



Universiteit
Leiden
The Netherlands

Regulation of DNA damage and immune response pathways by post-translational protein modification

Dijk, M.

Citation

Dijk, M. (2020, September 9). *Regulation of DNA damage and immune response pathways by post-translational protein modification*. Retrieved from <https://hdl.handle.net/1887/136522>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/136522>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/136522> holds various files of this Leiden University dissertation.

Author: Dijk, M.

Title: Regulation of DNA damage and immune response pathways by post-translational protein modification

Issue Date: 2020-09-09

2

Potential targets of the CSA-based cullin-RING ubiquitin ligase in transcription-coupled DNA repair

Madelon Dijk, Mischa G. Vrouwe and Leon H. Mullenders

Abstract

Transcription-coupled nucleotide excision repair (TC-NER) is responsible for the fast removal of helix-distorting DNA lesions that interfere with elongating RNA polymerase II (RNAPII_o). Stalling of RNAPII_o at sites of DNA damage triggers the assembly of a repair complex, which is initiated by the recruitment of key TC-NER factors CSA and CSB. CSA is part of a larger complex, consisting of CSA, DDB1, CUL4A/B and RBX1, which together form the cullin-RING ubiquitin ligase CRL^{CSA}. Although CSA is essential for repair of UV-induced damage and subsequent resumption of transcription, the precise role of CRL^{CSA} during NER remains largely elusive. Here we show that NEDDylation, which is required for the activation of cullin-RING ligases, modulates the presence of CRL^{CSA} at the repair complex, as well as the CSA-dependent degradation of the phosphorylated RNAPII subunit RPB1 after high doses of UV. Together our data uncover both CSA and RPB1 as potential CRL^{CSA} ubiquitination targets, showing that CRL^{CSA} may contribute not only to repair, but also to the avoidance of persistently stalled RNAPII_o when TC-NER cannot be properly executed.

Introduction

2

The nucleotide excision repair (NER) pathway removes a wide variety of helix-distorting lesions, including the mutagenic and toxic cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4)pyrimidone photoproducts (6-4 PPs) that are caused by UV irradiation¹. These photolesions are repaired via either the global genome repair (GG-NER) or transcription-coupled repair (TC-NER) subpathway. The damage recognition complexes XPC-RAD23B and UV-DDB are designed to continuously probe the whole genome for damage, initiating GG-NER when needed². TC-NER is triggered by the encounter of lesions by elongating RNA polymerase II (RNAPII) and thus is responsible for the repair of transcription-blocking lesions in transcriptionally active DNA³. The recruitment of the SWI/SNF2 ATPase CSA and the CSB protein to the stalled RNA polymerase is essential for repair of UV-induced damage and ultimately, for resumption of transcription⁴⁻⁷. Furthermore, UVSSA and its associated factor USP7 specifically contribute to transcription-coupled repair, possibly by regulating the presence of CSB⁸. Upon lesion detection, the formation of a pre-incision complex that facilitates damage verification is common to both pathways. In this complex, TFIIH exploits the ATPase and helicase activities of its XPB and XPD subunits, respectively, to unwind the DNA. In this way it cooperates with XPA to verify the damage. Next to directly recognizing structurally altered ssDNA, XPA is thought to play a central role in NER as it interacts with a large range of NER proteins⁹. Subsequent incision on both sides of the lesion by XPF-ERCC1 (5') and XPG (3') removes a DNA stretch containing the damage². Using the undamaged strand as a template, a DNA polymerase fills the gap, after which NER is completed by DNA ligase-dependent sealing of the final nick⁹.

Defects in NER genes underlie several disorders that display broadly varying symptoms. While classic Cockayne syndrome, resulting from mutations in CSA or CSB, is characterized by severe neurological and developmental abnormalities, the defects in the gene encoding UVSSA detected to date only lead to the mild photo-sensitivity observed in UV sensitivity syndrome (UVsS) patients^{7,10,11}. Defective XP proteins mainly give rise to xeroderma pigmentosum (XP), which is marked by sensitivity to sun light and a strong cancer predisposition, although certain mutations in XPB, XPD, XPF or XPG can cause a combined CS/XP patient phenotype¹²⁻¹⁶. Furthermore, mutations in TFIIH subunits can lead to trichothiodystrophy (TTD) – a disorder characterized by brittle hair and nails and intellectual impairment¹⁷.

The variety in NER-associated disorders underscores the importance of correct execution of each step of the pathway. Several post-translational modifications have been shown to contribute to NER regulation, of which ubiquitination has been mostly studied¹⁸⁻²⁰. Notably, both the GG-NER factor DDB2, as well as the TC-NER protein CSA assemble into a larger complex with DDB1, CUL4A/B and RBX1, forming the cullin-RING ubiquitin ligases CRL^{DDB2} and CRL^{CSA}, respectively²¹. The activation of cullin-RING ligases typically requires NEDDylation of the cullin protein²². In unperturbed conditions, this is counteracted by the COP9 signalosome that keeps CRL^{DDB2} and CRL^{CSA} in an inactive state by removing NEDD8 from CUL4A/B. Upon UV irradiation the dissociation of COP9 is thought to enable activation of both ligases, although possibly at different stages²³. Whereas the ubiquitination targets of

CRL^{DDB2} have been extensively studied and were shown to include XPC, histones and DDB2 itself, the role of CRL^{CSA} during NER is less well described^{24,25,26}. Possibly CRL^{CSA} employs auto-ubiquitination of CSA or ubiquitinates CSB to regulate repair^{21,27}. Furthermore, a last resort mechanism that degrades RNA polymerase II subunit RPB1 when repair is compromised has been shown to depend on CSA²⁸⁻³⁰.

Here we investigate CSA and RPB1 as potential CRL^{CSA} targets. By using a NEDDylation inhibitor, we show that NEDDylation modulates the amount of CRL^{CSA} that interacts with RNAPII_o upon UV irradiation. Possibly this reflects CRL^{CSA} auto-ubiquitination as a mechanism to control CRL^{CSA} levels at sites of DNA damage. In addition, we reveal a NEDDylation-dependent reduction in the total cellular levels of the RPB1 subunit of elongating RNAPII after UV irradiation. Interestingly, this UV-induced degradation of RPB1 appears to also depend on CSB and CSA, but not on XPA or UVSSA. Although further research is required to fully uncover the role of CRL^{CSA} during NER, these findings propose a contribution of CRL^{CSA} to the regulation of TC-NER under normal conditions, as well as to an alternative solution that removes the stalled RNA polymerase when TC-NER fails.

Results

NEDDylation modulates the presence of CRL^{CSA} at the TC-NER complex

Given that CRL^{DDB2} ubiquitinates itself during GG-NER to coordinate its removal from the repair complex, we hypothesized that the structurally comparable CRL^{CSA} could control its own dissociation from the TC-NER complex in a similar manner³¹. To investigate this possibility, the composition of the TC-NER complex was studied 1 hour after UV irradiation by immunoprecipitation of the RPB1 subunit of active RNA polymerase II complexes, which are characterized by RPB1's heavily phosphorylated C-terminal domain (CTD). Either serine 5-phosphorylated RPB1 (p-S5-RPB1; Fig. 1), which is usually detected during early transcription, or serine 2-phosphorylated RPB1 (p-S2-RPB1; Supplementary Fig. 1), which is associated with the positioning of elongating RNAPII (RNAPII_o) further along the gene, was precipitated. As expected, both p-S5-RPB1 and p-S2-RPB1 showed a UV-specific increase in the interaction with core TC-NER factor CSB. Furthermore, the build-up of the TC-NER complex was reflected by UV-induced interactions between p-S5-RPB1 or p-S2-RPB1 and members of CRL^{CSA} (DDB1, CUL4A, CSA). Interestingly, when the activation of E3 ubiquitin ligases was inhibited by the NEDDylation inhibitor MLN4924 (NEDDi), we observed increased levels of the CRL^{CSA} components associated with p-S5-RPB1 or p-S2-RPB1 after UV irradiation (Fig. 1b). In contrast, although CSB has been suggested to be a CRL^{CSA} target as well, the amount of CSB interacting with p-S5-RPB1 or p-S2-RPB1 appeared to be unaffected by the inhibition of culling-RING ubiquitin ligases²⁷. Although the use of a general NEDDylation inhibitor constrains any conclusions related to the involved ubiquitin ligase, these observations are supportive of considering CRL^{CSA} itself as a putative ubiquitination target of CRL^{CSA} during TC-NER. In agreement with this, *in vitro* studies have shown that CRL^{CSA} is capable of auto-ubiquitination²¹.

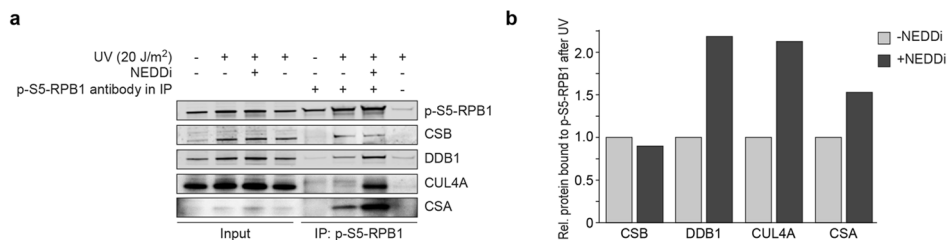


Figure 1. NEDDylation modulates the presence of CRL^{CSA} at the TC-NER complex

(a) Immunoprecipitation of the serine 5-phosphorylated RNAPII subunit RPB1 (p-S5-RPB1) from VH10-hTert cells 1 hour after mock treatment or UV-C irradiation (20 J/m²). Where indicated, global NEDDylation had been inhibited prior to UV-C irradiation by treatment with the NEDDylation inhibitor MLN4924. A similar experiment, in which p-S2-RPB1 was precipitated, is shown in Supplementary Fig. 1. (b) Relative amounts of CSB, DDB1, CUL4A and CSA that coprecipitated with p-S5-RPB1 1 hour after UV-C irradiation (20 J/m²).

Inhibition of CRL activation prevents UV-induced degradation of phosphorylated RPB1

Next to CRL^{CSA} itself, we envisaged RPB1 a possible target for CRL^{CSA}-mediated ubiquitination. RPB1 has been described to undergo CSA- and CSB-dependent ubiquitination upon induction of transcription-stalling DNA damage^{28,29}. Its subsequent eviction from the site of damage is considered to be a last resort mechanism when repair cannot be properly executed^{3,30,32,33}. To investigate the effect of NEDDylation on RPB1 degradation, total levels of p-S5-RPB1 were studied in non-irradiated or UV-irradiated non-dividing fibroblasts that were mock treated or treated with NEDDi (Fig. 2). Under unperturbed conditions, we observed a considerable decrease in total p-S5-RPB1 levels 6 hours after damage induction. In contrast, following treatment with NEDDi p-S5-RPB1 levels did not decrease and rather appeared to be substantially increased. Interestingly, a similar rise in total p-S5-RPB1 levels after UV has been shown for CSB- or CSA-deficient cells without inhibition of NEDDylation²⁹. Together these results indicate that p-S5-RPB1 is indeed subjected to NEDDylation-dependent degradation after UV, and open up the possibility of CRL^{CSA}-mediated ubiquitination of RPB1.

UV-induced degradation of phosphorylated RPB1 is dependent on CSA and CSB

To further study a potential role for CRL^{CSA} in RPB1 ubiquitination, the effect of UV damage on total p-S5-RPB1 levels was examined in different human cell lines. In accordance with our previous observations (Fig. 2), p-S5-RPB1 levels were reduced after UV in wildtype VH10-hTert cells (Fig. 3). A distinct degradation of p-S5-RPB1 was also observed in UV sensitive syndrome (UVsS) patient-derived UVSSA-deficient cells. In contrast, CSA-deficient cells derived from a Cockayne syndrome (CS) patient displayed no reduction in total p-S5-RPB1 levels after UV, but rather showed an increase in the p-RPB1 levels. The effect of CSA deficiency on p-S5-RPB1 stability mimicked the effect of inactivation of CRL^{CSA} and other cullin-RING E3 ligases by NEDDi treatment (Fig. 2). Similarly, in the absence of CSB, which is

essential for the recruitment of CRL^{CSA} to the TC-NER complex, no reduction in p-S5-RPB1 levels upon UV irradiation could be detected³⁴. Moreover, CS-B cells, similarly to CS-A cells, also displayed an increase of p-S5-RPB1 after UV. Together these observations hint towards CSA-dependent degradation of p-S5-RPB1 upon UV-induced transcription stalling. However, further research should elucidate whether the E3 ligase activity of CRL^{CSA} itself, or another ubiquitin ligase that is dependent on CSA is responsible for p-S5-RPB1 degradation.

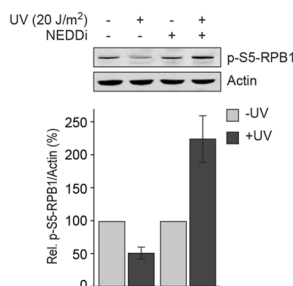


Figure 2. Inhibition of CRL activation prevents UV-induced degradation of p-RPB1

Total levels of serine 5-phosphorylated RPB1 (p-S5-RPB1) in VH10-hTert cells 6 hours after mock treatment or UV-C irradiation (20 J/m²). Where indicated, NEDDylation had been inhibited prior to UV irradiation. Bar graphs show p-S5-RPB1 levels relative to those in non-irradiated cells and represent the average \pm SEM of 2 independent experiments.

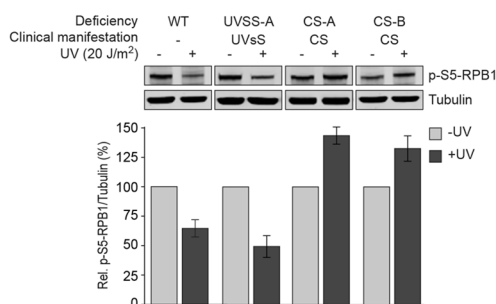


Figure 3. UV-induced degradation of p-RPB1 is dependent on CSA

Total levels of serine 5-phosphorylated RPB1 (p-S5-RPB1) in VH10-hTert (WT), KPS3-hTert (UVSS-A), CS3BE-hTert (CS-A) and CS1AN-hTert (CS-B) cells 6 hours after mock treatment or UV-C irradiation (20 J/m²). Bar graphs show p-S5-RPB1 levels relative to those in non-irradiated cells and represent the average \pm SEM of 2 independent experiments.

UV-induced degradation of p-RPB1 is similar in XP and combined XP/CS patient cell lines

UV-irradiated cells from UV sensitive syndrome patients and Cockayne syndrome patients display defective TC-NER and UV hypersensitivity, yet differ in the ability to degrade p-S5-RPB1 (Fig. 3). Displacement of abortive TC-NER complexes trapped at UV-inflicted photolesions has been suggested as one of the mechanisms to enable DNA damage repair via alternative pathways². Furthermore, avoidance of persistently stalled RNAPII is crucial in the prevention of accelerated cell death via p53-mediated apoptosis^{35,36}. Therefore, we hypothesized that the inability to remove stalled RNAPII from damaged DNA could cause, or contribute to the development of the more severe neurodevelopmental phenotype of Cockayne syndrome patients. To investigate this possibility, we first studied p-S5-RPB1 degradation in cells derived from a xeroderma pigmentosum (XP) patient harboring a mutation in the *XPA* gene. In agreement with our hypothesis, p-S5-RPB1 was efficiently degraded upon UV irradiation of these cells (Fig. 4). Accordingly, a distinct level of UV-induced p-S5-RPB1 degradation could also be detected in cells obtained from an XP patient with a mutation in the *XPD* gene. However, we observed a similar extent of UV-induced degradation in cells from a patient that suffered from combined XP/CS, caused by a mutation in the *XPD* gene that differs from the *XPD* mutations underlying classic xeroderma pigmentosum. Notably, XP and XP/CS cell lines harboring mutations in the *XPG* gene degraded RPB1 upon UV to an extent that was comparable to that in WT cells, as well as in the investigated *XPD*-deficient cell lines (data not shown). Together, our observations in UVsS, CS, XP, and XP/CS patient cell lines indicate that the ability to remove stalled RNAPII and/or repair complexes may contribute, but is likely not sufficient to prevent the development of the CS phenotype. Other mechanisms may play a role as well, such as the participation of CS proteins in mitochondrial repair and base excision repair³⁷⁻³⁹. Further research should elucidate the exact significance of RNAPII degradation and additional factors in determining the clinical outcome of genetic NER defects.

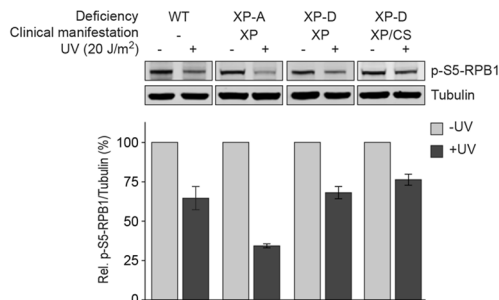


Figure 4. UV-induced degradation of p-RPB1 is similar in XP and combined XP/CS patient cell lines

Total levels of serine 5-phosphorylated RPB1 (p-S5-RPB1) in VH10-hTert (WT), XP25RO-hTert (XP-A), XP1DU-hTert (XP-D) and XP8BR-hTert (XP-D/CS) cells 6 hours after mock treatment or UV-C irradiation (20 J/m²). Bar graphs show p-S5-RPB1 levels relative to those in non-irradiated cells and represent the average \pm SEM of 2 independent experiments.

Discussion

Tight regulation of each step of NER is essential for its proper activation, as well as for faithful repair of the damage and restoration of the cellular state prior to DNA damage occurrence. Post-translational modifications are a powerful tool to control repair proteins by affecting their localization, function and stability, in addition to regulating their crosstalk with proteins involved in other processes. For example, ubiquitination has been described to contribute to the regulation of NER in various manners¹⁸⁻²⁰. The significance of this modification and its different biological applications is illustrated by the E3 ubiquitin ligase CRL^{DDB2}, which is important for GG-NER². CRL^{DDB2} ubiquitinates both DDB2 and XPC, thereby altering the faith of these proteins in different ways. Whereas auto-ubiquitination induces proteasomal degradation of DDB2, ubiquitinated XPC shows increased affinity for damaged DNA³¹. Moreover, CRL^{DDB2} ubiquitinates core histones and DDB2 associates with PARP1 to stimulate chromatin remodeling^{25,26,40,41}.

In contrast to the extensively studied CRL^{DDB2} complex, the exact function of the structurally similar CRL^{CSA}, which is crucial for TC-NER, remains largely elusive. Notably, CRL^{CSA} has been described to function differently in terms of activation of its ligase activity. The COP9 signalosome, which keeps cullin-RING ligases in an inactive state by preventing NEDDylation of the cullin, has been shown to dissociate from CRL^{DDB2} directly after damage induction. In contrast, COP9 dissociation from CRL^{CSA} was suggested to occur at later times, when repair has already been completed²³. Alternatively, *in vitro* studies suggest that CSB might replace the COP9 signalosome, enabling CRL^{CSA} to become activated by NEDD8²¹. In support of this, we clearly observed a UV-induced interaction between RNAPII α and CSA, but have never been able to detect the COP9 signalosome in the TC-NER complex (unpublished data, Fig. 1, Supplementary Fig. 1).

To investigate to which extent the function of CRL^{CSA} during TC-NER resembles that of CRL^{DDB2} during GG-NER, we have studied the composition of the TC-NER complex under conditions of unperturbed NEDDylation or after inhibiting NEDD8 E1 activating enzyme (NAE) by MLN4924. The increased association of CRL^{CSA} factors with p-S5-RPB1 and p-S2-RPB1 after UV and NEDDi treatment shows that NEDDylation regulates the stoichiometry of the TC-NER complex. Possibly this implies that CRL^{CSA}, comparably to CRL^{DDB2}, in conditions that allow normal activation of cullin-RING ligases (partly) dissociates from the repair complex. Interestingly, the association of DDB1, CUL4A and CSA with p-S5-RPB1 or p-S2-RPB1 increased to approximately the same extent, showing that the protein stoichiometry within CRL^{CSA} is maintained.

Another explanation for the increased levels of CRL^{CSA} proteins that coprecipitated with p-S2-RPB1 after NEDDylation inhibition would be that only non-ubiquitinated or non-NEDDylated CRL^{CSA} is able to become part of the TC-NER complex. In this case, the elevated amounts of CRL^{CSA} in this repair complex after NEDDi would result from the increased availability of unmodified CRL^{CSA}. However, CRL^{CSA} levels in chromatin (Fig. 1, input) appeared to be unaffected by NEDDylation, which suggest that ubiquitination of CRL^{CSA} might occur after its recruitment to the TC-NER complex. Moreover, under conditions of unperturbed NEDDylation we observed preferential binding of modified CUL4A to p-S5-

RPB1 (Fig. 1) or p-S2-RPB1 (Supplementary Fig. 1). Although probing with NEDD8-specific antibodies is required to confirm the modification, this most likely represents the NEDDylated form of CUL4A, which would also further support the presence of activated CRL^{CSA} in the TC-NER complex. Since NEDDylation is a general mechanism of cullin-RING ligase activation, we cannot ascribe the observed effects to the activity of CRL^{CSA}²². However, given the structural similarities to CRL^{DDB2} and the ability of CRL^{CSA} to ubiquitinate itself *in vitro*, coordination of CRL^{CSA} dissociation from the TC-NER complex by auto-ubiquitination seems a plausible method to regulate repair²¹.

CRL^{CSA} has also been proposed to ubiquitinate CSB²⁷. Interestingly, we did not observe increased levels of CSB in the TC-NER complex upon inhibiting NEDDylation. Nevertheless, these results do not completely exclude CSB as a possible CRL^{CSA} ubiquitination target. CSB has been shown to be deubiquitinated by USP7, which is recruited via UVSSA in a CSA-dependent manner^{11,42}. In this way, the eviction of CSB from the complex can be tightly regulated by the balanced actions of a ubiquitin ligase and a deubiquitinating enzyme. Thus, ubiquitination of CSB by CRL^{CSA} might have been counteracted by USP7. Alternatively, the effect of CRL^{CSA} ubiquitination might have gone unnoticed in these experiments if ubiquitination of CSB affects the protein in a way other than guiding its proteasomal degradation, as described for XPC ubiquitination by CRL^{DDB2}.

The actual dissociation of CSB has been suggested to occur at a later stage, after repair of the damage, enabling resumption of transcription²⁷. Remarkably, inhibition of the VCP/p97 segregase that mediates its dissociation was shown to enhance the recovery of RNA synthesis after UV⁴³. Importantly, VCP/p97 associates with CRL^{CSA} and interacts with both unmodified and ubiquitinated CSB⁴³. In this way, catalytically inactive CRL^{CSA} may still contribute to the degradation of CSB.

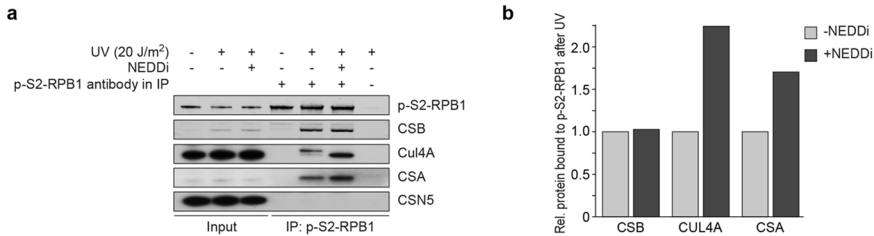
Apart from controlling the presence of (TC-NER) factors to regulate repair under normal circumstances, CRL^{CSA} may also assist in the removal of stalled RNAPII when the TC-NER pathway for some reason cannot be properly and timely executed. Despite this removal is considered a last resort response to transcription-stalling damage, it is crucial in the prevention of persistently stalled RNAPII that activates a signaling cascade eventually leading to p53-dependent cell death^{3,30,32,33,35,36}. As described previously, we observed CSA-dependent degradation of p-S5-RPB1 upon high doses of UV, although we cannot conclude whether the ligase activity of CRL^{CSA} itself contributes to this effect^{7,28,29}. Next to CRL^{CSA}, NEDD4, elongin A/B/C, Von-Hippel Lindau and BRCA1 have all been described to promote ubiquitination and subsequent degradation of stalled RNAPII⁴⁴⁻⁴⁸. Possibly, depending on for instance cell cycle stage, time after UV exposure and tissue type, multiple mechanisms are employed. The eventual degradation of RPB1 might result from the interplay between different ubiquitinating and deubiquitinating enzymes, ensuring that RNAPII is only degraded if other solutions fail. In agreement with this concept, NEDD4, its associated ubiquitin protease UBP2 and the elongin A/B/C complex have been shown to act sequentially in the step-wise poly-ubiquitination of RPB1⁴⁹. Furthermore, degradation is likely stimulated by proteins that contribute to the disassembly of RNAPII. Accordingly, VCP/p97 has also been shown to remove RPB1 from the elongation complex and to directly channel it into the 26S proteasome⁵⁰.

Future studies should reveal whether CSA itself, CSB and/or RPB1 are true CRL^{CSA} ubiquitination targets and potentially identify additional substrates. Ideally, experiments should be done after specific inhibition of CRL^{CSA}. That is, its ligase activity should be inactivated without compromising its incorporation into the TC-NER complex and its ability to recruit other factors. However, given the numerous compositions of cullin-RING ligases and their implications in even more cellular processes, general CUL4A/B inactivating mutations are not suitable to study CRL^{CSA}-specific responses, as is also the case for NEDDylation inhibitors^{51,52}. Alternatively, interfering with the interaction between CSA and the other CRL factors, with the aim to recruit solely CSA to the TC-NER complex, is equally complicated, as CSA stability and localization greatly depend on the establishment of the CRL^{CSA} complex⁵³. CRL^{CSA} ubiquitination targets may therefore be more straightforwardly identified by employing mass spectrometry-based approaches, uncovering differentially ubiquitinated proteins in WT and CSA-deficient cells.

Given the versatile roles of CRL^{DDB2} in the regulation of repair(-facilitating) factors during GG-NER, it is unlikely that the function of CRL^{CSA} is restricted to the ubiquitination of a single target. Almost certainly, uncovering CRL^{CSA}'s ubiquitination targets and revealing new interaction partners will expand our understanding of TC-NER regulation by CRL^{CSA} and show that CSA contributes to the UV response in a broader manner than previously anticipated.

Supplementary information

Supplementary figures



Supplementary Figure 1. NEDDylation modulates the presence of CRL^{CSA} at the TC-NER complex

Immunoprecipitation of the serine 2-phosphorylated RNAPII subunit RPB1 (p-S2-RPB1) from VH10-hTert cells 1 hour after mock treatment or UV-C irradiation (20 J/m²). Where indicated, global NEDDylation had been inhibited prior to UV-C irradiation by treatment with the NEDDylation inhibitor MLN4924. A similar experiment, in which p-S5-RPB1 was precipitated, is shown in Fig. 1. (b) Relative amounts of CSB, DDB1, CUL4A and CSA that coprecipitated with p-S2-RPB1 1 hour after UV-C irradiation (20 J/m²).

Methods

Cell culture

VH10-hTert (WT), CS1AN-hTert (CS-B), CS3BE-hTert (CS-A), KPS3-hTert (UVSS-A), XP1DU-hTert (XP-D; XP phenotype), XP8BR-hTert (XP-D; XP/CS phenotype) and XP25RO-hTert (XP-A) cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Bodinco BV) and penicillin/streptomycin (Sigma).

UV-C irradiation

UV damage was induced using a 254 nanometer TUV PL-S 9W lamp (Philips).

Inhibition of NEDDylation

NEDDylation was inhibited by the addition of 1 μ M MLN4924 (BostonBiochem) to the culturing medium. The inhibitor was added 3 to 4 hours before UV irradiation and kept in the medium during recovery.

Western blotting

Proteins were separated in 4-12% Bis-Tris NuPAGE® gels (Invitrogen) or hand casted 6% acrylamide gels in MOPS (Life Technologies). Separated proteins were blotted onto PVDF membranes (Millipore), which were incubated with the following primary antibodies: rabbit α -p-S2-RPB1 (CTD YSPTSPS repeat phospho S2, Abcam, ab5095); mouse α -p-S5-RPB1 (CTD YSPTSPS repeat phospho S5, Abcam, ab5408); rabbit α -CSB (Santa Cruz Biotechnology, sc-25370); goat α -DDB1 (Abcam, ab9194); rabbit α -CUL4A (Bethyl Laboratories, A300-739A); rabbit α -CSA (Abcam, ab137033); rabbit α -H2B (Millipore, 07-371), rabbit α -actin (Sigma, A2066), mouse α -tubulin (Sigma, T6199). Protein bands were visualized using the Odyssey® Imaging System (LI-COR) after incubation with CFTM dye-labelled secondary antibodies (Sigma), or detected by the ECLTM Prime Western blotting system (GE Healthcare) following incubation with horseradish peroxidase-conjugated secondary antibodies (Dako). Protein band intensities were quantified in ImageJ (<https://imagej.nih.gov/ij/>) or using the Odyssey® Imaging System software (LI-COR).

Immunoprecipitations

Cells were lysed in IP buffer (30 mM HEPES pH 7.5, 130 mM NaCl, 2 mM MgCl₂, 0.5 % Triton X-100, protease inhibitor cocktail (Roche)) during 1.5 hours at 4 °C. The pellet obtained by centrifugation was resuspended in IP buffer supplemented with 300 U/mL benzonase® nuclease (Novagen) and 1.5 µl mouse α-p-S5-RPB1 antibody (Abcam; ab5408) or rabbit α-p-S2-RPB1 antibody (Abcam, ab5095) and incubated during 3 hours at 4 °C. After another round of centrifugation, protein complexes were pulled down from the supernatant (solubilized chromatin) during 2 hours of incubation with Protein A Agarose beads (Millipore).

Determination of total p-S5-RPB1 levels

Cells were mock treated or UV irradiated at 20 J/m² and allowed to recover in medium supplemented with 25 µg/mL cycloheximide (Sigma) during 6 hours. Subsequently, cells were pelleted and lysed in 30 mM HEPES pH 7.5, 130 mM NaCl, 2 mM MgCl₂, 0.5% Triton X-100, protease inhibitor cocktail (Roche) and 125 U/mL benzonase® nuclease during 45 minutes at room temperature. Laemmli-SDS sample buffer was added 1:1 to the supernatant obtained after centrifugation (soluble fraction plus solubilized chromatin) and samples were heated at 95 °C for 10 minutes prior to Western blot analysis.

References

1. Svejstrup, J.Q. Mechanisms of transcription-coupled DNA repair. *Nat Rev Mol Cell Biol* 3, 21-9 (2002).
2. Marteijn, J.A., Lans, H., Vermeulen, W. & Hoeijmakers, J.H. Understanding nucleotide excision repair and its roles in cancer and ageing. *Nat Rev Mol Cell Biol* 15, 465-81 (2014).
3. Dijk, M., Typas, D., Mullenders, L. & Pines, A. Insight in the multilevel regulation of NER. *Exp Cell Res* 329, 116-23 (2014).
4. van Hoffen, A. et al. Deficient repair of the transcribed strand of active genes in Cockayne's syndrome cells. *Nucleic Acids Res* 21, 5890-5 (1993).
5. Venema, J., Mullenders, L.H., Natarajan, A.T., van Zeeland, A.A. & Mayne, L.V. The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA. *Proc Natl Acad Sci U S A* 87, 4707-11 (1990).
6. Mayne, L.V. & Lehmann, A.R. Failure of RNA synthesis to recover after UV irradiation: an early defect in cells from individuals with Cockayne's syndrome and xeroderma pigmentosum. *Cancer Res* 42, 1473-8 (1982).
7. Nakazawa, Y. et al. Mutations in UVSSA cause UV-sensitive syndrome and impair RNA polymerase II processing in transcription-coupled nucleotide-excision repair. *Nat Genet* 44, 586-92 (2012).
8. Schwertman, P., Vermeulen, W. & Marteijn, J.A. UVSSA and USP7, a new couple in transcription-coupled DNA repair. *Chromosoma* 122, 275-84 (2013).
9. Scharer, O.D. Nucleotide excision repair in eukaryotes. *Cold Spring Harb Perspect Biol* 5, a012609 (2013).
10. Laugel, V. et al. Mutation update for the CSB/ERCC6 and CSA/ERCC8 genes involved in Cockayne syndrome. *Hum Mutat* 31, 113-26 (2010).
11. Zhang, X. et al. Mutations in UVSSA cause UV-sensitive syndrome and destabilize ERCC6 in transcription-coupled DNA repair. *Nat Genet* 44, 593-7 (2012).
12. Oh, K.S. et al. Phenotypic heterogeneity in the XPB DNA helicase gene (ERCC3): xeroderma pigmentosum without and with Cockayne syndrome. *Hum Mutat* 27, 1092-103 (2006).
13. Andressoo, J.O. et al. An Xpd mouse model for the combined xeroderma pigmentosum/Cockayne syndrome exhibiting both cancer predisposition and segmental progeria. *Cancer Cell* 10, 121-32 (2006).
14. Ito, S. et al. XPG stabilizes TFIIH, allowing transactivation of nuclear receptors: implications for Cockayne syndrome in XP-G/CS patients. *Mol Cell* 26, 231-43 (2007).
15. Lehmann, A.R. DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. *Biochimie* 85, 1101-11 (2003).

16. Kashiyama, K. et al. Malfunction of nuclease ERCC1-XPF results in diverse clinical manifestations and causes Cockayne syndrome, xeroderma pigmentosum, and Fanconi anemia. *Am J Hum Genet* 92, 807-19 (2013).
17. Giglia-Mari, G. et al. A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. *Nat Genet* 36, 714-9 (2004).
18. Li, J., Bhat, A. & Xiao, W. Regulation of nucleotide excision repair through ubiquitination. *Acta Biochim Biophys Sin (Shanghai)* 43, 919-29 (2011).
19. Nospikel, T. Multiple roles of ubiquitination in the control of nucleotide excision repair. *Mech Ageing Dev* 132, 355-65 (2011).
20. van Cuijk, L., Vermeulen, W. & Marteijn, J.A. Ubiquitin at work: the ubiquitous regulation of the damage recognition step of NER. *Exp Cell Res* 329, 101-9 (2014).
21. Fischer, E.S. et al. The Molecular Basis of CRL4(DDB2/CSA) Ubiquitin Ligase Architecture, Targeting, and Activation. *Cell* 147, 1024-1039 (2011).
22. Enchev, R.I., Schulman, B.A. & Peter, M. Protein neddylation: beyond cullin-RING ligases. *Nat Rev Mol Cell Biol* 16, 30-44 (2015).
23. Groisman, R. et al. The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. *Cell* 113, 357-67 (2003).
24. Sugasawa, K. UV-induced ubiquitylation of XPC complex, the UV-DDB-ubiquitin ligase complex, and DNA repair. *J Mol Biol* 37, 189-202 (2006).
25. Guerrero-Santoro, J. et al. The cullin 4B-based UV-damaged DNA-binding protein ligase binds to UV-damaged chromatin and ubiquitinates histone H2A. *Cancer Res* 68, 5014-22 (2008).
26. Wang, H. et al. Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage. *Mol Cell* 22, 383-94 (2006).
27. Groisman, R. et al. CSA-dependent degradation of CSB by the ubiquitin-proteasome pathway establishes a link between complementation factors of the Cockayne syndrome. *Genes Dev* 20, 1429-34 (2006).
28. Bregman, D.B. et al. UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells. *Proc Natl Acad Sci U S A* 93, 11586-90 (1996).
29. McKay, B.C. et al. UV light-induced degradation of RNA polymerase II is dependent on the Cockayne's syndrome A and B proteins but not p53 or MLH1. *Mutat Res* 485, 93-105 (2001).
30. Wilson, M.D., Harreman, M. & Svejstrup, J.Q. Ubiquitylation and degradation of elongating RNA polymerase II: the last resort. *Biochim Biophys Acta* 1829, 151-7 (2013).
31. Sugasawa, K. et al. UV-induced ubiquitylation of XPC protein mediated by UV-DDB-ubiquitin ligase complex. *Cell* 121, 387-400 (2005).
32. Lommel, L., Bucheli, M.E. & Sweder, K.S. Transcription-coupled repair in yeast is independent from ubiquitylation of RNA pol II: implications for Cockayne's syndrome. *Proc Natl Acad Sci U S A* 97, 9088-92 (2000).
33. Woudstra, E.C. et al. A Rad26-Def1 complex coordinates repair and RNA pol II proteolysis in response to DNA damage. *Nature* 415, 929-33 (2002).
34. Saijo, M. The role of Cockayne syndrome group A (CSA) protein in transcription-coupled nucleotide excision repair. *Mech Ageing Dev* 134, 196-201 (2013).
35. Lagerwerf, S., Vrouwe, M.G., Overmeer, R.M., Fousteri, M.I. & Mullenders, L.H. DNA damage response and transcription. *DNA Repair (Amst)* 10, 743-50 (2011).
36. Ljungman, M. & Zhang, F. Blockage of RNA polymerase as a possible trigger for u.v. light-induced apoptosis. *Oncogene* 13, 823-31 (1996).
37. Aamann, M.D. et al. Cockayne syndrome group B protein promotes mitochondrial DNA stability by supporting the DNA repair association with the mitochondrial membrane. *FASEB J* 24, 2334-46 (2010).
38. Menoni, H., Hoeijmakers, J.H. & Vermeulen, W. Nucleotide excision repair-initiating proteins bind to oxidative DNA lesions in vivo. *J Cell Biol* 199, 1037-46 (2012).
39. Scheibye-Knudsen, M., Croteau, D.L. & Bohr, V.A. Mitochondrial deficiency in Cockayne syndrome. *Mech Ageing Dev* 134, 275-83 (2013).
40. Luijsterburg, M.S. et al. DDB2 promotes chromatin decondensation at UV-induced DNA damage. *J Cell Biol* 197, 267-81 (2012).

41. Pines, A. et al. PARP1 promotes nucleotide excision repair through DDB2 stabilization and recruitment of ALC1. *J Cell Biol* 199, 235-49 (2012).
42. Fei, J. & Chen, J. KIAA1530 protein is recruited by Cockayne syndrome complementation group protein A (CSA) to participate in transcription-coupled repair (TCR). *J Biol Chem* 287, 35118-26 (2012).
43. He, J., Zhu, Q., Wani, G., Sharma, N. & Wani, A.A. Valosin-containing Protein (VCP)/p97 Segregase Mediates Proteolytic Processing of Cockayne Syndrome Group B (CSB) in Damaged Chromatin. *J Biol Chem* 291, 7396-408 (2016).
44. Anindya, R., Aygun, O. & Svejstrup, J.Q. Damage-induced ubiquitylation of human RNA polymerase II by the ubiquitin ligase Nedd4, but not Cockayne syndrome proteins or BRCA1. *Mol Cell* 28, 386-97 (2007).
45. Yasukawa, T. et al. Mammalian Elongin A complex mediates DNA-damage-induced ubiquitylation and degradation of Rpb1. *EMBO J* 27, 3256-66 (2008).
46. Aune, G.J. et al. Von Hippel-Lindau-coupled and transcription-coupled nucleotide excision repair-dependent degradation of RNA polymerase II in response to trabectedin. *Clin Cancer Res* 14, 6449-55 (2008).
47. Kleiman, F.E. et al. BRCA1/BARD1 inhibition of mRNA 3' processing involves targeted degradation of RNA polymerase II. *Genes Dev* 19, 1227-37 (2005).
48. Starita, L.M. et al. BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II. *J Biol Chem* 280, 24498-505 (2005).
49. Harreman, M. et al. Distinct ubiquitin ligases act sequentially for RNA polymerase II polyubiquitylation. *Proc Natl Acad Sci U S A* 106, 20705-10 (2009).
50. He, J., Zhu, Q., Wani, G. & Wani, A.A. UV-induced proteolysis of RNA polymerase II is mediated by VCP/p97 segregase and timely orchestration by Cockayne syndrome B protein. *Oncotarget* (2016).
51. Petroski, M.D. & Deshaies, R.J. Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* 6, 9-20 (2005).
52. Jackson, S. & Xiong, Y. CRL4s: the CUL4-RING E3 ubiquitin ligases. *Trends Biochem Sci* 34, 562-70 (2009).
53. Pines, A. et al. TRiC controls transcription resumption after UV damage by regulating Cockayne syndrome protein A. *Nat Commun* 9, 1040 (2018).

