner, 1980). The ubiquitin molecule is attached to lysine 119 of histone H2A (Bohm et al., 1980). Although earlier studies have implicated a role for H2A ubiquitylation in gene activation (Jason et al., 2002), the recent identification of the H2A ubiquitin ligase has linked H2A ubiquitylation to Hox gene silencing (Cao et al., 2005; Wang et al., 2004). Interestingly, H2A ubiquitylation also appears to participate in X inactivation (de Napoles et al., 2004; Fang et al., 2004).

In addition to H2A and H2B, histones H3, H2A.Z, and H1 have also been reported as substrates for ubiquitin modification (Jason et al., 2002). The enzyme responsible for H1 ubiquitylation was identified as TAF_{II} 250 in *Drosophila* (Pham and Sauer, 2000), and ubiquitylation of H1 has been linked to gene repression in the Dorsal pathway (Pham and Sauer, 2000). Histone H3 was reported to be polyubiquitylated in elongating rat spermatids (Chen et al., 1998). The function of H3 ubiquitylation was proposed to mediate its degradation after its replacement by transition proteins during spermatogenesis (Chen et al., 1998). Recently, a testis-specific UBC4 isoform, UBC4-testis (Wing et al., 1996), and a HECT domain-containing protein, LASU1 (Liu et al., 2005), were reported to be capable of ubiquitylating histones in vitro. However, the physiological relevance of these proteins in histone ubiquitylation remains to be shown. The

*Correspondence: hbwang@uab.edu (H.W.); yi_zhang@med.unc.edu (Y.Z.)

[4] These authors contributed equally to this work.

histone H2A variant H2A.Z was also reported to be ubiquitylated in developing trout testis (Nickel et al., 1987), but neither the enzyme responsible for the modification nor its function is known.

Do histone H3 and H4 ubiquitylations occur in vivo? If they do, what are the responsible enzymes and what are the functions of these modifications? In this report, we demonstrate that both histones H3 and H4 are ubiquitylated in vivo. Moreover, we provide evidence that a ubiquitin ligase complex, which is composed of CUL4A, CUL4B, DDB1, DDB2, and ROC1 (RBX1), is responsible for H3 and H4 ubiquitylation. Importantly, UV irradiation results in a CUL4-DDB-ROC1-dependent increase in the levels of H3 and H4 ubiquitylation. Finally, we provide evidence indicating that CUL4-DDB-ROC1-mediated H3 and H4 ubiquitylation causes histone release from nucleosomes that in turn facilitates recruitment of repair proteins to the damaged foci. Thus, our studies identify CUL4-DDB-ROC1 as a histone ubiquitin ligase and link H3 and H4 ubiquitylation to cellular response to DNA damage.

Results

Purification of a Previously Unidentified Histone Ubiquitin Ligase Complex

In an effort to understand the function of histone ubiquitylation in mammalian cells, we set out to purify the histone ubiquitin E3 ligase enzyme(s) by using an in vitro assay coupled with chromatography. By monitoring the histone ubiquitin ligase activity, we previously purified and characterized a histone H2A-specific ubiquitin ligase complex, hPRC1L (Wang et al., 2004). During the purification, we noticed a ubiquitin ligase activity, which appears to be capable of ubiquitylating all histones, was present in the flowthrough from the Mono Q column. This activity was completely separated from the H2A-specific ligase activity (Figure 1B).

To purify this activity, the samples were fractionated on a Mono S column. Silver staining and ubiquitin ligase assays of the fractions derived from the Mono S column allowed us to correlate the enzymatic activity to five polypeptides (Figure 1C). To confirm that the five polypeptides are indeed components of the same protein complex and responsible for the activity, the active fractions (41–47) were combined and further fractionated on a Superdex 200 gel filtration column. Analysis of the fractions derived from this column indicated that the five polypeptides cofractionate as a protein complex of 300–400 kDa (Figure 1D, top) with histone ubiquitin ligase activity (Figure 1D, bottom). Therefore, we conclude that the five-component protein complex is responsible for the ubiquitin ligase activity.

To identify the polypeptides, we concentrated fractions 37–39 of the Superdex 200 column (Figure 1D) and resolved them on an SDS-PAGE gel (Figure 1E). After Coomassie staining, the protein bands were excised and subjected to in-gel tryptic digestion. Mass spectrometry analysis revealed that the five polypeptides were damaged DNA binding protein 1 (DDB1), Cullin 4B, Cullin 4A, damaged DNA binding protein 2 (DDB2), and ROC1/RBX1. Based on its polypeptide composition, we have named the protein complex CUL4-DDB-ROC1.

In Vitro Characterization of the CUL4-DDB-ROC1 Complex

The identification of CUL4-DDB-ROC1 as histone ubiquitin ligase is intriguing. Previous studies have demonstrated the ubiquitin ligase activity of CUL4A-containing complexes for a variety of substrates (Higa et al., 2003; Sugasawa et al., 2005; Wertz et al., 2004), but the ubiquitin ligase activity toward histones has not been reported. To determine whether histones are physiological substrates of the CUL4-DDB-ROC1 ligase, we attempted to analyze the substrate specificity of the purified CUL4-DDB-ROC1 complex. To this end, individual recombinant histones, histone octamers, mononucleosomes, and oligonucleosomes were used in the ubiquitylation assay. Results shown in Figure 2A indicate that CUL4-DDB-ROC1 ubiquitylated all forms of histones with a similar efficiency. Interestingly, CUL4-DDB-ROC1 could ubiquitylate histones to mono, di-, tri-, or multimer forms (Figure 2A, top).

The appearance of multiple ubiquitin molecules on a particular histone could be due to the formation of a multiubiquitin chain at a single lysine residue of a particular histone or due to the attachment of a single ubiquitin molecule to multiple lysine residues of a particular histone. To distinguish between these two possibilities, we performed a histone ubiquitin ligase assay with methyl ubiquitin, which is not capable of serving as a substrate for subsequent attachment of additional ubiquitin molecules (Liu et al., 2005). Results show in Figure 2B (lanes 2, 4, 6, and 8) indicate that multiubiquitylated forms of histones are still detected, although less efficiently, when methyl-ubiquitin is used in the reactions. This result indicates that histone ubiquitylation by CUL4-DDB-ROC1 occurs at multiple lysine residues of all four histones in vitro. A similar property was reported for a testis-specific histone ubiquitin ligase LASU1 in vitro (Liu et al., 2005).

Histones H3 and H4 Are Ubiquitylated In Vivo

Although histone H3 ubiquitylation has been reported in rat elongating spermatids (Chen et al., 1998), there is no report that histone H4 is ubiquitylated in vivo. To explore the physiological relevance of CUL4-DDB-ROC1-mediated histone ubiquitylation, we determined whether histones H3 and H4 are ubiquitylated in vivo. To this end, we transfected HeLa cells with DNA encoding Flag-H3.1 or HA-ubiquitin individually or in combination. Cell extracts derived from the transfected cells were subjected to immunoprecipitation with M2 anti-Flag antibody under denaturing conditions (Shiio and Eisenman, 2003). Western blot analyses with anti-Flag and anti-HA antibodies demonstrate that cells cotransfected with plasmids encoding Flag-H3.1 and HA-ubiquitin displayed slower-migrating bands, which correlate with the size of the ubiquitylated form of Flag-H3.1 (Figure 3A, lane 8, also see Figure 4C, lane 2). These results indicate that exogenously expressed histone H3 is subjected to ubiquitylation in HeLa cells.

To determine whether histones H3 and H4 are ubiquitylated under more physiological conditions, we generated a HeLa cell line that carries an integrated, HA-tagged ubiquitin gene. To avoid potential artifacts caused by overexpression of the tagged protein, we compared the levels of ubiquitylated H2A that contains HA-ubiquitin to endogenous ubiquitin by using the

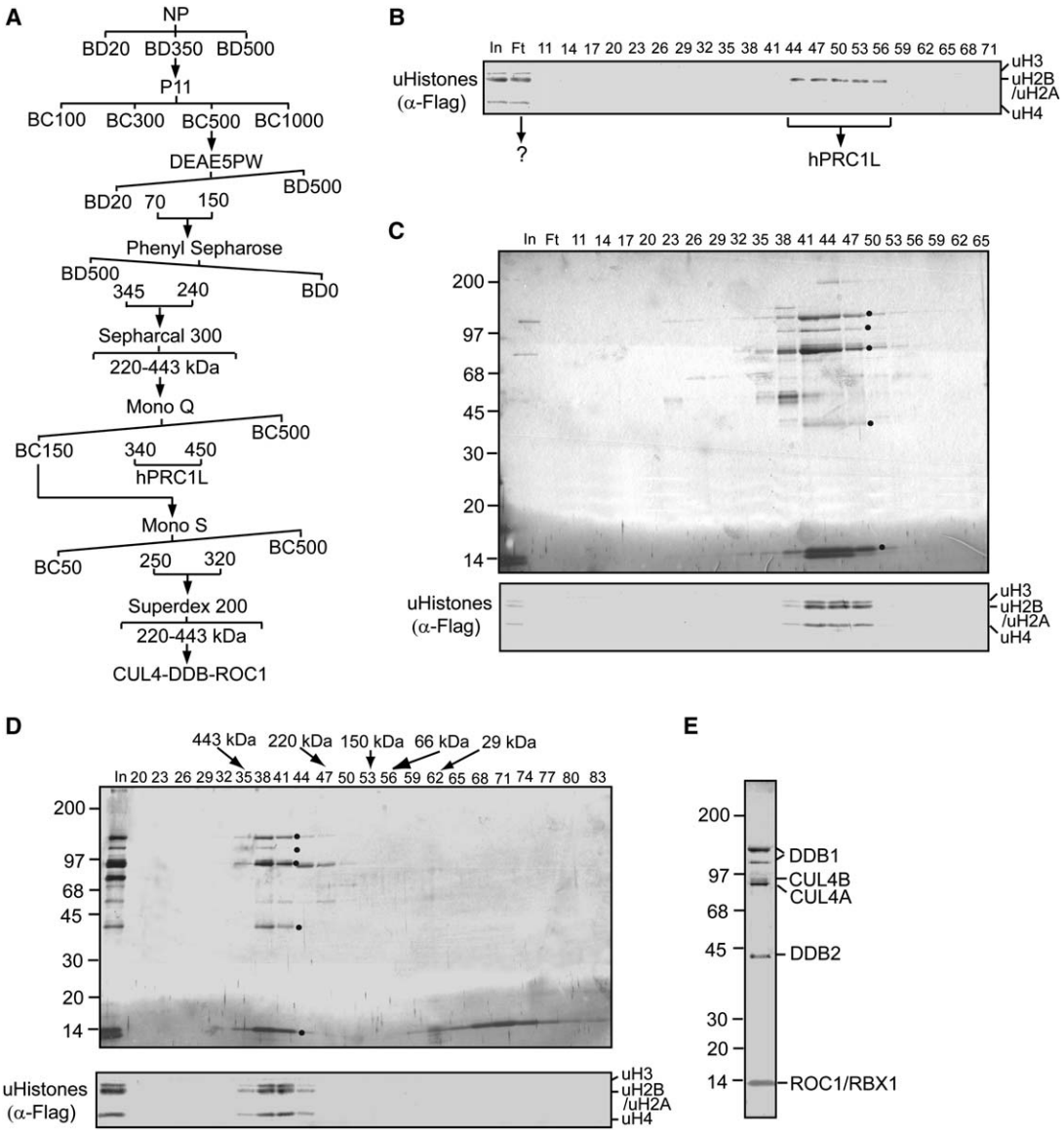

Figure 1. Purification of a Previously Unidentified Histone Ubiquitin E3 Ligase Complex

(A) Schematic representation of the steps used to purify the histone ubiquitin E3 ligase complex. Numbers represent the salt concentrations (mM) at which the E3 ligase activity elutes from the columns.

(B) Histone ubiquitin ligase assay of protein fractions derived from a Mono Q column. In addition to the ubiquitin ligase activity specific for histone H2A (hPRC1L), a previously unidentified ubiquitin ligase activity for all the core histones was observed in the flowthrough (Ft).

(C) Silver staining of a polyacrylamide-SDS gel (top) and histone ubiquitin ligase activity (bottom) of fractions derived from a Mono S column. The protein bands that cofractionated with the histone ubiquitin E3 ligase activity are indicated by an asterisk (*). The protein size marker is indicated on the left side of the panel.

(D) Silver staining of a polyacrylamide-SDS gel (top) and histone ubiquitin ligase activity (bottom) of fractions derived from a Superdex 200 column. The protein bands that cofractionated with the histone ubiquitin E3 ligase activity are indicated by an asterisk. The elution profile of the protein size markers is indicated on top of the panels.

(E) Coomassie staining of an 8%–15% gradient polyacrylamide-SDS gel containing the purified histone ubiquitin ligase complex. The individual protein bands were excised and identified by mass spectrometry and are indicated. The protein size marker is indicated on the left side of the panel.

uH2A-specific antibody (Vassilev et al., 1995). Western blotting analysis revealed that the levels of HA-uH2A were not higher than uH2A (data not shown). This result indicates that the HA-ubiquitin gene was expressed at a level similar to that of endogenous ubiquitin. When analyzed by immunoblotting with the anti-HA antibody, a signal of about 25 kDa that corresponds to HA-uH2A was detected in the HA-ubiquitin-integrated cells, but not in the control HeLa cells (Figure 3B, compare lanes 3 and 4). Importantly, we detected specific signals at positions that correspond to the ubiquitylated histones H3 and H4, although at much lower levels than that of uH2A (Figure 3B, lane 4). Consistent with the presence of ubiquitylated H3 and H4, Western blotting with antibodies against histone H3 or H4 detected signals at positions corresponding to uH3 (Figure 3B, lane 6) and uH4

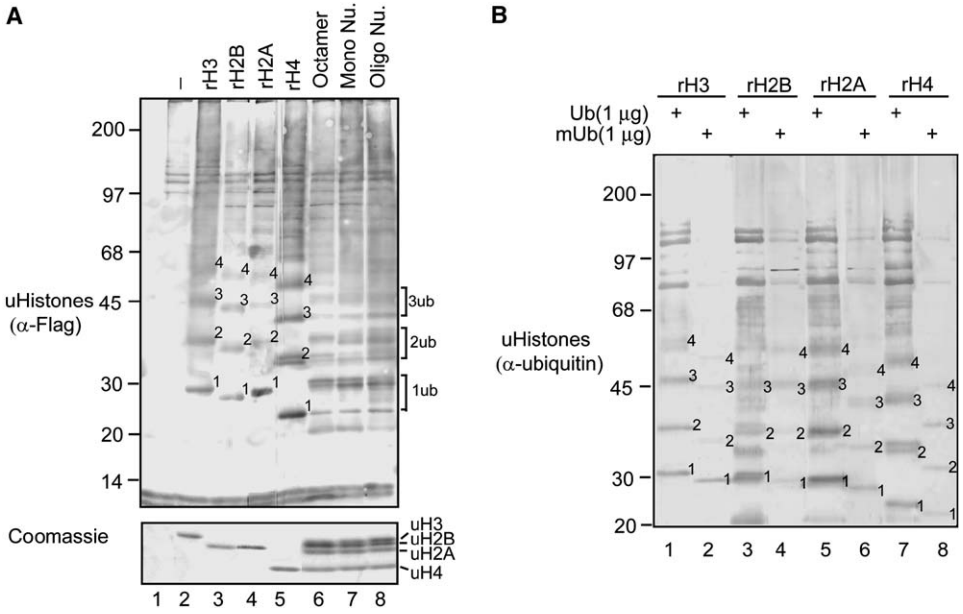

Figure 2. Characterization of the Histone Ubiquitylation Ligase Activity In Vitro

(A) In vitro characterization of the substrate specificity of the purified CUL4-DDB-ROC1 complex. Different histone substrates (bottom) were subjected to a ubiquitylation assay. The ubiquitylated histones were detected by Western blotting (top). Numbers between the lanes indicate ubiquitin molecule numbers on a particular histone molecule. Protein size markers are indicated on the left side of the top panel.

(B) Ubiquitylation of different recombinant histones by the CUL4-DDB-ROC1 complex in the presence of ubiquitin (1 μg) (lanes 1, 3, 5, and 7) or methyl ubiquitin (1 μg) (lanes 2, 4, 6, and 8). The numbers between the lanes indicate the numbers of ubiquitin molecules. Protein size markers are also indicated.

(Figure 3B, lane 8). Based on these results, we conclude that histones H3 and H4 are ubiquitylated in vivo.

We have also determined the relative levels of uH3 and uH4 in HeLa cells by Western blotting using antibodies against H3 and H4. Based on the results shown in Figure 3C, we estimate that only about 0.3% of total histone H3 is ubiquitylated (top, compare lanes 1 and 5), whereas less than 0.1% of total histone H4 is ubiquitylated (bottom, compare lanes 1 and 5).

Histone H3 and H4 Ubiquitylation Requires CUL4 and DDB1

After demonstrating that histone H3 and H4 ubiquitylation occurs in vivo, we set out to determine whether their ubiquitylation is catalyzed by the CUL4-DDB-ROC1 complex. Previous studies have indicated that CUL4A, CUL4B, and DDB1 are required for the ubiquitin ligase activity of a CUL4-containing protein complex (Higa et al., 2003; Hu et al., 2004). To address the potential

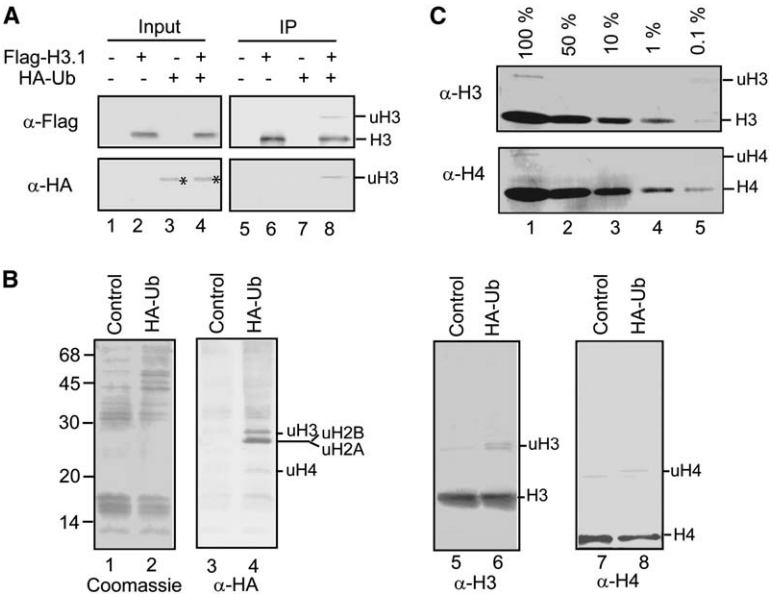

Figure 3. Histones H3 and H4 Are Ubiquitylated In Vivo

(A) Transfected histone H3 is subjected to ubiquitylation in vivo. HeLa cells were transfected with expression plasmids as indicted on the top of the panel. Cell lysates (lanes 1–4) were subjected to immunoprecipitation (lanes 5–8) with anti-Flag antibody under denaturing conditions and were analyzed by Western blotting. Antibodies used are indicated on the left side of the panels. Ubiquitylated H2A is indicated by an asterisk (*).

(B) Histones H3 and H4 are ubiquitylated in vivo. Coomassie staining (lanes 1 and 2) and Western blot assays (lanes 3–8) of a 18% polyacrylamide-SDS gel containing proteins derived from control cells and cells harboring a stably integrated HA-ubiquitin gene. Antibodies used are indicated on the bottom of the panels.

(C) Quantification of the endogenous histone H3 and H4 ubiquitylation levels. Western blot assay of proteins derived from HeLa cells. Antibodies used are indicated on the left side of the panels.

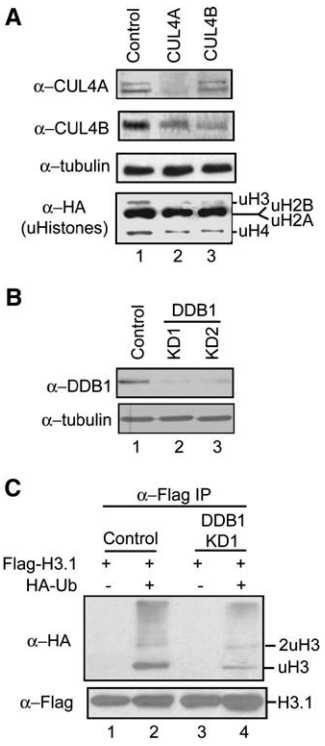

Figure 4. siRNA-Mediated Knockdown of CUL4 and DDB1 Affects Histone H3 and H4 Ubiquitylation Levels In Vivo

(A) Western blot analysis of proteins derived from control cells (lane 1) and cells treated with CUL4A and CUL4B siRNA (lanes 2 and 3). Antibodies used are indicated on the left side of the panels. Tubulin was used as a loading control.

(B) Western blot analysis of proteins derived from control cells (lane 1) and cells with stable knockdown of DDB1 (lanes 2 and 3). KD1 and KD2 are two cell lines independently derived from two cell clones. Antibodies used are indicated on the left side of the panels. Tubulin was used as a loading control.

(C) Western blot analysis of proteins immunoprecipitated from control cells (lanes 1 and 2) and DDB1-knockdown KD1 cells (lanes 3 and 4) transfected with the indicated combinations of expression plasmids. Antibodies used are indicated on the left side of the panels.

role of CUL4 proteins in histone H3 and H4 ubiquitylation, we performed RNAi-mediated knockdown of CUL4A and CUL4B in the HA-ubiquitin-tagged cell line and examined the effects of CUL4A or CUL4B knockdown on the levels of H3 and H4 ubiquitylation. Results shown in Figure 4A demonstrate that knockdown of CUL4A or CUL4B significantly reduces H3 and H4 ubiquitylation levels (compare lanes 2 and 3 with 1), indicating that both CUL4A and CUL4B contribute to histone H3 and H4 ubiquitylation in vivo.

To further evaluate the contribution of the CUL4-DDB-ROC1 complex in histone H3 and H4 ubiquitylation in vivo, we generated two independent DDB1 stable knockdown cell lines by using a vector-based RNAi technique (Wang et al., 2004). Western blot analysis indicated that the DDB1 levels were significantly reduced in both cell lines (Figure 4B). To examine the effect of DDB1 knockdown on histone ubiquitylation, DNAs encoding Flag-H3.1 and HA-ubiquitin were transfected individually or in combination into control and DDB1-knockdown cells. Cell extracts were then subjected to immunoprecipitation with M2 anti-Flag antibody under denaturing conditions (Shiio and Eisenman, 2003). Western blot analysis indicated that the H3 ubiquitylation levels were greatly reduced in the DDB1-knockdown cells when compared with control cells (Figure 4C, compare lanes 2 and 4). Similar results were also obtained by using the second DDB1 knockdown cell line KD2 or the CUL4A stable knockdown cell lines (Figure 6A and data not shown). Collectively, the above results allow us to conclude that the CUL4-DDB-ROC1 complex is responsible for histone H3 and H4 ubiquitylation in vivo.

CUL4-DDB-ROC1-Mediated H3 and H4 Ubiquitylation Is Induced by UV Irradiation

Having established that CUL4-DDB-ROC1 complex functions as a bone fide histone ubiquitin ligase, we explored its physiological function. Two lines of evidence point to its potential role in DNA damage response. First, the complex contains the damage DNA binding proteins DDB1 and DDB2, which have been shown to relocate to damaged DNA foci after UV irradiation (Otrin et al., 1997). Second, a similar ubiquitin ligase complex has been reported to bind chromatin tightly after UV irradiation (Groisman et al., 2003). To test this possibility, we examined the effect of UV irradiation on histone ubiquitylation. Although the levels of H2A ubiquitylation were not changed in response to UV irradiation or CUL4A knockdown (Figure S1 available in the Supplemental Data with this article online), a time course study indicates that the levels of H3 and H4 ubiquitylation exhibit a dynamic change in response to UV irradiation (Figure 5A). The ubiquitylation levels increase quickly after UV irradiation, reach peaks between 1 and 2 hr, decrease after 4 hr, and return to the normal levels after 8 hr. This dynamic change in response to UV irradiation suggests that histone ubiquitylation may participate in cellular response to DNA damage. To determine whether the UV-induced increase in histone ubiquitylation depends on the CUL4-DDB-ROC1 complex, we performed the same experiments in cells treated with CUL4A siRNA. Consistent with previous results, transfection of CUL4A siRNA greatly reduced the H3 and H4 ubiquitylation levels (Figure 5B, compare lanes 1 and 3). Importantly, UV-induced increases in H3 and H4 ubiquitylation were not observed in cells treated with CUL4A siRNA (Figure 5B, compare lanes 2 and 4). This result indicates that the UV-induced H3 and H4 ubiquitylation is mediated by the CUL4-DDB-ROC1 complex.

To test whether UV irradiation directly contributes to histone ubiquitylation, we assembled mononucleosomes using DNA with or without UV irradiation (Figure 5C). When these nucleosomes were subjected to in vitro ubiquitylation assay, we found that nucleosomes assembled with UV-damaged DNA are a better substrate for the CUL4-DDB-ROC1 complex compared to nucleosomes assembled with undamaged DNA (Figure 5D, compare lanes 1–4 with lanes 5–8). Interestingly, the reaction proceeded quickly, with more than 80% of the total ubiquitylation completed within 10 min when UV-damaged nucleosomes were used as substrates (Figure 5D, bottom). Similar results were obtained with nucleosomes that were directly subjected to UV irradiation (Figure S2). These results support

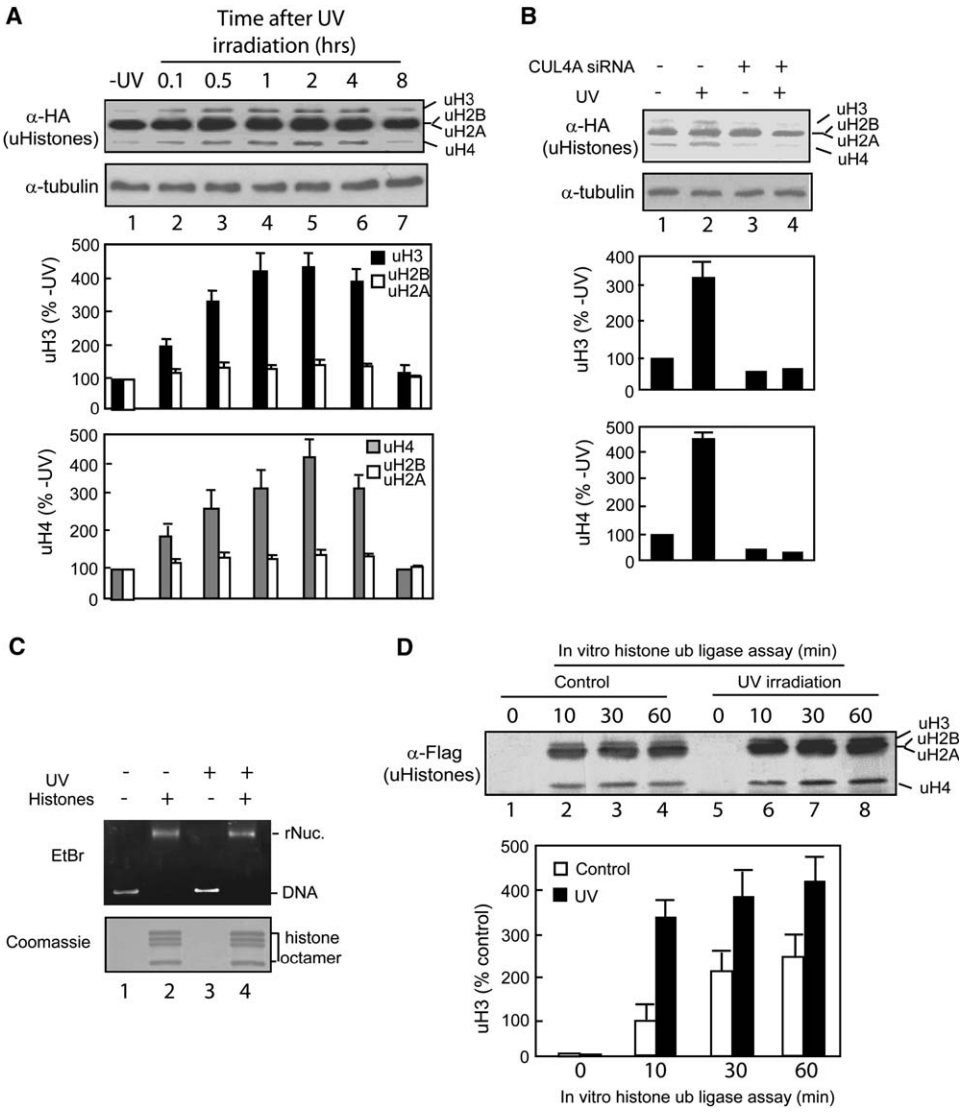

Figure 5. CUL4-DDB-ROC1-Mediated H3 and H4 Ubiquitylation Is Induced by UV Irradiation

(A) Dynamic changes of H3 and H4 ubiquitylation levels after UV irradiation. Western blot analysis (top two panels) of proteins derived from mock and UV-irradiated cells (lanes 2–7). Incubation time after UV irradiation is indicted on the top of the panel. Antibodies used are indicated on the left side of the panel. Tubulin was used as a loading control. The ubiquitylation levels of H3 and H4 (bottom two panels) were relative to the level before UV treatment. Variations of three independent experiments are indicated by error bars.

(B) UV-induced H3 and H4 ubiquitylation is dependent on CUL4A. Western blot analysis (top two panels) of proteins derived from UV irradiated control cells (lanes 1 and 2) or cells treated with CUL4A siRNA (lanes 3 and 4). Antibodies used are indicated on the left side of the panels. Tubulin was used as a loading control. The ubiquitylation levels of H3 and H4 (bottom two panels) were relative to the level before UV treatment. Variations of three independent experiments are indicated by error bars.

(C) Mononucleosome assembly with DNA fragments with or without UV irradiation. EtBr-stained 5% native polyacrylamide gel (top) and Coomassie staining of a 18% polyacrylamide-SDS gel (bottom) containing DNA (lanes 1 and 3) and reconstituted mononucleosomes (lanes 2 and 4) are shown. DNA was irradiated (lanes 3 and 4) or mock irradiated (lanes 1 and 2) with UV prior to the reconstitution.

(D) UV damage-containing nucleosomes are better substrates for CUL4-DDB-ROC1 ligase complex. Histone ubiquitin ligase assay (top) of the CUL4-DDB-ROC1 complex with mononucleosomes reconstituted with mock-irradiated DNA (lanes 1–4) and UV-irradiated DNA (lanes 5–8). Time points at which samples were taken from the reaction mixture are indicated on the top of the panel. Quantification of the ubiquitylated H3 levels is shown on the bottom. The ubiquitylation level at 10 min after the reaction with mock-irradiated DNA was arbitrarily set as 100%. Variations between three independent experiments are indicated by error bars.

a direct contribution of DNA damage by UV on CUL4-DDB-ROC1-mediated histone ubiquitylation.

CUL4A Knockdown Impairs Cellular Response to DNA Damage by UV

To examine the role of H3 and H4 ubiquitylation in cellular response to UV damage in vivo, we generated two stable cell lines with knockdown of CUL4A (Figure 6A, compare lane 1 with lanes 2 and 3). Knockdown of CUL4A reduced the ability of these cells to repair thymine dimers, especially within the first 1–2 hr (Figure 6B). Given that the H3 and H4 ubiquitylation levels increase quickly in response to UV irradiation (Figure 5D), we investigated whether CUL4A knockdown affects an early step in the

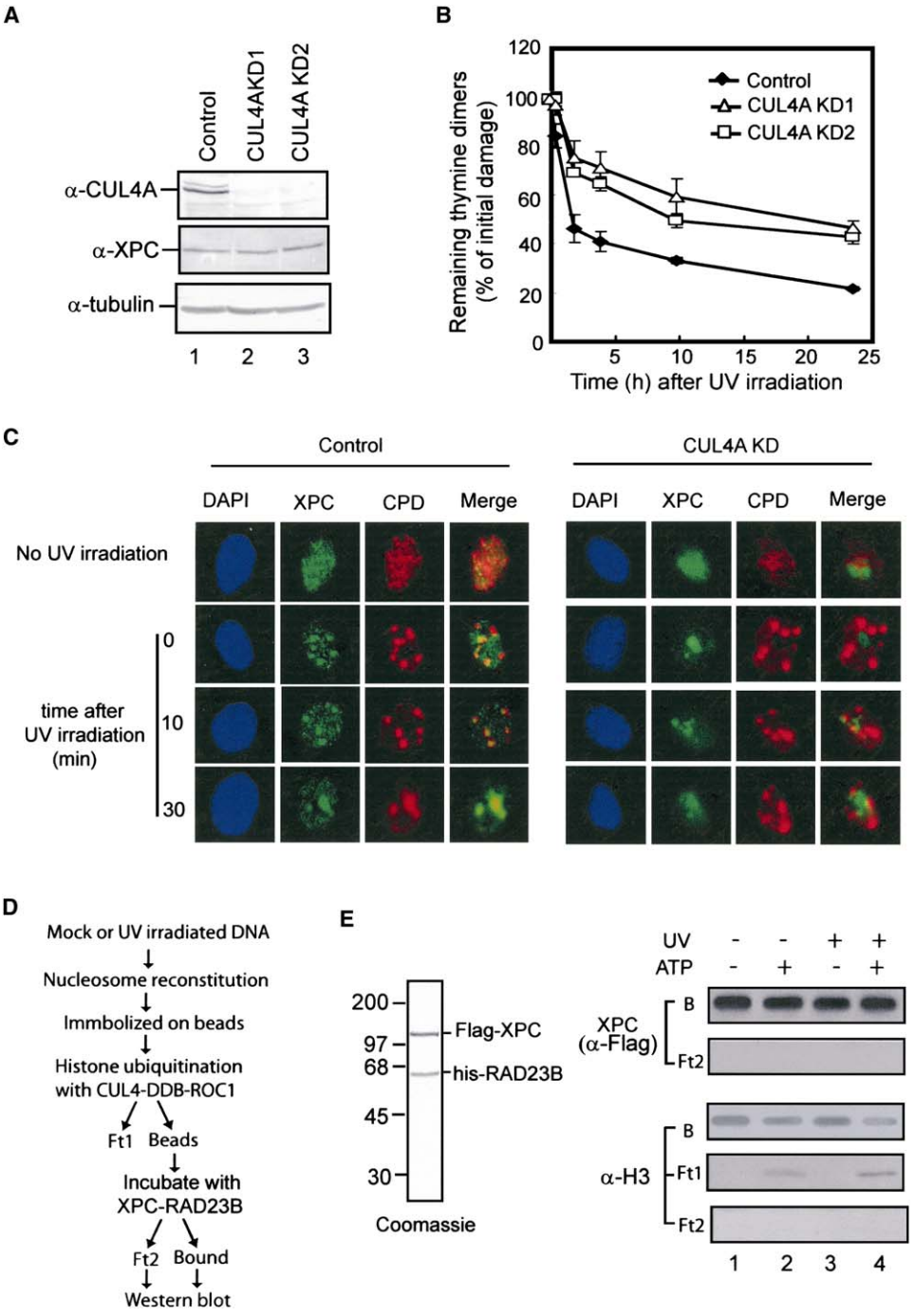

Figure 6. CUL4A Knockdown Impairs Cellular Response to UV-Induced DNA Damage

(A) Western blot analysis of proteins derived from control cells (lane 1) and two independent CUL4A knockdown cell lines (lanes 2 and 3). Antibodies used are indicated on the left side of the panel. Tubulin was used as a loading control.

(B) CUL4A knockdown impairs the capacity of cells to remove thymine dimers. Cells were irradiated with UV and lysed immediately (at time 0) or at the times indicated. The thymine dimers were analyzed by ELISA and presented as the percentage to those at time 0. Data presented are means ± SD of three independent experiments.

(C) CUL4A knockdown impairs recruitment of XPC to UV-damaged foci. Cells were UV irradiated through a polycarbonate UV-absorbing filter and incubated at different times as indicated on the left side of the panel. The recruitment of XPC to the damage sites was visualized by indirect immunofluorescence using a rabbit polyclonal antibody against XPC (green) and the mouse anti-thymine dimer monoclonal antibody (red).

(D) Schematic representation of the strategy used to determine the effects of histone ubiquitylation on the binding of XPC-RAD23B to nucleosomes.

(E) Histone ubiquitylation affects nucleosome stability in vitro. Recombinant XPC-RAD23B complex (left) was incubated with reconstituted nucleosomes with or without histone ubiquitylation. The effect of histone ubiquitylation on XPC-RAD23B binding was evaluated by Western blotting. Ft and B represent flowthrough and bound, respectively. Antibodies used are indicated on the left side of the panels.

repair process, namely the recruitment of the repair proteins such as XPC. Western blot analysis revealed that the levels of XPC are similar in control and CUL4A knockdown cells (Figure 6A). Immunostaining revealed that in control cells XPC started to relocate to the damaged foci, indicated by CPD staining, immediately after UV irradiation (0 min) and completely colocalized with the damaged foci 30 min after the irradiation (Figure 6C, left panels). However, colocalization of XPC and CPD was not observed under the same conditions in the CUL4A knockdown cells (Figure 6C, right panels). This result indicates that CUL4A, which is required for UV-induced H3 and H4 ubiquitylation (Figure 5B), plays an important role in the recruitment of repair proteins to the damaged foci.

To examine whether H3 and H4 ubiquitylation directly contributes to XPC recruitment, we analyzed whether the affinity of the XPC-RAD23B complex to nucleosomes is affected by DNA damage and/or histone ubiquitylation by using a strategy depicted in Figure 6D. Nucleosomes were first immobilized on streptavidin beads and then subjected to ubiquitylation reaction with the CUL4-DDB-ROC1 complex. After washing, the beads were incubated with the purified XPC-RAD23B complex (Figure 6E, left), which is capable of binding to the damaged DNA (Figure S3). Western blot analysis was then performed to determine the amount of XPC in supernatant and bound fractions. To our surprise, we found that neither UV damage nor histone ubiquitylation affected the affinity of XPC-RAD23B toward the nucleosomes (Figure 6E, right two top panels). Although this appears to be in conflict with a previous report (Yasuda et al., 2005), we note that the two experiments were carried out with nucleosomes containing different types of DNA damages. The nucleosomes used by Yasuda et al. all contained a 6-4PP damage site, whereas the nucleosomes used in this study were damaged by UV irradiation and contained both CPD and 6-4PP. In any case, our result indicates that H3 and H4 ubiquitylation may affect the recruitment of XPC-RAD23B to the damaged nucleosomes through an indirect mechanism.

To explore this possibility, we compared the amount of histone H3 in the supernatant (Ft1 and Ft2) and bound fractions. Results shown in Figure 6E indicate that the amounts of histone H3 retained on the beads were consistently lower in the presence of ATP than in the absence of ATP (Figure 6E, third panel, compare lanes 2 and 4 with lanes 1 and 3). Based on this result, we speculate that H3 and H4 ubiquitylation may interfere with histone-DNA interaction and cause core histones release from nucleosomes. The detection of H3 in the supernatant after histone ubiquitylation reaction is consistent with this notion (Figure 6E, fourth panel, compare lanes 2 and 4 with lanes 1 and 3).

UV-Induced Histone Ubiquitylation Facilitates Histone Release from Nucleosomes

To determine whether UV-induced histone ubiquitylation affects the association of histones with DNA in vivo, we fractionated HeLa cell proteins into cytoplasmic, nuclear extract, and nuclear pellet fractions. The nuclear pellet fraction contains proteins that are tightly associated with DNA. Western blot analysis indicated that the majority of histones was present in the nuclear pellet fraction (Figure 7A, lane 3). However, the ubiquitylated H3 is detectable in all three fractions, although the majority (83%) is present in the nuclear pellet fraction. As expected, upon UV irradiation, the levels of uH3 increased. Interestingly, a significant change in the distribution of uH3 was observed (Figure 7A, bottom). The uH3 in the nuclear pellet fraction dropped from 83% to 41%, whereas the uH3 in the nuclear extract and cytoplasm fractions increased from 12% to 40% and 5% to 19%, respectively. These results are consistent with the notion that UV-induced H3 and H4 ubiquitylation affects nucleosome stability.

To determine whether UV-induced redistribution of uH3 depends on H3 ubiquitylation, we repeated the experiments in CUL4A knockdown cells (Figure 7B). Consistent with our previous observations (Figure 5B), CUL4A knockdown reduced the uH3 levels (Figures 7A and 7B, compare second panels). Importantly, no significant change was observed in the distribution of uH3 in response to UV irradiation (Figure 7B, bottom). Together, our results support a model (Figure 7C) in which UV-induced H3 and H4 ubiquitylation weakens the interaction between histones and DNA, which results in partial or complete remodeling of the damaged nucleosomes so that the damaged DNA is accessible to the repair proteins such as XPC-RAD23B. Consistent with this model, XPC-RAD23B exhibited much higher affinity for naked DNA than for nucleosomes (Hara et al., 2000), although UV-damaged nucleosomes could still be bound by XPC-RAD23B (Yasuda et al., 2005).

Discussion

In this study, we identified and characterized the CUL4-DDB-ROC1 complex as a ubiquitin E3 ligase for histones H3 and H4. We provide evidence that CUL4-DDB-ROC1-mediated H3 and H4 ubiquitylation facilitates cellular response to UV damage by affecting nucleosome stability.

H3 and H4 Ubiquitylation Is Involved in DNA Damage Response

Of the four core histones, H2A and H2B have long been known to be modified by ubiquitin conjugation (Jason et al., 2002; Osley, 2004). Recent studies on H2B and H2A ubiquitylation in budding yeast and mammals have revealed a critical role for H2B and H2A ubiquitylation in regulating transcription and other cellular processes (Osley, 2004; Wang et al., 2004). However, whether histones H3 and H4 are ubiquitylated and what the function of histone H3 and H4 ubiquitylation is remained unknown.

Here, we present evidence that histone H3 and H4 are ubiquitylated in vivo. Compared with H2B and H2A ubiquitylation, H3 and H4 ubiquitylation occurs at substantially lower levels (0.05%–0.3%). Despite its low abundance, ubiquitylation of H3 and H4 may play important roles in regulating chromatin function. Several lines of evidence support the contention that CUL4-DDB-ROC1-mediated H3 and H4 ubiquitylation is an important aspect of cellular response to DNA damage. First, UV irradiation results in a marked increase in H3 and H4 ubiquitylation (Figure 5A). Importantly, this increase depends on CUL4A (Figure 5B). Second, CUL4A is not only required for efficient repair of the thymine dimers but also

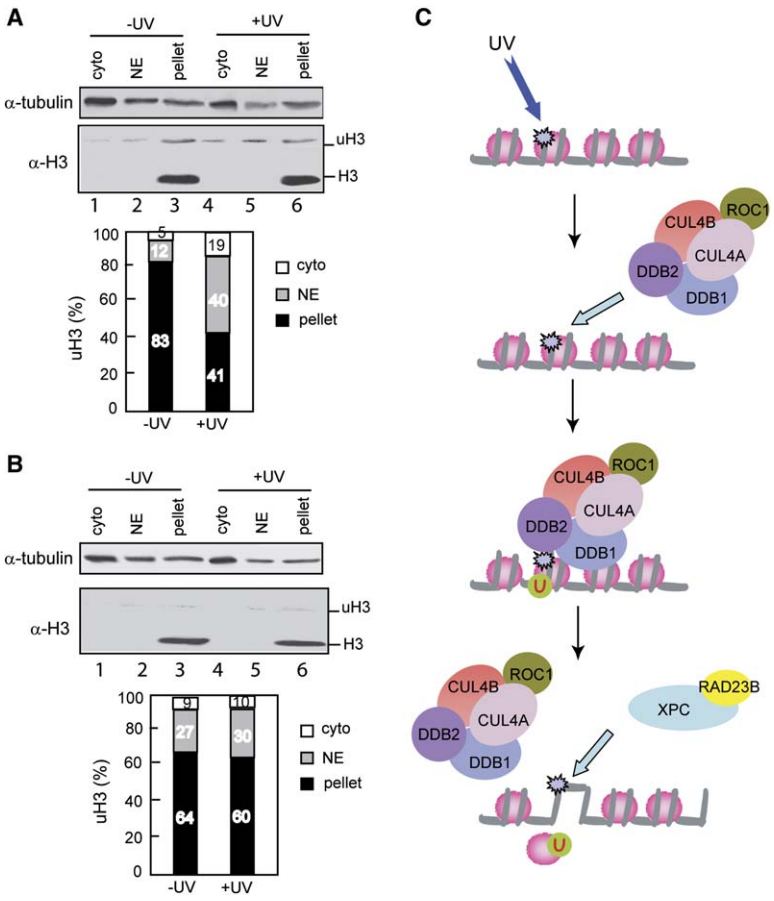

Figure 7. UV-Induced H3 and H4 Ubiquitylation Affects Nucleosome Stability

(A) Western blot analysis (top two panels) of proteins derived from different fractions of HeLa cells without (lanes 1–3) or with (lanes 4–6) UV irradiation. HeLa cells were fractionated as described in the Supplemental Experimental Procedures. Antibodies used are indicated on the left side of the panels. Tubulin was used as a loading control. Quantification of the distribution of the ubiquitinated histone H3 in each fraction is shown in the bottom panel.

(B) UV-induced redistribution of uH3 is dependent on CUL4A. Western blot analysis (top two panels) of proteins derived from different fractions of CUL4A KD1 cells without (lanes 1–3) or with (lanes 4–6) UV irradiation. CUL4A KD1 cells were fractionated as described in the Supplemental Experimental Procedures. Antibodies used are indicated on the left side of the panels. Tubulin was used as a loading control. Quantification of the distribution of the ubiquitinated histone H3 in each fraction is shown in the bottom panel.

(C) A model depicting the involvement of CUL4-DDB-ROC1-mediated histone ubiquitylation in cellular response to UV damage. Upon UV irradiation, CUL4-DDB-ROC1 histone ubiquitin ligase complex is recruited to the damaged chromatin. CUL4-DDB-ROC1-mediated histone ubiquitylation around the lesion causes histone eviction from the damaged nucleosomes, which exposes the damaged DNA to the repair proteins.

plays an important role in the recruitment of the repair proteins, such as XPC, to the damaged foci (Figure 6). Consistently, DDB2, a component of the CUL4-DDB-ROC1 complex, has been shown to be required for the recruitment of XPC to the damaged foci (Fitch et al., 2003). Third, we provide evidence that UV-induced histone ubiquitylation alters nucleosome stability, which can cause histone release from nucleosomes. Because XPC has been shown previously to have higher affinity for naked DNA than for nucleosomes (Hara et al., 2000), histone eviction from damaged nucleosomes may facilitate the recruitment of XPC to the damaged foci.

Our studies indicated that UV irradiation does not have a significant effect on the levels of uH2A (Figure S1). Although this result is consistent with our previous reports that hPRC1L is the major ubiquitin ligase for histone H2A (Wang et al., 2004), it is inconsistent with a recent report that DDB1-CUL4A-DDB2 ubiquitylates histone H2A during UV irradiation (Kapetanaki et al., 2006). While this manuscript was under review, Kapetanaki et al. (2006) reported that the DDB1-CUL4A-DDB2 complex coimmunoprecipitates with uH2A and that ubiquitylation of H2A is impaired after UV irradiation in DDB2-mutated xeroderma pigmentosum group E cells, indicating that histone H2A might be a physiological target for the DDB1-CUL4A-DDB2 complex during cellular response to UV damage (Kapetanaki et al., 2006). Although the reasons for these discrepancies are currently unknown, we note that the two studies used different cell lines.

Although our studies support a function for CUL4-DDB-ROC1-mediated histone ubiquitylation in DNA damage response, ubiquitylation of proteins other than histones by CUL4 has been reported to regulate genome stability (Higa et al., 2003; Hu et al., 2004; Zhong et al., 2003). For example, CTD-1, a licensing factor required for DNA replication in S phase, has been shown to be a substrate for CUL4. It is believed that ubiquitylation and subsequent degradation of CDT-1 ensures progression of the cell cycle. In addition to CDT1, CUL4 has also been shown to affect the stability of other proteins, including STAT1 and STAT3 (Andrejeva et al., 2002; Ulane et al., 2003), DDB2 (Chen et al., 2001), c-*jun* (Wertz et al., 2004), and XPC (Sugasawa et al., 2005). Although ubiquitylation of CDT1 or other nonhistone proteins may regulate their stability, ubiquitylation of histones may cause their eviction from nucleosomes. Whether the evicted ubiquitylated histones are targeted for degradation remains to be determined.

Role of Histone Ubiquitylation on Chromatin Function

In addition to participating in the cellular response to DNA damage, CUL4-mediated histone ubiquitylation may regulate other aspects of chromatin function, including heterochromatin silencing. For example, the fission yeast CUL4 ortholog Pcu4 was recently reported to be a component of a complex that also includes Rik1 and Clr4 (Horn et al., 2005; Jia et al., 2005). Rik1 is highly related to DDB1, whereas Clr4 is the H3K9

methyltransferase in fission yeast. Both proteins are involved in heterochromatin formation. Expression of a dominant-negative Pcu4 mutant interferences with heterochromatin function, and mutations of CUL4 result in defective localization of Clr4 and loss of heterochromatin silencing. Interestingly, these defects can be rescued by the expression of wild-type, but not by a mutant CUL4, which lacks sites for Nedd8 modification (Horn et al., 2005; Jia et al., 2005). Given that the ubiquitin ligase activity of this CUL4 complex requires Nedd8 modification (Liu et al., 2002), it is likely that the defective heterochromatin function that the CUL4 mutants exhibit is mediated by defective histone ubiquitylation.

Pcu4 and Rik1, the fission yeast homologs of the CUL4 and DDB, associate with the H3K9 methyltransferase Clr4, suggesting that H3 and H4 ubiquitylation might be connected to H3K9 methylation. Although the exact relationship between these two modifications remains to be elucidated, it is noteworthy to mention that a "transtail" relationship between H2B ubiquitylation and H3K4/H3K79 methylation has been established in both budding yeast and mammals (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002; Kim et al., 2005; Zhu et al., 2005). Genetic studies in budding yeast indicate that H2B ubiquitylation is required for subsequent methylation of H3K4 and H3K79. Recent studies indicate that H2B ubiquitylation may control H3K4 and H3K79 methylation by regulating the activity of the histone methyltransferases Set1 and Dot1 (Shahbazian et al., 2005).

Compared with other histone modifications, ubiquitylation involves the addition of a relatively large molecule that is two-thirds the mass of an individual histone. Due to the large size of this molecule, it has been postulated that ubiquitylation will have an impact on chromatin structure (Jason et al., 2002). However, in vitro nucleosome reconstitution experiments and in vivo studies on histone H2B ubiquitylation have thus far failed to obtain supporting evidence (Jason et al., 2002; Shahbazian et al., 2005). Chromatin fibers reconstituted with uH2A molecules have similar properties to the control chromatin with regard to folding and sedimentation (Jason et al., 2002). However, our studies suggest that CUL4-DDB-ROC1-mediated H3 and H4 ubiquitylation may affect nucleosome stability and cause histone eviction. Although our data do not support a major role of CUL4-DDB-ROC1 in global H2A or H2B ubiquitylation in vivo (Figure S1), our in vitro data suggest that CUL4-DDB-ROC1 is able to ubiquitylate all of the four core histones. It is possible that ubiquitylation of all histones in the same nucleosome may weaken the interaction between histones and DNA. Further structure studies on the nucleosomes ubiquitylated by CUL4-DDB-ROC1 may provide insight into this issue.

Experimental Procedures

Nucleosome Reconstitution and XPC-RAD23B Binding Assays

The 5s DNA fragments were PCR amplified from the XP-10 plasmid (provided by Dr. Jeffrey J. Hayes), and the 5′ primer used for PCR amplification was biotinylated. DNA fragments were gel purified and subjected to mock or UV irradiation at 500 J/m^2. The mononucleosomes were assembled by salt dilution (Dyer et al., 2004).

For XPC-RAD23B binding assay, reconstituted nucleosomes were first immobilized on streptavidin agarose (Introgen). After histone ubiquitylation, the beads were washed three times with buffer C (20 mM sodium phosphate [pH 7.8], 10% glycerol, 0.01% Nonidet P-40, and 0.25 mM PMSF) containing 0.6 M NaCl. The flowthrough and wash were combined and designated as Ft1, and proteins were precipitated with TCA. The XPC-RAD23B complex was reconstituted in vitro (Sugasawa et al., 2005) and incubated with streptavidin agarose beads at 30°C for 30 min. The beads were then washed three times with buffer C containing 100 mM NaCl. The flowthrough and wash solution were combined and designated as Ft2, and proteins were precipitated with TCA and analyzed by Western blotting.

Immnofluorescence and Measurement of Thymine Dimers

Coverslips were washed with PBS and CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES [pH 7.0], and 3 mM $MgCl_2$) twice. Cells were then permeablized with 0.5% Triton in CSK buffer at room temperature for 5 min. After washing with CSK buffer twice, cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. After three washes with PBS, DNA was denatured by incubation with 4 N HCl at room temperature for 10 min. Cells were then blocked by incubation with blocking buffer (PBS plus 1% BSA) for 30 min at room temperature. The primary antibodies, in blocking buffer, were incubated with the coverslips for 2 hr at room temperature. Cells were then washed three times with blocking buffer and incubated with second antibodies for 45 min at room temperature. After washing three times with PBS, DAPI at a concentration of 0.5 μg/ml was added and incubated for an additional 5 min. The coverslips were then washed with PBS twice and with water once before being mounted onto slides with vectorshield mounting media (Vector Laboratories). Images were captured with an Olympus microscope with 60× oil objective. Antibodies were used at the following dilutions: anti-thymine dimer mouse monoclonal (Kamiya Biomedical), 1:500; anti-XPC rabbit polyclonal (GeneTex), 1:500; and Texas Red conjugated anti-rabbit IgG and FITC conjugated anti-mouse IgG1 (GeneTex), 1:1000. The amounts of thymine dimers in UVC-irradiated cells were measured by ELISA as described previously (Zhai et al., 2005) using anti-CPD antibody.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and three figures and can be found with this article online at http://www.molecule.org/cgi/content/full/22/3/383/DC1/.

Acknowledgments

We thank K. Sugasawa for baculovirus expressing XPC and constructs for RAD23B; H. Zhang for CUL4B construct and antibody; and Y. Nakatani for H3.1 plasmid. We thank members of the T.R. Broker and L.T. Chow lab for technical assistance with microscopy; L. Lacomis and G. Boysen for help with mass spectrometry; and R. Klose for critically reading the manuscript. This work was supported by National Institutes of Health grant GM68804 (to Y.Z.), a start-up grant (to H.W.), and P30 CA08748 (to P.T.). Y.Z. is an investigator of the Howard Hughes Medical Institute.

Received: February 3, 2006
Revised: March 7, 2006
Accepted: March 30, 2006
Published: May 4, 2006

References

Andrejeva, J., Poole, E., Young, D.F., Goodbourn, S., and Randall, R.E. (2002). The p127 Subunit (DDB1) of the UV-DNA damage repair binding protein is essential for the targeted degradation of STAT1 by the V protein of the paramyxovirus simian virus 5. J. Virol. *76*, 11379–11386.

Bohm, L., Crane-Robinson, C., and Sautiere, P. (1980). Proteolytic digestion studies of chromatin core-histone structure. Identification of a limit peptide of histone H2A. Eur. J. Biochem. *106*, 525–530.

Briggs, S.D., Xiao, T., Sun, Z.-W., Caldwell, J.A., Shabanowitz, J., Hunt, D.F., Allis, C.D., and Strahl, B.D. (2002). Gene silencing: trans-histone regulatory pathway in chromatin. Nature *418*, 498.

Cao, R., Tsukada, Y.-i., and Zhang, Y. (2005). Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. Mol. Cell *20*, 845–854.

Chen, H.Y., Sun, J.-M., Zhang, Y., Davie, J.R., and Meistrich, M.L. (1998). Ubiquitination of histone H3 in elongating spermatids of rat testes. J. Biol. Chem. *273*, 13165–13169.

Chen, X., Zhang, Y., Douglas, L., and Zhou, P. (2001). UV-damaged DNA-binding proteins are targets of CUL-4A-mediated ubiquitination and degradation. J. Biol. Chem. *276*, 48175–48182.

de Napoles, M., Mermoud, J.E., Wakao, R., Tang, Y.A., Endoh, M., Appanah, R., Nesterova, T.B., Silva, J., Otte, A.P., and Vidal, M. (2004). Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev. Cell *7*, 663–676.

Dover, J., Schneider, J., Tawiah-Boateng, M.A., Wood, A., Dean, K., Johnston, M., and Shilatifard, A. (2002). Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. J. Biol. Chem. *277*, 28368–28371.

Dyer, P., Edayathumangalam, R., White, C., Bao, Y., Chakravarthy, S., Muthurajan, U., and Luger, K. (2004). Reconstitution of nucleosome core particles from recombinant histones and DNA. Methods Enzymol. *375*, 23–44.

Emre, N.C.T., Ingvarsdottir, K., Wyce, A., Wood, A., Krogan, N.J., Henry, K.W., Li, K., Marmorstein, R., Greenblatt, J.F., Shilatifard, A., and Berger, S.L. (2005). Maintenance of low histone ubiquitylation by Ubp10 correlates with telomere-proximal sir2 association and gene silencing. Mol. Cell *17*, 585–594.

Fang, J., Chen, T., Chadwick, B., Li, E., and Zhang, Y. (2004). Ring1b-mediated H2A ubiquitination associates with inactive X chromosomes and is involved in initiation of X inactivation. J. Biol. Chem. *279*, 52812–52815.

Fitch, M.E., Nakajima, S., Yasui, A., and Ford, J.M. (2003). In vivo recruitment of XPC to UV-induced cyclobutane pyrimidine dimers by the DDB2 gene product. J. Biol. Chem. *278*, 46906–46910.

Gardner, R.G., Nelson, Z.W., and Gottschling, D.E. (2005). Ubp10/Dot4p regulates the persistence of ubiquitinated histone H2B: distinct roles in telomeric silencing and general chromatin. Mol. Cell. Biol. *25*, 6123–6139.

Giannattasio, M., Lazzaro, F., Plevani, P., and Muzi-Falconi, M. (2005). The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1 and H3 methylation by Dot1. J. Biol. Chem. *280*, 9879–9886.

Goldknopf, I.L., Taylor, C.W., Baum, R.M., Yeoman, L.C., Olson, M.O., Prestayko, A.W., and Busch, H. (1975). Isolation and characterization of protein A24, a "histone-like" non-histone chromosomal protein. J. Biol. Chem. *250*, 7182–7187.

Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J.-i., Saijo, M., Drapkin, R., Kisselev, A.F., Tanaka, K., and Nakatani, Y. (2003). The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. Cell *113*, 357–367.

Hara, R., Mo, J., and Sancar, A. (2000). DNA damage in the nucleosome core is refractory to repair by human excision nuclease. Mol. Cell. Biol. *20*, 9173–9181.

Henry, K.W., Wyce, A., Lo, W.-S., Duggan, L.J., Emre, N.C.T., Kao, C.-F., Pillus, L., Shilatifard, A., Osley, M.A., and Berger, S.L. (2003). Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. Genes Dev. *17*, 2648–2663.

Higa, L.A.A., Mihaylov, I.S., Banks, D.P., Zheng, J., and Zhang, H. (2003). Radiation-mediated proteolysis of CDT1 by CUL4–ROC1 and CSN complexes constitutes a new checkpoint. Nat. Cell Biol. *5*, 1008–1015.

Horn, P.J., Bastie, J.-N., and Peterson, C.L. (2005). A Rik1-associated, cullin-dependent E3 ubiquitin ligase is essential for heterochromatin formation. Genes Dev. *19*, 1705–1714.

Hu, J., McCall, C.M., Ohta, T., and Xiong, Y. (2004). Targeted ubiquitination of CDT1 by the DDB1-CUL4A-ROC1 ligase in response to DNA damage. Nat. Cell Biol. *6*, 1003–1009.

Hwang, W.W., Venkatasubrahmanyam, S., Ianculescu, A.G., Tong, A., Boone, C., and Madhani, H.D. (2003). A conserved RING finger protein required for histone H2B monoubiquitination and cell size control. Mol. Cell *11*, 261–266.

Jason, L., Moore, S., Lewis, J., Lindsey, G., and Ausio, J. (2002). Histone ubiquitination: a tagging tail unfolds? Bioessays *24*, 166–174.

Jia, S., Kobayashi, R., and Grewal, S. (2005). Ubiquitin ligase component Cul4 associates with Clr4 histone methyltransferase to assemble heterochromatin. Nat. Cell Biol. *7*, 1007–1013.

Kao, C.-F., Hillyer, C., Tsukuda, T., Henry, K., Berger, S., and Osley, M.A. (2004). Rad6 plays a role in transcriptional activation through ubiquitylation of histone H2B. Genes Dev. *18*, 184–195.

Kapetanaki, M.G., Guerrero-Santoro, J., Bisi, D.C., Hsieh, C.L., Rapic-Otrin, V., and Levine, A.S. (2006). The DDB1-CUL4A^{DDB2} ubiquitin ligase is deficient in xeroderma pigmentosum group E and targets histone H2A at UV-damaged DNA sites. Proc. Natl. Acad. Sci. USA *103*, 2588–2593.

Kim, J., Hake, S., and Roeder, R. (2005). The human homolog of yeast Bre1 functions as a transcriptional coactivator through direct activator interactions. Mol. Cell *20*, 759–770.

Liu, J., Furukawa, M., Matsumoto, T., and Xiong, Y. (2002). NEDD8 modification of Cul1 dissociates p120CAND1, an inhibitor of Cul1-skp1 binding and SCF ligases. Mol. Cell *10*, 1511–1518.

Liu, Z., Oughtred, R., and Wing, S.S. (2005). Characterization of E3Histone, a novel testis ubiquitin protein ligase which ubiquitinates histones. Mol. Cell. Biol. *25*, 2819–2831.

Martin, C., and Zhang, Y. (2005). The diverse functions of histone lysine methylation. Nat. Rev. Mol. Cell Biol. *6*, 838–849.

Ng, H.H., Xu, R.-M., Zhang, Y., and Struhl, K. (2002). Ubiquitination of histone H2B by Rad6 is required for efficient Dot1-mediated methylation of histone H3 lysine 79. J. Biol. Chem. *277*, 34655–34657.

Nickel, B.E., Roth, S.Y., Cook, R.G., Allis, C.D., and Davie, J.R. (1987). Changes in the histone H2A variant H2A.Z and polyubiquitinated histone species in developing trout testis. Biochemistry *26*, 4417–4421.

Osley, M.A. (2004). H2B ubiquitylation: the end is in sight. Biochim. Biophys. Acta. *1677*, 74–78.

Otrin, V., McLenigan, M., Takao, M., Levine, A., and Protic, M. (1997). Translocation of a UV-damaged DNA binding protein into a tight association with chromatin after treatment of mammalian cells with UV light. J. Cell Sci. *110*, 1159–1168.

Peterson, C.L., and Laniel, M.-A. (2004). Histones and histone modifications. Curr. Biol. *14*, 546–551.

Pham, A.-D., and Sauer, F. (2000). Ubiquitin-activating/conjugating activity of TAFII250, a mediator of activation of gene expression in drosophila. Science *289*, 2357–2360.

Robzyk, K., Recht, J., and Osley, M.A. (2000). Rad6-dependent ubiquitination of histone H2B in yeast. Science *287*, 501–504.

Shahbazian, M.D., Zhang, K., and Grunstein, M. (2005). Histone H2B ubiquitylation controls processive methylation but not monomethylation by Dot1 and Set1. Mol. Cell *19*, 271–277.

Shiio, Y., and Eisenman, R.N. (2003). Histone sumoylation is associated with transcriptional repression. Proc. Natl. Acad. Sci. USA *100*, 13225–13230.

Sugasawa, K., Okuda, Y., Saijo, M., Nishi, R., Matsuda, N., Chu, G., Mori, T., Iwai, S., Tanaka, K., Tanaka, K., and Hanaoka, F. (2005). UV-induced ubiquitylation of XPC protein mediated by UV-DDB-ubiquitin ligase complex. Cell *121*, 387–400.

Sun, Z.-W., and Allis, C.D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature *418*, 104–108.

Ulane, C.M., Rodriguez, J.J., Parisien, J.-P., and Horvath, C.M. (2003). STAT3 ubiquitylation and degradation by mumps virus suppress cytokine and oncogene signaling. J. Virol. *77*, 6385–6393.

Vassilev, A., Rasmussen, H., Christensen, E., Nielsen, S., and Celis, J. (1995). The levels of ubiquitinated histone H2A are highly upregulated in transformed human cells: partial colocalization of uH2A clusters and PCNA/cyclin foci in a fraction of cells in S-phase. J. Cell Sci. *108*, 1205–1215.

Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R.S., and Zhang, Y. (2004). Role of histone H2A ubiquitination in Polycomb silencing. Nature *431*, 873–878.

Wertz, I.E., O'Rourke, K.M., Zhang, Z., Dornan, D., Arnott, D., Deshaies, R.J., and Dixit, V.M. (2004). Human de-etiolated-1 regulates c-Jun by assembling a Cul4A ubiquitin ligase. Science *303*, 1371–1374.

West, M., and Bonner, W. (1980). Histone H2B can be modified by the attachment of ubiquitin. Nucleic Acids Res. *8*, 4671–4680.

Wing, S., Bedard, N., Morales, C., Hingamp, P., and Trasler, J. (1996). A novel rat homolog of the Saccharomyces cerevisiae ubiquitin-conjugating enzymes UBC4 and UBC5 with distinct biochemical features is induced during spermatogenesis. Mol. Cell. Biol. *16*, 4064–4072.

Wood, A., Krogan, N.J., Dover, J., Schneider, J., Heidt, J., Boateng, M.A., Dean, K., Golshani, A., Zhang, Y., and Greenblatt, J.F. (2003). Bre1, an E3 ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter. Mol. Cell *11*, 267–274.

Yamashita, K., Shinohara, M., and Shinohara, A. (2004). Rad6-Bre1-mediated histone H2B ubiquitylation modulates the formation of double-strand breaks during meiosis. Proc. Natl. Acad. Sci. USA *101*, 11380–11385.

Yasuda, T., Sugasawa, K., Shimizu, Y., Iwai, S., Shiomi, T., and Hanaoka, F. (2005). Nucleosomal structure of undamaged DNA regions suppresses the non-specific DNA binding of the XPC complex. DNA Repair (Amst.) *4*, 389–395.

Zhai, L., Kita, K., Wano, C., Wu, Y., Sugaya, S., and Suzuki, N. (2005). Decreased cell survival and DNA repair capacity after UVC irradiation in association with down-regulation of GRP78/BiP in human RSa cells. Exp. Cell Res. *305*, 244–252.

Zhong, W., Feng, H., Santiago, F.E., and Kipreos, E.T. (2003). CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing. Nature *423*, 885–889.

Zhu, B., Zheng, Y., Pham, A.-D., Mandal, S.S., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2005). Monoubiquitination of human histone H2B: the factors involved and their roles in Hox gene regulation. Mol. Cell *20*, 601–611.