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

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

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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 (RNAPIIo). Stalling of RNAPIIo 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 culling-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 RNAPIIo when TC-NER cannot be properly executed.

Introduction

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 irradiation[1]. 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 needed[2]. TC-NER is triggered by the encounter of lesions by elongating RNA polymerase II (RNAPIIo) and thus is responsible for the repair of transcription-blocking lesions in transcriptionally active DNA[3]. The recruitment of the SWI/SNF2 ATPase CSB and the CSA protein to the stalled RNA polymerase is essential for repair of UV-induced damage and ultimately, for resumption of transcription[4-7]. Furthermore, UVSSA and its associated factor USP7 specifically contribute to transcription-coupled repair, possibly by regulating the presence of CSB[8]. 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 proteins[9]. Subsequent incision on both sides of the lesion by XPF-ERCC1 (5') and XPG (3') removes a DNA stretch containing the damage[2]. 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 nick[9].

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 phenotype[12-16]. Furthermore, mutations in TFIIH subunits can lead to trichothiodystrophy (TTD) – a disorder characterized by brittle hair and nails and intellectual impairment[17].

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 studied[18-20]. 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}, respectively[21]. The activation of cullin-RING ligases typically requires NEDDylation of the cullin protein[22]. 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 stages[23]. 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 CSA[28-30].

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 RNAPIIo 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 manner[31]. 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 (RNAPIIo) 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 ligases[27]. 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-ubiquitination[21].

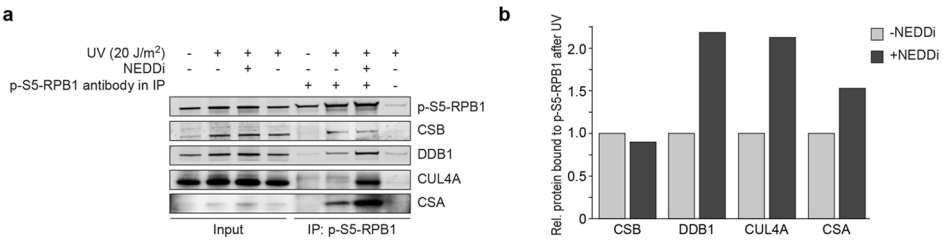


Figure 1. NEDDylation modulates the presence of CRL^{CSA} at the TC-NER complex
(a) Immunoprecipitation of the serine 5-phosphorylated RNAPII subunit RPB1 (p-S5-RPB1) from VH10-hTert cells 1 hour after mock treatment or UV-C irradiation (20 J/m^2). Where indicated, global NEDDylation had been inhibited 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/m^2).

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-RBP1 levels after UV has been shown for CSB- or CSA-deficient cells without inhibition of NEDDylation[29]. 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 detected[34]. 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.

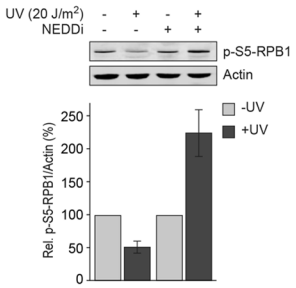


Figure 2. Inhibition of CRL activation prevents UV-induced degradation of p-RPB1
Total levels of serine 5-phosphorylated RPB1 (p-S5-RPB1) in VH10-hTert cells 6 hours after mock treatment or UV-C irradiation (20 J/m²). Where indicated, NEDDylation had been inhibited prior to UV irradiation. Bar graphs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

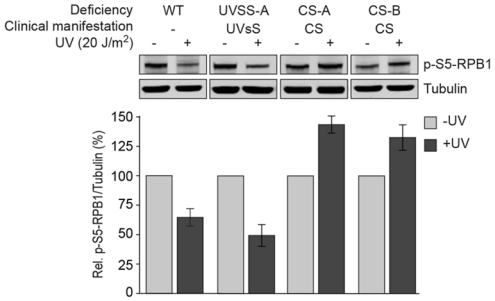


Figure 3. UV-induced degradation of p-RPB1 is dependent on CSA
Total levels of serine 5-phosphorylated RPB1 (p-S5-RPB1) in VH10-hTert (WT), KPS3-hTert (UVSS-A), CS3BE-hTert (CS-A) and CS1AN-hTert (CS-B) cells 6 hours after mock treatment or UV-C irradiation (20 J/m²). Bar graphs show p-S5-RPB1 levels relative to those in non-irradiated cells and represent the average ± 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 pathways[2]. Furthermore, avoidance of persistently stalled RNAPIIo 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 a well, such as the participation of CS proteins in mitochondrial repair and base excision repair[37-39]. Further research should elucidate the exact significance of RNAPII degradation and additional factors in determining the clinical outcome of genetic NER defects.

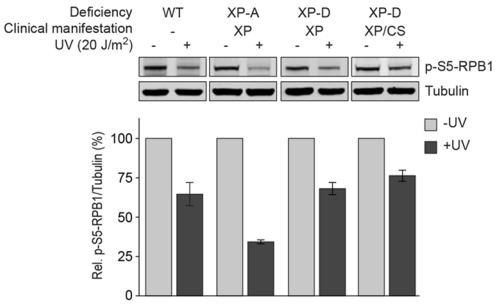


Figure 4. UV-induced degradation of p-RPB1 is similar in XP and combined XP/CS patient cell lines
Total levels of serine 5-phosphorylated RPB1 (p-S5-RPB1) in VH10-hTert (WT), XP25RO-hTert (XP-A), XP1DU-hTert (XP-D) and XP8BR-hTert (XP-D/CS) cells 6 hours after mock treatment or UV-C irradiation (20 J/m^2). Bar graphs show p-S5-RPB1 levels relative to those in non-irradiated cells and represent the average ± 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 manners[18-20]. The significance of this modification and its different biological applications is illustrated by the E3 ubiquitin ligase CRL^{DDB2}, which is important for GG-NER[2]. 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 DNA[31]. 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 completed[23]. Alternatively, in vitro studies suggest that CSB might replace the COP9 signalosome, enabling CRL^{CSA} to become activated by NEDD8[21]. In support of this, we clearly observed a UV-induced interaction between RNAPIIo 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 repair[21].

CRL^{CSA} has also been proposed to ubiquitinate CSB[27]. 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 transcription[27]. Remarkably, inhibition of the VCP/p97 segregase that mediates its dissociation was shown to enhance the recovery of RNA synthesis after UV[43]. Importantly, VCP/p97 associates with CRL^{CSA} and interacts with both unmodified and ubiquitinated CSB[43]. In this way, catalytically inactive CRL^{CSA} may still contribute to the degradation of CSB.

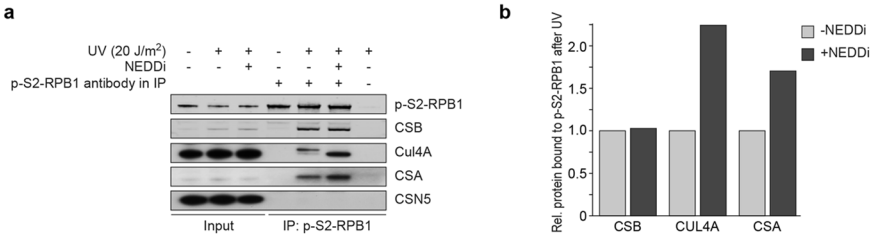
Apart from controlling the presence of (TC-NER) factors to regulate repair under normal circumstances, CRL^{CSA} may also assist in the removal of stalled RNAPIIo when the TC-NER pathway for some reason cannot be properly and timely executed. Despite this removal is considered a last resort response to transcription-stalling damage, it is crucial in the prevention of persistently stalled RNAPIIo 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 RNAPIIo[44-48]. 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 RNAPIIo 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 RPB1[49]. Furthermore, degradation is likely stimulated by proteins that contribute to the disassembly of RNAPIIo. Accordingly, VCP/p97 has also been shown to remove RPB1 from the elongation complex and to directly channel it into the 26S proteasome[50].

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} complex[53]. 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/m^2). 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/m^2).

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 μM 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/m^2 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.

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