Valosin-containing Protein (VCP)/p97 Segregase Mediates Proteolytic Processing of Cockayne Syndrome Group B (CSB) in Damaged Chromatin

Jinshan He†, Qianzheng Zhu†,‡, Gulzar Wani‡, Nidhi Sharma‡, and Altaf A. Wani†§ ‡

From the Departments of †Radiology and §Molecular and Cellular Biochemistry and ‡James Cancer Hospital and Solove Research Institute, Ohio State University, Columbus, Ohio 43210

Cockayne syndrome group A and B (CSB) proteins act in transcription-coupled repair, a subpathway of nucleotide excision repair. Here we demonstrate that valosin-containing protein (VCP)/p97 segregase functions in ultraviolet radiation (UVR)-induced ubiquitin-mediated CSB degradation. We show that VCP/p97 inhibition and siRNA-mediated ablation of VCP/p97 and its cofactors UFD1 and UBXD7 impair CSB degradation. VCP/p97 inhibition also results in the accumulation of CSB in chromatin. Moreover, VCP/p97 interacts with both native and ubiquitin-conjugated forms of CSB. The localized cellular UVR exposures lead to VCP/p97 accumulation at DNA damage spots, forming distinct UVR-induced foci. However, manifestation of VCP/p97 foci is independent of CSB and UBXD7. Furthermore, VCP/p97 and UBXD7 associate with the Cockayne syndrome group A-DDB1-Cul4A complex, an E3 ligase responsible for CSB ubiquitination. Compromising proteasome and VCP/p97 function allows accumulation of both native and ubiquitinated CSB and results in increases of UBXD7, proteasomal RPN2, and Sug1 in the chromatin compartment. Surprisingly, both biochemical inhibition and genetic defect of VCP/p97 enhance the recovery of RNA synthesis following UVR, whereas both VCP/p97 and proteasome inhibitions decrease cell viability. Our findings reveal a new role of VCP/p97 segregase in the timely processing of ubiquitinated CSB from damaged chromatin.

The ubiquitin-specific segregase valosin-containing protein (VCP)/p97,4 known as CDC48 in yeast, is an ATP-driven molecular chaperone that belongs to the ATPase associated with various cellular activities family (1). VCP/p97 has two characteristic properties: ATPase activity and the ability to bind to ubiquitin (2). It is believed that these properties enable VCP/p97 to segregate ubiquitinated proteins from their tightly bound partners within multiprotein complexes, membranes, and chromatin (3, 4). For different cellular functions, VCP/p97 cooperates with different sets of mutually exclusive cofactors and additional adaptors. UFD1-NPL4 is a known substrate-recruiting cofactor that is needed for extracting misfolded proteins from the endoplasmic reticulum membrane during endoplasmic reticulum-associated degradation (5–8). VCP/p97 also cooperates with the adaptors from the ubiquitin regulatory X (UBX) family. For example, UBXD7 has been shown to participate in ubiquitin-mediated degradation of hypoxia-inducible factor 1α (HIF1α) (9). Here UBXD7 possesses an ubiquitin-associated domain and an UBX domain that enable UBXD7 to bind both VCP/p97 and ubiquitinated clients.

Although the involvement of VCP/p97 in the ubiquitin-proteasome system is becoming clearer, the role of VCP/p97 in ubiquitin-dependent extraction of individual DNA damage response factors from chromatin is largely unknown (10). It has been observed that VCP/p97 mediates the extraction of Aurora B kinase from mitotic chromosomes (11) and the L3MBTL1 repressor from DNA double-strand breaks (12). Recently VCP/p97 has been shown to participate in the extraction and removal of early damage recognition factors, DDB2 and XPC, from DNA damage sites (13). Failure to remove DDB2 and XPC impairs the excision repair of photolesions and thereby leads to chromosome aberrations. In the case of RNA polymerase II (RNAPII), it was found that ultraviolet radiation (UVR)-induced turnover of the RNAPII Rpb1 subunit is dependent on CDC48-UFD1-NPL4 in yeast (14).

It is already established that DDB2, XPC, and RNAPII serve as the damage sensors for two alternative subpathways of nucleotide excision repair: global genomic repair (GGR) and transcription-coupled repair (TCR) (15, 16). In general, nucleotide excision repair eliminates a broad variety of helix-distorting DNA lesions, including cyclobutane pyrimidine dimers, 6–4 photoproducts, and other chemically induced bulky adducts. In particular, GGR operates throughout the genome, whereas TCR eliminates DNA damage from transcribing DNA

ubiquitin-specific protease; Dox, doxycycline; UVRIF, UV radiation-induced foci; CRL, Cullin-Ring ubiquitin ligase; NHF, normal human fibroblast.
strands of transcriptionally active genes (17, 18). The orderly sequential assembly of the preincision complex of both GGR and TCR has been studied (19, 20). GGR and TCR differ mainly in their early stage of the damage recognition phase but then follow a common pathway after damage verification by TFIIH (21, 22). In GGR, the DNA lesions are detected by DDB2 and XPC, both of which are capable of binding lesion-containing DNA. In TCR, lesion recognition is sensed from stalled RNAPII complexes on damaged chromatin. The proteolytic release of RNAPII requires the function of Cockayne syndrome complementary group A and B (CSA and CSB) proteins (23–25). Alternatively, CSA can use its translocase (DNA-dependent ATPase) activity to remodel the interface between RNAPII and damaged DNA to allow repair to take place (23, 26–30).

Although the mode of action of CSA and CSB in TCR is still unclear, it is known that the CSB protein itself undergoes proteasomal degradation via ubiquitination by the CSA-DDB1-Cul4A E3 ligase complex (31). The degradation of CSB is suggested as a prerequisite for post-TCR recovery of RNA synthesis (RRS). It is possible that the timely removal of lesion- arrested RNAPII or CSA from lesion sites facilitates the productive sequential assembly of the preincision complex. In our previous work, we have demonstrated that VCP/p97 is involved in XPC degradation in damaged chromatin when XPC is not protected by ubiquitin-specific protease 7 (USP7), which was shown to deubiquitinate XPC (32). In this study, we tested the hypothesis that CSB protein is subjected to regulation by the multifunctional VCP/p97 chaperone. Our data provide important insights into the clearance of ubiquitinated CSA from damaged chromatin via the cooperative processing by VCP/p97 and the proteasome. Failure to remove ubiquitinated CSB, e.g., because of VCP/p97 inhibition, leads to retention of CSA, UBXD7, and proteasomal proteins in chromatin. Compromising VCP/p97 function does not impair post-TCR RRS but severely affects cell viability upon UV exposure. Thus, our findings reveal a key role of VCP/p97 in processing ubiquitinated CSB during TCR and in the maintenance of cell viability by clearance of unstable subunit(s), including CSB, from macromolecular protein complexes on damaged chromatin.

**Experimental Procedures**

**Chemicals, Antibodies, and Cell Lines**—The VCP/p97 inhibitor N2,N4-dibenzyloxquinazoline-2,4-diamine (DBeQ), was obtained from Sigma-Aldrich (St. Louis, MO). The proteasome inhibitor MG132 was purchased from EMB Millipore (Billerica, MA). Anti-FLAG M2 and anti-Myc-agarose affinity gels were purchased from Sigma-Aldrich. Anti-VCP/p97, anti-UFD1, anti-NPL4, anti-h2AX, anti-ubiquitin FK2, anti-Myc, and the antibodies against RPN2/S1 and Sug1 were purchased from Abcam, BD Biosciences, Abnova/Novus Biologicals (Littleton, CO), Cell Signaling Technology (Danvers, MA), EMB Millipore, Santa Cruz Biotechnology (Dallas, TX) and Thermo Scientific (Rockford, IL), respectively.

HCT116 and HCT116-USP7-/- cells were obtained from the laboratory of Bert Vogelstein (33). U2OS cells lines, stably transfected with tetracycline-inducible DNA constructs expressing Myc-tagged WT or mutant EQ (E578Q)-VCP/p97, were provided by the Weih lab (34). The dominant-negative EQ mutant is deficient in ATP hydrolysis activity of VCP/p97 but still able to bind ubiquitinated substrates. The CSB-defective CS1AN cell line, which expresses CSA under control of the tetracycline-responsive promoter (CS1AN+icSB cells) (35), was a gift from Dr. Yonggang Zhou. HeLa cells stably expressing C-terminally FLAG-HA-tagged CSA (HeLa CSA.Com cells) (36) were provided by the Nakatani laboratory. The VCP/p97 mutant fibroblasts GM22764 and GM22041 from clinically affected donor subjects heterozygous for mutated VCP/p97 genes were obtained through the Coirell Cell Repository (Camden, NJ). GM22764 fibroblasts harbor a 475C>T transition in exon 5 of the VCP gene, resulting in the substitution of cysteine for arginine at codon 159 (R159C), whereas GM22041 fibroblasts harbor a 463C>T transition in exon 5 of the VCP/p97 gene, resulting in the substitution of cysteine for arginine at codon 155 (R155C). These VCP/p97 mutations cause inclusion body myopathy with early-onset Paget disease and frontotemporal dementia.

**RNA Interference**—The siRNA sequences for non-coding 5'-AAUUCUCGGA-ACGUGUCAGCU-3' (catalog no. SI03650325); UBXd1, 5'-GGCCCTCACTAGAAGATCTA-3' (catalog no. SI04209044); UBXd7, 5'-CAGC-ACGTGCATTTCTATTACGTTTA-3' (catalog no. SI00455364); UFD1, 5'-CAGTGGATG-ATGC-AGAACTTA-3' (catalog no. SI04132583); and VCP/p97, 5'-AACAGCAUCUUCAAAACAG-AA-3' (catalog no. SI03019730) were purchased from Qiagen (Valencia, CA). The siRNA for CSA/ERCC6, 5'-GTGTGCATGTCTTTCA-3' (Am16708), was obtained from Life Technology.

**Cell Culture, DNA, and siRNA Transfection—**HCT116 and HCT116-USP7-/- cells were grown in McCoy's 5A medium. CSB-defective CS1AN cell lines, U2OS, HeLa cells, and their derivatives were grown in DMEM. Human fibroblasts were grown in minimum Eagle’s medium with additional amino acids. All cells were grown in medium supplemented with 10% (or 15% if required) FBS, penicillin, and streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. Hygromycin B and zeocin were used for maintaining stably transfected CSB-defective CS1AN cell lines and Myc-tagged VCP/p97 U2OS cell lines. Plasmid DNAs were transfected into U2OS or CS1AN cell lines using FuGENE 6 transfection reagents (Promega, Madison, WI). The siRNA transfection was conducted using Lipofectamine 2000 reagent (Life Technologies) as described earlier (37, 38).

**Cellular Protein Fractionation**—The experiment was conducted as described by Anindya R. et al. (24) with modifications. Briefly, cells (~10⁶) were lysed with 1 ml (~5X cell volume) of cytoplasmic lysis buffer (10 mM Tris-Cl (pH 7.9), 0.34 M sucrose, 3 mM CaCl₂, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40, and a protease inhibitor mixture). Nuclei were pelleted by centrifugation at 3500 × g for 15 min and washed with cytoplasmic lysis buffer without Nonidet P-40 and then lysed in 1 ml of nuclear lysis buffer (20 mM HEPES (pH 7.9), 3 mM EDTA, 10% glycerol, 1.5 mM MgCl₂, 150 mM KOAc, and protease inhibitors). The nucleoplasmic fractions were separated by centrifugation at 15,000 × g for 30 min, and the pellets were designated as the chromatin fraction. For further processing, the pellets were resuspended in 0.2 ml of nuclease incubation buffer (150 mM HEPES (pH 7.9), 1.5 mM
VCP/p97 Extracts CSB for Proteolysis

MgCl₂, 150 mM KOAc, and protease inhibitors) and incubated with 50 units Benzonase (25 units/µl) for 30 min at room temperature. The soluble chromatin fraction was collected by centrifugation at 20,000 × g for 30 min, whereas the insoluble chromatin fraction was dissolved by boiling in SDS sample buffer.

**Immunoprecipitation and Affinity Purification of Myc-VCP/p97 and the FLAG-CSA Complex**—The immunoprecipitation was done using whole cell lysates in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and protease inhibitors) with anti-CSB or anti-VCP/p97 antibodies or control IgG at 4 °C overnight. The immunocomplexes were captured by protein A Plus G-agarose beads. The inducible Myc-WT or EQ-VCP/p97 or FLAG-CSA from whole cell lysates in RIPA buffer was immunoprecipitated with anti-Myc or anti-FLAG-agarose affinity gels. The proteins or ubiquitin conjugates were recovered by immunoprecipitation and analyzed by Western blotting. For affinity purification of the FLAG-CSA complex, the soluble chromatin fractions were made from parental HeLa and FLAG-HA-tagged CSA-expressing HeLa cells. The co-immunoprecipitation was done with anti-FLAG M2-agarose affinity gels in RIPA buffer at 4 °C overnight. After subsequent washing and eluting, the immunoprecipitates were analyzed by Western blotting.

**Micropore UV Irradiation and Immunofluorescence**—The experiments were conducted according to a method rigorously established in our laboratory (39). Briefly, the cells were grown on glass coverslips, induced for Myc-tagged VCP/p97 expression, and treated with siRNA. The cells were then washed once with PBS, and a 5-µm isopore polycarbonate filter was placed on top of the cell monolayer, followed by UV irradiation at a desired dose. The UV-irradiated cells were maintained in a suitable medium for the indicated times. For immunofluorescence staining, the cells were washed twice with cold PBS, treated with pre-extraction buffer (25 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM EDTA, 3 mM MgCl₂, 300 mM sucrose, and 0.5% Triton X-100) for 2.5 min at 4 °C as needed and/or fixed with 2% paraformaldehyde in 0.5% Triton X-100 at 4 °C for 30 min. The fixed cells were rinsed twice with cold PBS, blocked with 20% normal goat serum, and stained with an appropriate primary antibody as well as FITC-, Alexa Fluor 488-, or Texas Red-conjugated secondary antibodies. The coverslips were mounted in Vectashield mounting medium with DAPI. The fluorescence images were obtained with a Nikon fluorescence microscope (E80i, Tokyo, Japan) and processed with SPOT and ImageJ software.

**Nascent RNA Imaging**—NHF and VCP/p97 mutant fibroblasts were grown on coverslips and subjected to UV irradiation at 10 J/m², whereas CS1AN + iCSB cells were induced for CSB expression for 24 h before UV irradiation. After allowing DNA repair to occur in the presence or absence of 10 µM DBeQ for the indicated time periods, the treated and untreated cells were labeled with 1 mM 5-ethyluridine for 1 h, rinsed with cold PBS, and then fixed with 2% paraformaldehyde. The 5-ethyluridine incorporation was revealed with a Click-iT RNA imaging kit (Invitrogen) using Alexa Fluor 488 dye according to the instructions of the manufacturer. The images were captured with a Nikon fluorescence microscope (E80i). ImageJ software was used for measuring pixel and integrated density of interested area of fluorescence images.

**Cell Viability Assay**—Cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The CS1AN-derived cells were plated at ~ 8000 cells/well in 96-well plates and incubated for 16–24 h. In the case of CSB induction, the plating cell numbers were adjusted to ~5000 cells/well, and the plated cells were incubated with 1 µg/ml doxycycline for an additional 24 h. The cells were then UV-irradiated at 10 J/m² and immediately treated with 10 µM DBeQ, MG132, or vehicle for 8 or 24 h. The cells were incubated with 100 µl of fresh medium and 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide stock (5 mg/ml), and the incubation continued for 4 h. The media were carefully removed, the cells were rinsed once with PBS and dissolved in 200 µl of dimethyl sulfoxide, and then the absorbance was read at 540 nm. The relative cell viability was calculated from the ratios of absorbance of treated to untreated cells, each from an average of 6–8 wells.

**Results**

**VCP/p97 Inhibition Affects CSB Degradation**—To test whether VCP/p97 is involved in DNA damage-induced proteasomal degradation of CSB, we first applied a small-molecule inhibitor, DBeQ, which has been identified as a potent, reversible, and specific inhibitor of VCP/p97 ATPase (40). The levels of CSB were monitored under the influence of DBeQ-mediated VCP/p97 or MG132-mediated proteasome inhibition following cellular UVR exposure. The CSB levels in HCT116 cells at 50 J/m² exhibited an apparent decrease at 8 h following UVR. Both DBeQ and MG132 treatments blocked the reduction of CSB (Fig. 1A). The slowly migrating ubiquitin-modified CSB bands were clearly seen at 2 h regardless of inhibitor treatment, consistent with previous observations that the CSA-dependent CSB ubiquitination occurs after UV irradiation (41). At 10 J/m², CSB levels exhibited a decrease at 2 and 4 h but a recovery at 8 h following UVR. Thus, a higher UV dose induced more consistent CSB degradation at the expense of attenuated CBS recovery (Fig. 1, A and B). The decrease in ubiquitinated CSB at 8 h may reflect the processing of CSB Ub conjugates by the proteasome or ubiquitin-specific proteases (USPs) or both systems. Because USP7 is implicated in protecting CSB from proteasomal degradation (42, 43), we next compared the dynamics of CSB levels after UVR in parental and USP7-deficient HCT116 cells (Fig. 1C). The results showed that the CSB level was relatively higher in parental HCT116 cells. At 10 J/m², CSB was clearly reduced at the 0-, 2-, and 4-h time points, indicating that CSB degradation occurs immediately after DNA damage. The CSB level, however, was re-established at 8 and 24 h. Without USP7 protection, no significant recovery of CSB was seen at 8 or even 24 h post-UVR. As expected, the CSB degradation in USP7-deficient cells was similarly blocked by proteasomal inhibition and also impaired by VCP/p97 inhibition (Fig. 1D). Although the exact role of USP7 in CSB protection remains to be explored, it can be concluded that VCP/p97 has an obvious role in ubiquitin-mediated CSB degradation.
VCP/p97 Components Are Functionally Required for Proteasome-mediated CSB Degradation—The homeostasis of cellular CSB was further investigated using corrected CSB-deficient CS1AN cells harboring doxycycline (Dox)-inducible CSB transgenes. At the 2-, 4-, and 6-h time points, UVR exposure reduced the steady-state CSB levels, which were otherwise unaffected by Dox withdrawal at the same time periods (Fig. 2A). Again, DBeQ treatment clearly blocked the UVR-induced CSB reduction. The VCP/p97 levels, however, were not affected either by the CSB induction from Dox or by DBeQ inhibition. These results reinforced the findings that VCP/p97 regulates the steady-state CSB level post-transcription (Fig. 2A). Next we used siRNA-mediated functional ablation to examine the requirement of the VCP/p97 cofactor UFD1 and the adapter UBXD7 in CSB degradation. Control or target-specific siRNAs were transfected before CSB induc-
VCP/p97 Extracts CSB for Proteolysis

Ubiquitin-conjugated CSB Species from Chromatin—Following the introduction of UVR-induced photolesions in cells, CSB is promptly engaged in TCR to efficiently remove DNA lesions from actively transcribing DNA strands. Thus, we isolated chromatin by cellular protein fractionation and determined CSB degradation in UV-damaged chromatin. As expected, the characteristic markers, e.g. DNA origin recognition complex 2 subunit (ORC2), resided exclusively in chromatin, whereas proliferating cell nuclear antigen, an auxiliary protein of DNA polymerase, was detected in both the cytoplasm and chromatin (Fig. 3A). The CSB protein was primarily seen to exist in chromatin, and its level was reduced following UVR. Conversely, analogous to proliferating cell nuclear antigen, the bulk of VCP/p97 primarily resides in the cytoplasm. Nevertheless, a small fraction of VCP/p97 could indeed be seen in the chromatin fraction. Because CSB protein primarily exists in chromatin (Figs. 3A and 7B), we next examined the effect of DBeQ on CSB protein within chromatin. The VCP/p97 inhibition exhibited a prominent blockage of CSB degradation, leading to persistence of CSB in UVR-damaged chromatin (Fig. 3B). Thus, VCP/p97 is actively involved in the degradation of TCR-engaged CSB within chromatin.

To investigate how VCP/p97 regulates ubiquitin-mediated CSB degradation, we next examined CSB ubiquitination and the interaction between VCP/p97 and CSB/ubiquitinated CSB proteins. The results showed that both proteasome and VCP/p97 inhibition allowed an accumulation of total ubiquitin conjugates (Fig. 4A, left panel). In anti-CSB recovered immunoprecipitates, the ubiquitin conjugates of CSB exhibited a slight increase upon UVR that was further augmented upon proteasome as well as VCP/p97 inhibition (Fig. 4A, right panel). More importantly, VCP/p97 protein was shown to exist in association with CSB. Accordingly, in reciprocal immunoprecipitation with VCP/p97 antibody, both CSB and ubiquitinated CSB were detected using anti-CSB antibody (Fig. 4B). Consistent with the ability of VCP/p97 to bind ubiquitin conjugates, anti-VCP/p97 immunoprecipitation enriched distinctive and CSB antibody-recognizable ubiquitinated CSB form, as this species was undetectable in 10% input lanes without the help of extended film exposures (Fig. 4B, also see Fig. 1, A–C). As expected, UFD1 and NPL4 were also shown to associate with VCP/p97. Taken together, the results suggested that both native and ubiquitin-conjugated forms of CSB associate with VCP/p97.

The binding of VCP/p97 to ubiquitin conjugates and ubiquitinated CSB species was further investigated using genetically engineered U2OS cells harboring an inducible Myc-tagged WT-VCP/p97 (Fig. 5A). The ubiquitin conjugates, labeled by an HA tag, were specifically detected in 10% input samples of HA-ubiquitin-expressing cells. Notably, the ubiquitin conjugates were present in anti-Myc precipitates regardless of UV irradiation, indicating the binding of VCP/p97 to ubiquitin conjugates. Anti-CSB Western blotting showed that CSB proteins and ubiquitinated CSB species were present in anti-Myc (VCP/p97) precipitates from VCP/p97-induced cells, indicating the specific CSB-VCP/p97 association. This protein-protein association was further confirmed by similar experiments in U2OS cells harboring an inducible Myc-tagged EQ-VCP/p97 (Fig. 5B). Interestingly, the ubiquitinated CSB forms were clearly enriched in anti-Myc (VCP/p97) precipitates, presumably because of the dominant-negative binding of mutant VCP/p97 to ubiquitin conjugates. Taken together, these results indicated that VCP/p97 not only binds ubiquitin conjugates but also specifically associates with CSB and the ubiquitinated CSB species. It can be hypothesized that VCP/p97 binds ubiquitinated CSB protein species and removes them from UV-induced lesion sites to facilitate CSB proteolysis.

VCP/p97 Is Recruited to UV Lesions, but Manifestation of Local UVR-induced Foci (UVRIFF) of VCP/p97 Is Independent of CSB and UBXD7—To assess the recruitment of VCP/p97 to DNA damage sites, UVR was delivered through the micropores
of polycarbonate filters for generating localized subnuclear DNA damage spots, and VCP/p97 recruitment was examined by immunofluorescent staining with cognate antibodies. In Myc-tagged VCP/p97-induced U2OS cells, both WT- and EQ-VCP/p97 could form UVRIF, which co-localized with an authentic damage indicator, γH2AX foci (supplemental Fig. S1). The presence of the ATPase-inactive VCP/p97 at the damage spots is consistent with the ability of the mutant form to bind ubiquitin conjugates (Fig. 5B). The VCP/p97 UVRIF were also detected in HeLa cells, suggesting an ubiquitous nature of VCP/p97 involvement in the UV damage response.

The recruitment of VCP/p97 has previously been shown to be completely dependent on the presence of UFD1 and NPL4 (13), which are two cofactors present in the VCP/p97 complex. Therefore, we next investigated the role of CSB and the adaptor UBXD7 in the recruitment of VCP/p97. The CSB and UBXD7 proteins were depleted by siRNA, and VCP/p97 UVRIF were visualized by immunofluorescence. Although accumulation of Myc-VCP/p97 can be seen at DNA damage spots generated by micropore UV irradiation at 100 J/m², and the cell lysates in RIPA were made 2 h after UV irradiation, CSB, VCP/p97, NPL4, and UFD1 were detected in input (~10%) and immunoprecipitates recovered by anti-VCP/p97 or control antibody. The quantification of VCP/p97 and CSB blots was done by ImageJ software. The relative VCP/p97 or CSB amount (including ubiquitinated forms) was calculated in reference to each 10% input lane.
FIGURE 5. VCP/p97 binds to CSB and ubiquitin-conjugated CSB. A, U2OS cells harboring inducible Myc-tagged-WT-VCP/p97 transgenes (iMyc-WT-VCP/p97) were transfected with expression constructs for HA-tagged ubiquitin (HA-Ub) for 48 h, and during the last 24 h, Dox was added for transgene induction. The transfected cells were then UV-irradiated at 50 J/m² and maintained for the indicated times. The cell lysates were made in RIPA buffer and subjected to immunoprecipitation (IP). The indicated Ub conjugates, CSB, and VCP/p97 proteins in the input (~10%) and immunoprecipitates were detected by Western blotting (WB). B, U2OS cells harboring inducible Myc-tagged-EQ-VCP/p97 transgenes (iMyc-EQ-VCP/p97) were used in experiments similar to that shown in A. (Ub)n-CSB represents the ubiquitin-conjugated CSB forms. Blotting of Lamin B served as the loading control. The quantification of CSB blots was done by ImageJ software. The relative CSB amount (including ubiquitinated forms) was calculated in reference to each 10% input lane.
not affect the frequency of γH2AX-positive foci (Fig. 6, C and D). Interestingly, UBXD7 depletion caused a modest but statistically significant decrease in the formation of VCP/p97 UVRIF at a 400 J/m² UVR dose. Thus, it seems that the manifestation of VCP/p97 UVRIF is independent of CSB and UBXD7, suggesting that CSB-dependent VCP/p97 recruitment does not significantly contribute to the manifestation of VCP/p97 UVRIF. Because of the relatively modest effect, it may be argued that UBXD7 simply acts as an accessory factor for VCP/p97 recruitment.

**The CSA-DDB1-Cul4A E3 Ligase Complex Associates with VCP/p97 and UBXD7 in Chromatin**—Several Cullin-Ring ubiquitin ligases (CRLs), including Cullin 4A (Cul4A)-based CRLs, were reported to interact with VCP/p97 and the UBXD7 adapter (9, 44). Therefore, we explored the association of VCP/p97 and UBXD7 with a particular CRL, the CSA-DDB1-Cul4A E3 ligase complex, that could affect CSB homeostasis and function. As mentioned above, CSA acts as substrate acceptor for the E3 ligase complex responsible for CSB ubiquitination and subsequent degradation (31). When RIPA buffer whole cell extracts were used for immunoprecipitation, only a very small fraction of VCP/p97 and CSB could be detected to physically associate with CSA (Fig. 7A). To clearly define the nature of this association, we utilized the isolated soluble chromatin fraction for immunoprecipitation (Fig. 7B). As expected, Cul4A and DDB1 were seen to associate with CSA. Importantly, CSB, VCP/p97, and UBXD7 were also clearly recovered by anti-FLAG-CSA immunoprecipitation, indicating that the CSA-DDB1-Cul4A E3 ligase complex not only binds to its substrate CSB but also harbors VCP/p97 and adaptor UBXD7 in the complex irrespective of cellular UVR exposure.

Next we investigated whether UBXD7 and the proteasome bind to damaged chromatin under conditions of VCP/p97 inhibition (Fig. 7C). HCT116 cells were pretreated with DBeQ or MG132 prior to UVR exposure. UVR incited a reduction of CSB and DDB2 in chromatin, which was blocked by DBeQ or MG132 treatment. The examination of DDB2 confirmed the involvement of VCP/p97 in promoting the degradation of chromatin bound DDB2 (13). As expected, the nuclear protein ORC2 was detected in the nucleoplasmic fraction and enriched in the soluble chromatin fraction. More importantly, UBXD7 was unmistakably present in the nucleoplasmic fraction, and UVR exposure did not induce appreciable chromatin accumulation of UBXD7. On the other hand, both DBeQ and MG132 pretreatments caused a clear UBXD7 accumulation following UVR exposure. The VCP/p97 inhibitor pretreatment also enhanced the presence of 19S proteasomal RPN2 and Sug1 but abolished the presence of VCP/p97 itself in chromatin. Nonetheless, we noticed that, when VCP/p97 inhibitor was applied after UVR (Fig. 3), VCP/p97 was still present in chromatin.
VCP/p97 Extracts CSB for Proteolyis

VCP/p97 inhibitor pretreatment probably reduced the availability of VCP/p97 for processing chromatin-associated ubiquitin conjugates. Given that the accumulation of ubiquitin conjugates occurs under conditions of VCP/p97 and proteasome inhibition, the results indicate that, when VCP/p97 function is compromised, the ubiquitin conjugates attract UBXD7 and the proteasome to chromatin. The results support the hypothesis that VCP/p97 and UBXD7 are recruited to ubiquitin conjugates independent of each other (Fig. 6).

Compromising VCP/p97 Function Enhances RRS but Decreases Cell Viability upon DNA Damage—VCP/p97 inhibition-mediated persistence of CSB in chromatin (Fig. 3B) could affect TCR and/or RRS occurring as a consequence of successful DNA repair, especially TCR. To explore the function of VCP/p97-mediated CSB clearance from chromatin, we first monitored nascent RNA synthesis via Click-iT RNA imaging in primary normal human fibroblasts (NHF) and in primary human GM22764 and GM22014 fibroblasts obtained from clinically affected patients with VCP/p97 mutations (Fig. 8, A–C). When compared with normal VCP/p97, two mutants did not seem to significantly affect global RNA synthesis, as indicated by similar fluorescence pixel densities in three cell types (Fig. 8B). Cellular UVR exposures at 10 J/m² decreased nascent RNA synthesis in NHFs to ~38% at 2 h, which recovered to ~45% at 8 h and ~59% at 24 h (Fig. 8C). Similarly, UVR inhibited nascent RNA synthesis in primary VCP/p97 mutant fibroblasts. However, nascent RNA synthesis recovered much faster in both mutant cells. For example, nascent RNA synthesis recovered to 70% at 8 h and recovered almost fully in 24 h in GM22014 cells, suggesting that VCP/p97 mutation causing inclusion body myopathy with early-onset Paget disease and frontotemporal dementia does not impair but enhances post-TCR RRS following UV-induced DNA damage.

We further monitored nascent RNA synthesis in CSB-deficient CS1AN cells harboring inducible CSB transgenes (Fig. 8, D and E). As expected, CSB induction increased nascent RNA synthesis without DNA damage (Fig. 8E). This observation may reflect an overall impact of CSB transient re-expression on nascent RNA synthesis and is consistent with the role of CSB as a chromatin remodeler in transcription in numerous genes (45, 46). VCP/p97 inhibition increased RRS even in the absence of CSB induction (Fig. 8F). Moreover, CSB induction led to an overall increase in RRS, as determined by relative pixel density, although transient CSB re-expression failed to support full RRS. Notably, RRS was significantly increased by DBeQ treatment with CSB induction compared with that without DBeQ treatment or that without CSB induction. These results indicated that compromising VCP/p97 function enhances post-TCR RRS, largely dependent on CSB.

We next examined cell viability under VCP/p97 inhibition in the presence or absence of CSB (Fig. 8G). VCP/p97 inhibition considerably decreased cell viability at 8 and 24 h post-UVR, as did proteasome inhibition. Remarkably, VCP/p97 inhibition caused a significantly greater cytotoxicity upon CSB induction compared with that without CSB induction. Likewise, proteasome inhibition also increased the cytotoxicity of UVR upon CSB induction. Thus, transient CSB re-expression enhanced DNA damage-induced cytotoxicity when VCP/p97 or proteasome function was compromised. Taken together, these results suggested that, although VCP/p97 function is not required for post-TCR RRS, the clearance of ubiquitinated proteins, including CSB, from damaged chromatin by the cooperative action of VCP/p97 and the proteasome is important for the maintenance of cell viability following DNA damage.

**Discussion**

The Pathway for Proteolytic Clearance of CSB in UV-damaged Chromatin—In processing of DNA damage by TCR, CSB is believed to recruit CSA as well as other TCR factors (20). Conspicuously, however, CSB itself undergoes ubiquitin-mediated proteasomal degradation during TCR (31, 41, 42). Besides the CSA–DDB1–Cul4A E3 ligase, the BRCA1-BARD1 complex has also been found to mediate CSB ubiquitination (47). Nevertheless, the nature and scope of paradoxical CSB breakdown have remained unexplained. In this report, we identify the VCP/p97 segregase machinery as an important regulatory partner of the proteasome in processing CSB within chromatin. From a mechanistic standpoint, CSB serves as the critical sensor of RNAPII stalled at the sites of DNA photolesions (48, 49).
In case the arrest of RNAPII is irreversible, a failure to dislodge RNAPII by CSB inevitably triggers the ubiquitin-mediated proteolytic clearance of both RNAPII and CSB. When ubiquitinated, however, CSB becomes a *bona fide* substrate of VCP/p97 segregase for the targeted extraction from chromatin (Fig. 9).

Because RNAPII and CSB are involved in sensing and repairing many kinds of helix-distorting DNA lesions, the role of VCP/p97 segregase in proteolytic clearance of CSB may not be limited to UV-induced photolesions but applicable in general to the lesions processed by TCR.
Many studies support a role of CSB in transcription elongation where CSB monitors the elongation status of transcribing RNA polymerases. For example, CSB was found to reside in an RNAPII-containing complex (48). DNA damage was also indicated to stabilize the interaction of CSB with transcription elongation machinery containing RNAPII (49). An analysis of the composition of the UV-stalled transcription elongation complex revealed that UV irradiation stabilizes the interaction and/or recruits CSB to lesion-installed RNAPII, which subsequently recruits TFIIF, XPG, RPA, and the CSA-DDB1-Cul4A E3 ligase complex with the COP9 signalosome and other core nucleotide excision repair components (20). Conceivably, a failed attempt of CSB to dislodge irreversibly stalled RNAPII at lesion sites eventually leads to CSB ubiquitination by the CSA-DDB1-Cul4A E3 ligase complex. In this study, the data not only affirm the association of the CSA-DDB1-Cul4A complex with CSB (31) but also uncover a hitherto unknown association of such a complex with VCP/p97 and its adapter UBXD7 (Fig. 7). To our knowledge, this is the first report of a specific CRL interacting with both VCP/p97 and its adapter UBXD7 (9, 44). The interaction between the CRL4 complex with CSB as well as that between VCP/p97 and CSB and may be physically dynamic. However, it raises the interesting possibility that CSB ubiquitination is coupled with VCP/p97-mediated extraction of cognate factors. In support of this, UBXD7 was shown to interact exclusively with the active/NEDD8-modified form of Cullins, including Cul4A (44). We infer that the coupling of ubiquitination to VCP/p97-mediated substrate extraction adds another layer of selective control over proteolysis of a specific substrate.

Depletion of UFD1 or UBXD7 stabilizes CSB in UV-irradiated cells, as observed for VCP/p97 depletion, and points to a positive role of these adaptors in the degradation of ubiquitinated CSB at lesion sites (Fig. 2). Unexpectedly, the depletion of UBXD7 had only a minor effect on the formation of VCP/p97 UVRIF (Fig. 6). Nonetheless, both proteasome and VCP/p97 inhibition led to UBXD7 accumulation in damaged chromatin. In a previous study, the formation of VCP/p97 UVRIF was completely abolished by depletion of UFD1 and NPL4 (13). Thus, UFD1 and NPL4 may have a different function than UBXD7 in VCP/p97 machinery. We speculate that UBXD7 plays an accessory role in stabilizing VCP/p97-substrate interaction, when UBXD7 itself is attracted to the ubiquitinated substrates independent of the VCP/p97 segregase complex. It is also noteworthy that functional ablation of CSB had no effect on the formation of VCP/p97 UVRIF. Given the binding of VCP/p97 to ubiquitin-conjugated CSB (Figs. 4 and 5), it would seem that VCP/p97 attracted by CSB species does not have a significant contribution to the manifestation of local VCP/p97 UVRIF.

The two VCP/p97 mutants tested in this study did not impair but rather enhanced post-TCR RRS. In accord, VCP/p97 inhibition increased post-TCR RRS in a partially CSB-dependent manner (Fig. 8). This is in agreement with a previously demonstrated role of CSB in transcription. However, this apparently diverges from the suggestion that CSB elimination by CSA-DDB1-Cul4A E3 ligase is important for post-TCR RRS (31). In a previous study, post-TCR RRS was examined after proteasome inhibition by MG132. Proteasome inhibition can have a broad effect on the degradation of many other proteins, including RNAPII, which could contribute to post-TCR RRS. That said, paradoxical enhancement of post-TCR RRS by VCP/p97 inhibition may be explained by the possible deubiquitination of CSB-ubiquitin conjugates by USP7. Because USP7 is required for re-establishment of CSB after UV irradiation (Fig. 3C), it is conceivable that deubiquitinating CSB by USP7 converts ubiquitinated CSB to its native forms, which can remodel the RNAPII-DNA interface during TCR. Regardless of how CSB may function, both proteasome and VCP/p97 inhibitions severely diminish cell viability following UVR exposure. The toxicity of the VCP/p97 inhibitor was greater during the earlier post-UVR hours in the presence of CSB. Thus, the proteolytic removal of ubiquitinated proteins, including CSB and RNAPII, from damaged chromatin may be the last resort for allowing normal cell survival.
In summary, we uncovered a new role of VCP/p97 segregase machinery in proteolytic clearance of CSB from damaged chromatin (Fig. 9). The finding has serious implications for the future direction of the field. For example, questions may be asked about the functionality of UBXD7 or the interactions of VCP/p97 segregase machinery with other CRLs, e.g. the DDB2-DDB1-Cul4A E3 ligase complex. Insights into the involvement of other substrates and adaptors of VCP/p97 segregase would also provide a better understanding of the biology of VCP/p97 segregase acting in the context of chromatin.

Author Contributions—J. H., G. W., and N. S. performed the experiments and analyzed the data. Q. Z. and A. A. W. conceived the experiment, analyzed the data, and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank Bert Vogelstein (Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD) for the HCT116 and HCT116-USP7 cells. Eric Verma (Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD) for the DDB1-Cul4A E3 ligase complex. Insights into the involvement of other substrates and adaptors of VCP/p97 segregase would also provide a better understanding of the biology of VCP/p97 segregase acting in the context of chromatin.

References

1. Stolz, A., Hilt, W., Buchberger, A., and Wolf, D. H. (2011) Cdc48: a power machine in protein degradation. Trends Biochem. Sci. 36, 515–523
2. Ye, Y. (2006) Diverse functions with a common regulator: ubiquitin takes command of an AAA ATPase. J. Struct. Biol. 156, 29–40
3. Meyer, H., Bug, M., and Bremer, S. (2012) Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. Nat. Cell Biol. 14, 117–123
4. Meyer, H. (2012) p97 complexes as signal integration hubs. BMC Biol. 10, 48
5. Bays, N. W., Wilhouisky, S. K., Goradia, A., Hodgkiss-Harlow, K., and Hampton, R. Y. (2001) HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. Mol. Biol. Cell 12, 4114–4128
6. Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (2002) Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates. EMBO J. 21, 615–621
7. Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D. H., and Sommer, T. (2002) Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. Nat. Cell Biol. 4, 134–139
8. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature 414, 652–656
9. Alexandru, G., Graumann, J., Smith, G. T., Kolawa, N. J., Fang, R., and Deshaies, R. J. (2008) UBXD7 binds multiple ubiquitin ligases and implicates p97 in HIF1α turnover. Cell 134, 804–816
10. Meerang, M., Ritz, D., Palival, S., Garajova, Z., Bosshard, M., Nallain, J., Janscak, P., Hübser, U., Meyer, H., and Ramadan, K. (2011) The ubiquitin-selective segregase VCP/p97 orchestrates the response to DNA double-strand breaks. Nat. Cell Biol. 13, 1376–1382
11. Ramadan, K., Bruderer, R., Spiga, F. M., Popp, O., Baur, T., Gotta, M., and Meyer, H. H. (2007) Cdc48/p97 promotes reformation of the nucleus by extracting the kinase Aurora B from chromatin. Nature 450, 1258–1262
12. Acs, K., Luijsterburg, M. S., Ackermann, L., Salomons, F. A., Hoppe, T., and Dantuma, N. P. (2011) The AAA-ATPase VCP/p97 promotes 53BP1 recruitment by removing LMBTL1 from DNA double-strand breaks.

Author Contributions—J. H., G. W., and N. S. performed the experiments and analyzed the data. Q. Z. and A. A. W. conceived the experiment, analyzed the data, and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank Bert Vogelstein (Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD) for the HCT116 and HCT116-USP7 cell lines. Conrad C. Wehl (Department of Neurology and Cell Biology and Physiology, Washington University of Medicine, St. Louis, MO) for the Myc-DDB1-Cul4A E3 ligase complex. Insights into the involvement of other substrates and adaptors of VCP/p97 segregase would also provide a better understanding of the biology of VCP/p97 segregase acting in the context of chromatin.

References

1. Stolz, A., Hilt, W., Buchberger, A., and Wolf, D. H. (2011) Cdc48: a power machine in protein degradation. Trends Biochem. Sci. 36, 515–523
2. Ye, Y. (2006) Diverse functions with a common regulator: ubiquitin takes command of an AAA ATPase. J. Struct. Biol. 156, 29–40
3. Meyer, H., Bug, M., and Bremer, S. (2012) Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. Nat. Cell Biol. 14, 117–123
4. Meyer, H. (2012) p97 complexes as signal integration hubs. BMC Biol. 10, 48
5. Bays, N. W., Wilhouisky, S. K., Goradia, A., Hodgkiss-Harlow, K., and Hampton, R. Y. (2001) HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. Mol. Biol. Cell 12, 4114–4128
6. Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (2002) Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates. EMBO J. 21, 615–621
7. Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D. H., and Sommer, T. (2002) Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. Nat. Cell Biol. 4, 134–139
8. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature 414, 652–656
9. Alexandru, G., Graumann, J., Smith, G. T., Kolawa, N. J., Fang, R., and Deshaies, R. J. (2008) UBXD7 binds multiple ubiquitin ligases and implicates p97 in HIF1α turnover. Cell 134, 804–816
10. Meerang, M., Ritz, D., Palival, S., Garajova, Z., Bosshard, M., Nallain, J., Janscak, P., Hübser, U., Meyer, H., and Ramadan, K. (2011) The ubiquitin-selective segregase VCP/p97 orchestrates the response to DNA double-strand breaks. Nat. Cell Biol. 13, 1376–1382
11. Ramadan, K., Bruderer, R., Spiga, F. M., Popp, O., Baur, T., Gotta, M., and Meyer, H. H. (2007) Cdc48/p97 promotes reformation of the nucleus by extracting the kinase Aurora B from chromatin. Nature 450, 1258–1262
12. Acs, K., Luijsterburg, M. S., Ackermann, L., Salomons, F. A., Hoppe, T., and Dantuma, N. P. (2011) The AAA-ATPase VCP/p97 promotes 53BP1 recruitment by removing LMBTL1 from DNA double-strand breaks.
32. He, J., Zhu, Q., Wani, G., Sharma, N., Han, C., Qian, J., Pentz, K., Wang, Q. E., and Wani, A. A. (2014) Ubiquitin-specific protease 7 regulates nucleotide excision repair through deubiquitinating XPC protein and preventing XPC protein from undergoing ultraviolet light-induced and VCP/p97 protein-regulated proteolysis. *J. Biol. Chem.* **289**, 27278–27289.

33. Cummins, J. M., Rago, C., Kohli, M., Kinzler, K. W., Lengauer, C., and Vogelstein, B. (2004) Tumour suppression: disruption of HAUSP gene stabilizes p53. *Nature* **428**, 1.

34. Ju, J. S., Miller, S. E., Hanson, P. I., and Weihl, C. C. (2008) Impaired protein aggregate handling and clearance underlie the pathogenesis of p97/VCP-associated disease. *J. Biol. Chem.* **283**, 30289–30299.

35. Yuan, X., Feng, W., Imhof, A., Grummt, I., and Zhou, Y. (2007) Activation of RNA polymerase I transcription by Cockayne syndrome group B protein and histone methyltransferase G9a. *Mol. Cell* **27**, 585–595.

36. Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kissel, 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.

37. Sharma, N., Zhu, Q., Wani, G., He, J., Wang, Q. E., and Wani, A. A. (2014) USP3 counteracts RNF168 via deubiquitinating H2A and /H2AX at lysine 13 and 15. *Cell Cycle* **13**, 106–114.

38. Zhu, Q., Sharma, N., He, J., Wani, G., and Wani, A. A. (2015) USP7 deubiquitinase promotes ubiquitin-dependent DNA damage signaling by stabilizing RNF168. *Cell Cycle* **14**, 1413–1425.

39. Wang, Q. E., Zhu, Q., Wani, G., El-Mahdy, M. A., Li, J., and Wani, A. A. (2005) DNA repair factor XPC is modified by SUMO-1 and ubiquitin following UV irradiation. *Nucleic Acids Res.* **33**, 4023–4034.

40. Chou, T. F., Brown, S. J., Minond, D., Nordin, B. E., Li, K., Jones, A. C., Chase, P., Porubsky, P. R., Stoltz, B. M., Schoenien, F. J., Patricelli, M. P., Hodder, P., Rosen, H., and Deshaies, R. J. (2011) Reversible inhibitor of p97, DBeQ, impairs both ubiquitin-dependent and autophagic protein clearance pathways. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 4834–4839.

41. Fei, J., and Chen, J. (2012) KIAA1530 protein is recruited by Cockayne syndrome complementation group protein A (CSA) to participate in transcription-coupled repair (TCR). *J. Biol. Chem.* **287**, 35118–35126.

42. Zhang, X., Horihata, K., Saijo, M., Ishigami, C., Ukai, A., Kanno, S., Tahara, H., Nellen, E. G., Honma, M., Nohmi, T., Yasui, A., and Tanaka, K. (2012) Mutations in UVSSA cause UV-sensitive syndrome and destabilize ERCC6 in transcription-coupled DNA repair. *Nat. Genet.* **44**, 593–597.

43. Schwertman, P., Lagarou, A., Dekkers, D. H., Raams, A., van der Hoek, A. C., Laffeber, C., Hoeijmakers, J. H., Demmers, J. A., Fousteri, M., Vermeulen, W., and Martein, J. A. (2012) UV-sensitive syndrome protein UVSSA recruits USP7 to regulate transcription-coupled repair. *Nat. Genet.* **44**, 598–602.

44. den Besten, W., Verma, R., Kleger, G., Oania, R. S., and Deshaies, R. J. (2012) NEDD8 links cullin-RING ubiquitin ligase function to the p97 pathway. *Nat. Struct. Mol. Biol.* **19**, 511–516.

45. Newman, J. C., Bailey, A. D., and Weiner, A. M. (2006) Cockayne syndrome group B protein (CSB) plays a general role in chromatin maintenance and remodeling. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 9613–9618.

46. Wang, Y., Chakravarty, P., Ranes, M., Kelly, G., Brooks, P. J., Nellen, E., Stewart, A., Shiavo, G., and Svejstrup, J. Q. (2014) Dysregulation of gene expression as a cause of Cockayne syndrome neurological disease. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 14454–14459.

47. Wei, L., Lan, L., Yasui, A., Tanaka, K., Saijo, M., Matsuazawa, A., Kashwagi, R., Maseki, E., Hu, Y., Parvin, J. D., Iahioka, C., and Chiba, N. (2011) BRCA1 contributes to transcription-coupled repair of DNA damage through polyubiquitination and degradation of Cockayne syndrome B protein. *Cancer Sci.* **102**, 1840–1847.

48. van Gool, A. J., Citterio, E., Rademakers, S., van Os, R., Vermeulen, W., Constantinou, A., Egly, J. M., Bootsma, D., and Hoeijmakers, J. H. (1997) The Cockayne syndrome B protein, involved in transcription-coupled DNA repair, resides in an RNA polymerase II-containing complex. *EMBO J.* **16**, 5955–5965.

49. van den Boom, V., Citterio, E., Hoogstraten, D., Zoster, A., Egly, J. M., van Cappellen, W. A., Hoeijmakers, J. H., Houtsmsuller, A. B., and Vermeulen, W. (2004) DNA damage stabilizes interaction of CSB with the transcription elongation machinery. *J. Cell Biol.* **166**, 27–36.