

# Regulation of DNA damage and immune response pathways by posttranslational protein modification

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# 4

# Regulation of the DNA damage response by the SUMO E3 ligase Zimp7

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#### Abstract

The DNA damage response covers a network of signaling cascades and DNA repair pathways that serve to protect genome stability. Post-translational modifications of the involved proteins profoundly contribute to regulation of these processes. For example, protein SUMOylation has been described to be important in the response to different types of DNA lesions, including those caused by UV irradiation that are removed via nucleotide excision repair (NER). A focused siRNA screen that examines the effect of several proteins involved in (de)SUMOylation on RNA synthesis recovery upon exposure to UV, identified the PIAS-like protein Zimp7 as a potentially important factor in the transcription-coupled NER subpathway. While PIAS proteins are capable of catalyzing SUMOylation reactions by means of their highly conserved SP-RING domain, this function had not been demonstrated for Zimp7. We reveal that the SP-RING-like motif in Zimp7 confers true SUMOylating activity, uncovering Zimp7 as a new SUMO E3 ligase. Moreover, Zimp7 is recruited to laser-induced DNA damage and interacts with elongating RNA polymerase, as well as with PCNA. Together these findings suggest potential roles of Zimp7 in the DNA damage response, transcription and DNA replication.

#### Introduction

DNA lesions caused by endogenous processes or exogenous insults such as radiation and chemical agents, continuously pose a threat to genome stability and may lead to ageing-related diseases and cancer if left unattended. To maintain genome integrity, DNA damage occurrence triggers the activation of a variety of repair and signaling cascades, collectively referred to as the DNA damage response (DDR)<sup>1,2</sup>.

Post-translational modifications of the involved proteins have been shown to significantly contribute to the regulation of these pathways, thereby facilitating accurate repair and cell cycle progression. For example, ubiquitination and SUMOylation have been described to be broadly implicated in the DDR<sup>3-5</sup>. These modifications involve the reversible covalent attachment of the structurally similar ubiquitin or small ubiquitin-like modifier (SUMO), respectively, to the target protein, altering protein functions and interactions.

Analogously to ubiquitination, SUMOylation is established in a cascade of enzymatic reactions executed by E1, E2 and E3 proteins, which yet differ from those responsible for ubiquitin conjugation. In short, a SUMO precursor protein is C-terminally cleaved by one of the sentrin-specific proteases (SENPs), which is followed by its activation by the dimeric E1 protein SUMO activating enzyme 1 and 2 (SAE1/2). Subsequently, the SUMO moiety is transferred to the E2 conjugating enzyme UBC9, which couples it to the acceptor lysine of the target protein. Importantly, although this is sufficient for the SUMOylation of several substrates, SUMO attachment to many targets requires coordination by one of the E3 SUMO ligase proteins that can catalyze the reaction and provide target specificity<sup>6</sup>.

The protein inhibitor of activated STAT (PIAS) proteins 1-4 comprise a class of SUMO E3 ligases that have been described to both enhance and negatively regulate transcription. This is not merely dependent on their SUMOylation activities but may also rely on their SUMO interacting motifs (SIMs) that modulate interactions with other proteins or DNA<sup>7-10</sup>. Their function as SUMOylation catalyzers depends on the highly conserved Siz/PIAS-RING (SP-RING) motif that resembles the RING domain in ubiquitin E3 ligase proteins<sup>7,11</sup>. Particularly, their roles in the response to DNA damage have been extensively studied. For example, the accumulation of PIAS1 and PIAS4 at double-strand breaks induces SUMOylation and/or recruitment of numerous repair factors, including BRCA1, RAP80, 53BP1 and RNF168, and modulates repair complex disassembly by regulating RNF4 recruitment<sup>12-16</sup>. Moreover, overexpression of PIAS3 can enhance homologous recombination as well as nonhomologous end-joining<sup>17</sup>. Apart from DSB repair, protein modification by SUMO conjugation is crucial in the response to several other types of DNA damage. During base excision repair (BER), SUMOylation of damage recognition factor TDG reduces its interaction with abasic sites and enables its turnover<sup>18,19</sup>. Furthermore, in yeast the recruitment of the anti-recombinogenic helicase Srs2 to SUMOylated PCNA not only regulates replication events during uncompromised DNA synthesis, but also coordinates repair pathway choice upon stalling of replication forks by for instance MMS- or UV-induced DNA damage<sup>20,21</sup>. Protein modification by SUMO hence comprises an important mechanism to control various aspects of the DNA damage response.

UV irradiation triggers the SUMOylation of PCNA, as well as that of factors that are essential for faithful nucleotide excision repair (NER). Removal of DNA helix-destabilizing lesions via NER is initiated by the recognition of damage either specifically in transcribed DNA, referred to as transcription-coupled repair (TC-NER), or throughout the whole genome during global genome repair (GG-NER)<sup>22</sup>. In both subpathways, DNA damage detection is followed by the excision of a lesion-containing single-stranded stretch of DNA, and subsequent DNA synthesis and gap sealing<sup>22,23</sup>.

Whereas stalling of elongating RNA polymerase II at the lesion serves as a damage signal for the recruitment of CSA/CSB and activation of TC-NER, lesion recognition by GG-NER requires the damage sensor proteins XPC and DDB2. Notably, UV-induced SUMOylation of CSB appears to be critical for the repair of transcription-blocking lesions<sup>24</sup>. Similarly, it has been demonstrated that SUMOylation of XPC upon UV irradiation triggers its recognition by the SUMO-targeted ubiquitin ligase (STUBL) RNF111 and the consequential K63-linked ubiquitination that regulates its recruitment to lesions<sup>25,26</sup>. Although these findings underscore the importance of SUMOylation during both subpathways, the exact mechanisms by which SUMO ligases and proteases regulate NER remain to be established.

Here we study the contribution of (de)SUMOylation enzymes to TC-NER. We identify the PIAS-like protein Zimp7 (Zinc finger containing, Miz1, PIAS-like protein on chromosome 7) to be important for the recovery of RNA synthesis upon UV irradiation of VH10-hTert cells. Resembling the PIAS proteins, Zimp7 has been shown to regulate transcription in multiple ways. It has been described to augment transcription that is mediated by Wnt/β-catenin, the androgen receptor and a number of other nuclear hormone receptors. Its function as a transcriptional regulator is further supported by the presence of a C-terminal transactivation domain (TAD)<sup>27-29</sup>. In addition, Zimp7 contains an SP-RING-like motif, which explains its classification as a PIAS-like protein<sup>27</sup>. We show that this domain confers true SUMOylating activity, thereby revealing Zimp7 as a novel SUMO E3 ligase. Moreover, its in vivo SUMOylation and interaction with PIAS3 confirm Zimp7's involvement in the SUMO conjugation system. Finally, Zimp7 is recruited to laser-induced DNA damage and interacts with elongating RNA polymerase II and PCNA in the absence of DNA damage. These findings uncover Zimp7 as a promising SUMO E3 ligase in the context of the DNA damage response and DNA replication.

#### Results

#### Zimp7 may play a role in the DNA damage response

To identity factors involved in (de)SUMOylation that could play a role in transcription-coupled nucleotide excision repair (TC-NER), we performed a small screen in VH10-hTert cells that examined the effect of siRNA-mediated knockdown of candidate proteins on the recovery of RNA synthesis after UV irradiation (Supplementary Fig. 1a). Of the 27 proteins that were studied, knockdown of the PIAS-like protein Zimp7 led to the greatest impairment in RNA synthesis recovery when normalized to siGFP-treated control cells. Notably, the effect was comparable to that caused by depletion of CSB (Fig. 1a, Supplementary Fig. 1a).



#### Figure 1. Zimp7 may play a role in the DNA damage response

(a) An siRNA-based screen targeting 27 different proteins potentially involved in (de)SUMOylation identified Zimp7 as a factor that is important for the recovery of RNA synthesis upon UV damage induction. VH10-hTert cells were transfected with siRNA, UV-C-irradiated at 10 J/m<sup>2</sup> and allowed to recover for 24 hours. RNA synthesis was determined by means of EU incorporation. Data represent the increase in RNA synthesis relative to non-irradiated cells between 2 and 24 hours after UV irradiation, normalized to that in siGFP-treated cells. (b) Loss of Zimp7 renders cells sensitive to UV irradiation. VH10-hT cells were transfected with the indicated siRNAs before UV irradiation at different doses. Clonogenic survival was determined after 2 weeks. Data represent mean ± SEM of 2 independent experiments. (c) Zimp7 is recruited to DNA damage created by the multiphoton laser. DNA damage was inflicted in U2OS cells stably expressing GFP-Zimp7 by multiphoton laser micro-irradiation. Upon pre-extraction, cells were fixed and stained by DAPI and antibodies against the indicated proteins. Length of scale bar: 5 µm. (d) Zimp7 interacts with RNAPIIo. GFP or GFP-Zimp7 was pulled down from HAP1 Zimp7 KO cells stably expressing these proteins at the indicated times after UV-C irradiation at 20 J/m<sup>2</sup>. (e) Zimp7 knockdown slightly affects XPA levels, but none of the other studied NER proteins. VH10-hTert cells were treated with the indicated siRNAs before preparation of whole cell extracts for determination of the presented protein levels.

Importantly, clonogenic survivals showed that knockdown of Zimp7 by 4 different siRNAs markedly increased UV sensitivity of VH10-hTert cells when compared to control cells (Fig. 1b). We therefore hypothesized that Zimp7 could be an important factor in the UV response. To study whether the contribution of Zimp7 would require the presence of the protein at the site of the damage, we stably expressed GFP-tagged Zimp7 in U2OS cells and created DNA damage locally by using a multiphoton laser (Fig. 1c). Interestingly, we could indeed detect recruitment of GFP-Zimp7 to sites of laser-induced DNA damage that were decorated with DDB2. Furthermore, pulldown of GFP-Zimp7 from HAP1 cells revealed that Zimp7 interacts with elongating RNA polymerase II (RNAPIIo), although this appeared to be independent of UV damage (Fig. 1d).

The observation that Zimp7 already interacts with RNAPIIo in the absence of damage, may be explained by a more general role of Zimp7 in transcription. For example, Zimp7 has been described to enhance androgen receptor-mediated transcription and augment Wnt/β-catenin-mediated transcription<sup>27,28,29</sup>. To determine whether regulation of TC-NER by Zimp7 could occur via controlling transcription of TC-NER genes, we studied the effect of Zimp7 depletion on the levels of several of the main NER factors (Fig. 1e). While most of the studied proteins remained unaffected by Zimp7 knockdown, we observed a substantial decrease in the levels of XPA upon treatment with siZimp7-1,-2 or -4. In contrast, siZimp7-3 did not negatively affect the abundance of XPA and only induced a minor increase in UV sensitivity when compared to the other siRNAs, while depleting Zimp7 with similar efficiency (Fig. 1b, Fig. 1e). These findings not only suggest that the loss of XPA was (at least partly) causative for the observed increase in UV sensitivity, but also indicate a possible off-target effect of siZimp7-1,-2 and -4 on XPA. Although our results may implicate a potential role for Zimp7 in the DNA damage response, siRNA-independent approaches are needed to further support this conclusion.

#### Knockout of Zimp7 does not increase UV sensitivity

To further study the effect of the absence of Zimp7 on XPA levels and NER, we generated Zimp7 knockout U2OS and RPE-1 cells. Analyzing 3 different clones in each cellular background, we were unable to verify the small decrease in XPA expression levels observed after siRNA-mediated Zimp7 depletion (Fig. 2a,c). We subsequently performed clonogenic survival assays following UV exposure of wildtype and Zimp7 knockout cells. Strikingly, we could not detect increased UV sensitivity in either U2OS (Fig. 2b) or RPE-1 (Fig. 2d) Zimp7 KO cells as compared to wildtype cells.

To circumvent the use of siRNAs and overcome the potential adaptation of Zimp7 knockout cells during their generation, we next studied the effect of 2 different shRNAs that target Zimp7 in U2OS cells. No reduction in XPA levels was detected upon shRNA-mediated Zimp7 depletion (Fig. 2e). Furthermore, neither of these shRNAs increased UV sensitivity as compared to control shRNA (Fig. 2f). Although several additional experiments are required to exclude other explanations for the observed data, these results suggest that the siRNAs used in VH10-hTert cells indeed caused an off-target effect on XPA, explaining the observed RNA synthesis recovery phenotype.











## Figure 2. Knockout of Zimp7 does not increase UV sensitivity

(a) Zimp7 was successfully knocked out in U2OS cells. 3 different Zimp7 KO clones and the wildtype pool were examined for Zimp7 and XPA expression. Tubulin serves as a loading control. (b) Zimp7 KO U2OS cells are not sensitive to UV damage when compared to the wildtype control. Cells, shown in a, were UV-C-irradiated at the indicated doses and clonogenic survival was determined after 9 days. (c) Zimp7 was successfully knocked out in RPE-1 cells. As in a, but in RPE-1 cells. (d) Zimp7 KO RPE-1 cells are not sensitive to UV damage when compared to the wildtype control. As in b, but in RPE-1 cells shown in c. (e) Knockdown of Zimp7 using shRNA. Cells were treated with the indicated shRNAs and examined for expression of Zimp7, XPA and the loading control tubulin. (f) shRNA-mediated knockdown of Zimp7 does not render U2OS cells UV sensitive. Cells from e were UV-C-irradiated at different doses and assayed for clonogenic survival.

#### Zimp7 interacts with PCNA

Although we were not able to confirm a role for Zimp7 in the response to UV in U2OS cells, we had detected recruitment of GFP-Zimp7 to multiphoton laser-tracks in these cells (Fig. 1c). Since the laser that we used does not solely create DNA lesions that are normally induced by UV (6-4-PPs or CPDs), it is possible that GFP-Zimp7 is recruited to other types of DNA damage. These may for instance include DNA breaks or oxidative damage, raising the possibility that Zimp7 plays a role in DNA damage responses other than the UV damage response. To study this hypothesis, we first performed clonogenic survivals upon IR irradiation, which primarily inflicts DNA breaks (Fig. 3a-b). Knockout of Zimp7 did not increase the sensitivity of U2OS or RPE-1 cells to ionizing radiation-induced DNA damage. Similarly, proliferation assays on cells treated with the DNA alkylating agent MMS (Fig. 3c-d) did not reveal elevated drug sensitivity of Zimp7-depleted cells. Interestingly, several replication stress factors have been shown to accumulate in laser tracks<sup>30-34</sup>. Importantly, Zimp7 has been described to colocalize with PCNA at replication foci and was found to be enriched at hydroxyurea-stalled replication forks in an iPOND study<sup>11,27,28</sup>. We therefore studied a potential role for Zimp7 in the replication stress response. Although knockout of Zimp7 did not render RPE-1 cells more sensitive to hydroxyurea, which causes replication stress by depleting the dNTP pool (Fig. 3e), we were able to observe a clear interaction between Zimp7 and PCNA<sup>35</sup>. Pulldown of GFP-Zimp7 from HAP1 cells convincingly coprecipitated PCNA (Fig. 3f). Considering this interaction, in addition to the reported observation that Zimp7 depletion can cause severe defects in cell proliferation, we hypothesize that Zimp7 may play an important role in the response to replication stress as well as DNA replication in general<sup>29</sup>.

#### Zimp7 is a true SUMO E3 ligase

To facilitate our study of the roles of Zimp7 in the DNA damage response and DNA replication, we decided to examine its molecular function as a PIAS-like protein. Originally identified as inhibitors of STAT transcription factors, PIAS proteins have been shown to broadly function as transcriptional coregulators and, by means of their highly conserved SP-RING-type domain, act as SUMO E3 ligases in different processes<sup>7</sup>. Next to a transactivation domain important in transcription, Zimp7 contains 2 SUMO interacting motifs and an SP-RING-like domain (Fig. 4a) <sup>28</sup>. Indeed, alignment of Zimp7's SP-RING-like domain to that of PIAS1-4 showed a high degree of similarity (Fig. 4b). However, actual SUMOylating activity by Zimp7 has never been demonstrated. To study this potential activity, we expressed amino acids 419-920, containing the complete SP-RING-type domain, in E. coli and eased its purification by adding an N-terminal GST-tag. Next to the wildtype protein (GST-Zimp7 WT), we also purified the recombinant protein harboring a C616A mutation in the SP-RING domain (GST-Zimp7 CD) (Fig. 4a). As the respective cysteine in PIAS4 has been described to be required for its SUMO E3 ligase activity, this mutation would most likely render GST-Zimp7 CD catalytically dead<sup>36,37</sup>.



#### Figure 3. Zimp7 interacts with PCNA

(a) IR sensitivity of U2OS cells is not increased by Zimp7 knockout. Clonogenic survival of wildtype and Zimp7 KO U2OS cells was determined upon IR irradiation at the indicated doses. (b) IR sensitivity of RPE-1 cells is not increased by Zimp7 knockout. As in a, but using RPE-1 cells. (c) Zimp7 knockout does not negatively affect the proliferation of MMS-treated U2OS cells. Cells were incubated with the indicated concentrations of MMS during 24 hours. Proliferation was measured 48 hours after MMS removal. (d) Zimp7 knockout does not negatively affect the proliferation of MMS-treated RPE-1 cells. As in c, but using RPE-1 cells. (e) Zimp7 KO RPE-1 cells are not more sensitive to HU than the wildtype control pool. Cells were treated with different concentrations of hydroxyurea and clonogenic survival was determined. (f) Zimp7 interacts with PCNA. GFP-Zimp7 was pulled down from HAP1 Zimp7 KO cells. Among the coprecipitated proteins were RNAPIIo and PCNA.

We next tested these proteins for their SUMOylating activity in an in vitro assay. SUMOylation of Zimp7 became apparent from a ladder of additional bands on top of wildtype Zimp7 as detected by Western blotting, upon addition of both the SAE1/2 and UBC9 proteins and either SUMO-1 or SUMO-2/3 to the reaction (Fig. 4c-d). Importantly, we did not observe this laddering pattern for GST-Zimp7 CD, suggesting that wildtype, but not CD ZIMP7 possesses (auto-)SUMOylating activity in vitro. To subsequently study (auto-)SUMOylation of Zimp7 in vivo, we pulled down GFP-Zimp7 WT and CD (Fig. 4a) from HAP1 cells under denaturing conditions. Probing the precipitated and Western blotted proteins with antibodies against GFP and SUMO-2/3, revealed that Zimp7 is SUMOylated in vivo as well (Fig. 4e). Strikingly however, we did not only detect SUMOylated GFP-Zimp7 WT, but also observed SUMOylation of GFP-Zimp7 CD. As we determined that the latter is not capable of auto-SUMOylation in vitro (Fig. 4c-d), this indicates that SUMOylation of Zimp7 in vivo may not (solely) result from its own SUMO E3 ligase activity. We then hypothesized that one of the PIAS proteins could be involved in Zimp7 SUMOylation, as interactions between Zimp7 and PIAS proteins have been described<sup>38</sup>. In agreement, pulldown of GFP-Zimp7 WT from HAP1 cells showed a clear interaction between GFP-Zimp7 and PIAS3 (Fig. 4f). The relevance of this interaction remains elusive, although it has been suggested to contribute to Zimp7's stability and/or its transcriptional activity<sup>38</sup>.

Together these data show that Zimp7 is a bona fide SUMO E3 ligase. Whether this function contributes to its role in the DNA damage response is yet to be determined. Furthermore, the fact that ZIMP7 interacts with PCNA (Fig. 3f, 4f) provides a promising foundation to investigate the involvement of Zimp7 as a SUMO E3 ligase in DNA replication.

#### Discussion

Zimp7 has mainly been studied as a transcriptional regulator. Initially identified as an enhancer of androgen receptor-mediated transcription, Zimp7 was found to also augment transcription that is moderated by a number of other nuclear hormone receptors and to act as a coactivator in the Wnt/ $\beta$ -catenin signaling pathway<sup>28,29</sup>. These roles are supported by physical interactions between Zimp7 and the hormone receptor or  $\beta$ -catenin, respectively, and most likely facilitated by Zimp7's C-terminal transactivation domain (TAD) that possesses intrinsic transcriptional activity<sup>28</sup>. In contrast, much less is known about the contribution of the SP-RING- or Miz-like motif to Zimp7's cellular functions. Strikingly, despite the high sequence similarity to the SP-RING domains of the PIAS SUMO E3 ligases, the actual ability to catalyze SUMO conjugation had thus far never been demonstrated for Zimp7.

In this study, Zimp7 was revealed as a factor potentially implicated in the DNA damage response. Its classification as a PIAS-like protein made Zimp7 an attractive candidate in our screen, which examined the effects of knockdown of several (de)SUMOylating proteins on the recovery of RNA synthesis upon UV irradiation. This read-out specifically investigated a role for candidate proteins in transcription-coupled nucleotide excision repair and/or subsequent resumption of transcription.

а



#### b

#### SP-RING type domain:

Zimp7 585 GEDGVEQTAIKVSLKCPITFRRIQLPARGHDCRHIQCFDLESYLQLNCERGTWRCPVCNKTALLEGLEVDQYMLGILI 662 VSL CP R PR СНQFD ΥQΝ TW CPVC K A Т L D IL PIAS1 320 PDSEIATTSLRVSLLCPLGKMRLTIPCRALTCSHLQCFDATLYIQMNEKKPTWVCPVCDKKAPYEHLIIDGLFMEILK 397 PIAS2 331 PDSEIATTSLRVSLMCPLGKMRLTIPCRAVTCTHLQCFDAALYLQMNEKKPTWICPVCDKKAAYESLILDGLFMEILN 408 PIAS3 312 PDSEVATTSLRVSLMCPLGKMRLTVPCRALTCAHLQSFDAALYLQMNEKKPTWTCPVCDKKAPYESLIIDGLFMEILS 389 PIAS4 311 PDSEIATTGVRVSLICPLVKMRLSVPCRAETCAHLQCFDAVFYLQMNEKKPTWMCPVCDKPAPYDQLIIDGLLSKILS 388



#### Figure 4. Zimp7 is a true SUMO E3 ligase

(a) Schematic overview of the Zimp7 protein, showing the SUMO interacting motifs (SIMs), the SP-RINGlike domain important for SUMO E3 ligase activity and the transactivation domain (TAD) important for transcription. GST- and GFP-tagged Zimp7 constructs were created as shown, containing either the wildtype SP-RING domain (WT) or harboring mutation C616A (CD). (b) Alignment of Zimp7's SP-RINGtype domain to that of PIAS1-4. The asterisk indicates residue C616 in Zimp7. (c) Zimp7 is capable of auto-SUMOylation using SUMO-1. Recombinantly produced GST-Zimp7 WT or CD, shown in a, was added to an in vitro reaction containing SUMO-1, the E1 enzyme and different amounts of the SUMO E2. After 3 hours, Zimp7 modifications were examined by Western blotting. The asterisk indicates nonmodified GST-Zimp7. (d) Zimp7 is capable of auto-SUMOylation using SUMO-2/3. As in c, but using SUMO-2/3. (e) Zimp7 is SUMOylated in vivo. GFP-Zimp7 WT and CD were pulled down from HAP1 Zimp7 KO cells under denaturing conditions and examined for SUMOylation on a Western blot. The asterisk indicates the size of non-modified GFP-Zimp7. (f) Zimp7 interacts with PIAS3. GFP-Zimp7 WT was pulled down from HAP1 Zimp7 KO cells. Among the coprecipitated proteins were PCNA and PIAS3. Our screen revealed Zimp7 as a promising factor that may regulate TC-NER. However, whereas the increased UV sensitivity of VH10-hTert cells upon siRNA-mediated depletion of Zimp7 confirmed a role for this protein in TC-NER, these observations could not be verified by knockout of Zimp7 in U2OS or RPE-1 cells, or by Zimp7 depletion using shRNAs in U2OS cells. The discrepancy between results that were obtained in different experimental set-ups did not warrant further mechanistic studies and demonstrates the need for additional validation experiments to determine which approach is most suitable to study Zimp7's cellular functions. The use of siRNAs seems questionable, as the observed effects of siZimp7-1,-2 and -4 on XPA levels and UV sensitivity were most likely caused by off-target effects. Ectopic expression of siRNA-resistant Zimp7 would therefore most likely not restore XPA levels nor rescue the UV sensitivity upon siRNA-mediated Zimp7 depletion, yet comprises a straightforward method that is required to affirm our interpretation of the data.

To circumvent the use of siRNAs, we generated Zimp7 knockout cell lines by CRISPR/Cas9mediated targeting of the *Zimp7* gene. Although this is a frequently used approach to study phenotypes in the complete absence of a protein of interest, our observations indicate that it is critical to validate the obtained cell lines prior to their further use. Zimp7 depletion has been described to negatively affect proliferation and thus already leads to growth defects in unperturbed conditions<sup>29</sup>. In contrast, major effects of Zimp7 knockout on cell growth were not evident in our experiments. This raises the possibility that the clones that were used had adapted to Zimp7 depletion, which potentially has also affected the outcomes of our experiments. Proliferation of the individual clones should therefore be evaluated more precisely, for example by cell count measurements or FAQS analyses. Similarly, the effect of Zimp7 depletion on transcriptional regulation of Wnt signaling genes could aid in the characterization of individual knockout clones<sup>29</sup>. Considering the off-target effects of siRNAs and possible adaptation of Zimp7 knockout cells, the preferred approach to study Zimp7's cellular roles could involve the use of shRNAs, although it is recommended to first assess their application for example by performing complementation experiments.

Regardless of the need for additional validation experiments to establish the optimal experimental set-up, we have made several novel findings related to Zimp7's biological functions. The challenging question is whether these are related to its role as a transcriptional regulator, to its newly identified function as a SUMO E3 ligase or should even be ascribed to other, yet to be determined, activities. The first of these possible functions could be further studied by for instance RNA-seq experiments with control and Zimp7-depleted cells to examine which genes are regulated by Zimp7. Importantly, these will also indicate to what extent phenotypes that are observed in subsequent experiments are to be explained by an indirect role of Zimp7, that is via regulating levels of certain proteins, including those involved in the DDR. Evidently, the suggested experiments should be accompanied by studying the effects of DNA damage induction on the expression (and potential modification) of Zimp7 itself.

The recruitment of Zimp7 to multiphoton laser-inflicted damage, on the other hand, illustrates a potential direct role for Zimp7 at sites of DNA damage. Notably, Zimp7 was found to interact with the BAF57 and BRG1 components of the highly conserved SWI/SNF-like chromatin remodeling complexes<sup>28</sup>. By destabilizing histone-DNA interactions and

thereby repositioning nucleosomes, these complexes allow binding of transcription factors to the DNA and facilitate transcription of the respective genes<sup>39</sup>. Importantly, SWI/SNF-like factors have also been found implicated in the DDR, for example by promoting the repair of double-strand DNA breaks and UV-induced DNA damage<sup>40-43</sup>. We therefore speculate that Zimp7 could contribute to the chromatin remodeling that is required to increase accessibility of the lesion during DNA damage repair.

The emerging question concerning Zimp7's possible direct and indirect roles in the DDR, is whether these depend on its ability to catalyze SUMO conjugation. Future endeavors to unravel Zimp7's cellular functions, could therefore include pulldowns of SUMOylated proteins in the absence and presence of Zimp7. Analysis of the precipitated proteins by mass spectrometry could reveal Zimp7-specific SUMOylation targets that potentially shed light on the processes that Zimp7 participates in. Similarly, Zimp7 itself can be precipitated to examine (directly) interacting proteins.

Evidently, the sliding clamp PCNA and other DNA replication factors would be interesting proteins to look for in the hereby obtained data. Strengthening the observation that Zimp7 colocalizes with PCNA and newly synthesized DNA at replication foci in S-phase, we detected a clear interaction between these proteins in unperturbed conditions<sup>28</sup>. This suggests that Zimp7 might play a role in DNA replication and/or the replication stress response. Notably, SUMOylation of PCNA, which is triggered by its loading onto DNA during uncompromised DNA replication, appears to play an important role in influencing pathway choice upon replication stress. In yeast, binding of the anti-recombinogenic protein Srs2 to PCNA, which is increased by its SUMOylation, prevents the formation of RAD51 filaments that could otherwise cause unwanted homologous recombination between the newly formed sister chromatids<sup>20,21,44</sup>. Although levels of SUMOylated PCNA are much lower in mammalian cells, the human helicase PARI likewise interacts with modified PCNA via its PIP and SIM motifs and seems to function analogously to Srs2<sup>45,46</sup>. The interaction between Zimp7 and PCNA and the reported colocalization at replication foci in S-phase, make it worthwhile to study whether Zimp7 contributes to regulation of DNA replication (stress pathways) and, if so, whether this can be ascribed to Zimp7-mediated SUMOylation of PCNA or other replication (stress) factors<sup>28</sup>. An iPOND study, examining the presence of Zimp7 on nascent DNA in unperturbed conditions as well as under conditions of replication stress, could greatly improve our understanding of the spatiotemporal coordination of Zimp7 and its interaction with PCNA. Regulation of the SUMOylated PCNA status by Zimp7 in a DNA damagedependent manner could suggest an important contribution of Zimp7 to the replication stress response and argue for investigating its role in modulating the PARI-PCNA interaction. Overall, studying Zimp7's biological functions might be difficult in case of redundancy between Zimp7 and for instance the PIAS proteins or its homolog Zimp10, which shares important domains such as the TAD and the SP-RING type motif<sup>27</sup>. Despite their high sequence similarity and comparable nuclear localization, there are indications that Zimp7 and Zimp10 play different cellular roles. They have been described to have different expression profiles and possibly regulate different subsets of nuclear hormone receptors and other transcription factors. Furthermore, Zimp7 is not capable of fully compensating for loss of Zimp10 function in Zimp10 knockout mice, resulting in embryonic lethality<sup>27,28</sup>. However,

potentially overlapping or complementing functions in the DDR and/or DNA replication, for example by catalyzing SUMOylation of target proteins, have yet to be determined. In support of this, both Zimp7 and Zimp10 colocalize with SUMO-1 at replication foci in S-phase<sup>28,47</sup>. Zimp10 would therefore be a promising factor to include in future studies.

Interestingly, Zimp10 interacts with the tumor suppressor and DDR protein p53, thereby altering its transcriptional activity<sup>48</sup>. Although the relevance of p53-mediated transcription regulation by Zimp10 for the DDR remains to be established, it may explain how Zimp10 contributes to genome stability maintenance. Given their potential roles in the DDR and genome stability maintenance, Zimp7 and Zimp10 are both attractive proteins to study in the context of cancer development and ageing-related diseases.

#### Supplementary information

#### Supplementary figures



### Supplementary Figure 1. Zimp7 is a promising hit in a screen for factors involved in RNA synthesis recovery upon UV irradiation

(a) An siRNA-based screen targeting 27 different proteins potentially involved in (de)SUMOylation identified Zimp7 as a factor that may be important for the recovery of RNA synthesis upon UV damage induction. VH10-hTert cells were transfected with siRNA, UV-C-irradiated at 10 J/m<sup>2</sup> and allowed to recover for 24 hours. RNA synthesis was determined by means of EU incorporation. Data represent the increase in RNA synthesis relative to non-irradiated cells between 2 and 24 hours after UV irradiation, normalized to that in siGFP-treated cells.

#### Methods

#### Cell culture

Cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Bodinco BV) and penicillin/streptomycin (Sigma). The following cell lines were used: VH10-hTert, U2OS, RPE-1 and HAP1.

#### Generation of stable cell lines

Stable expression of GFP-Zimp7 in U2OS cells was established by cloning Zimp7 cDNA into the multiple cloning site of pEGFP-C1, which is followed by an IRES and puromycin resistance gene that had previously been added to the plasmid. This construct was transfected using Lipofectamine® 2000 (Invitrogen) in Opti-MEMTM (Gibco) containing 10% FBS. Stable integrands were obtained by selection on puromycin (ThermoFisher Scientific). HAP1 Zimp7 KO cells were obtained from Horizon. For stable expression of GFP-tagged Zimp7, wildtype GFP-Zimp7 or that containing a C616A mutation (created by overlap PCR) was transferred from pEGFP-C1 into pLX304 (Addgene). Lentivirus was produced using the pCMV-VSV-G, pMDLg-RRE and pRSV-REV plasmids

(Addgene) and used to infect cells with Polybrene® (Sigma). Stable integrands were obtained after selection in medium containing blasticidin (ThermoFisher Scientific).

U2OS and RPE-1 Zimp7 KO cells were generated by CRISPR/Cas9-mediated targeting of the Zimp7 gene. Cells were transfected with pX458 (Addgene) into which the following guideRNA was cloned: GCTGAAGGCGGCGCCAACAA. 48 hours after transfection, GFP-expressing cells were obtained by sorting using a BD FACSAria III Sorter (BD Biosciences) and seeded at low density. Single clones were examined for Zimp7 protein levels by Western blotting. Mutations in the Zimp7 gene were determined by Sanger sequencing of a PCR-amplified fragment of genomic DNA and analyzed by TIDE (NKI). The clones in this study all acquired premature stop codons, producing only 20% or less of the full-length Zimp7 protein.

#### Recovery of RNA synthesis screen

Transfections with SMARTpool siRNAs (Dharmacon) were performed in 96-well plates using Lipofectamine® RNAiMAX in Opti-MEMTM (Gibco) containing 10% FBS. siRNAs against the following targets were used: CBX4, COPS5, CSB, FLJ32440, GFP, MDM2, PCGF2, PIAS1, PIAS2, PIAS3, PIAS4, PLP2, RAI17, RANBP2, SAE1, SENP1, SENP2, SENP3, SENP5, SENP6, SENP7, SENP8, TOPORS, UBA1, UBE2I, UBE4B, UCHL5, UFD1L, ZIMP7. Cells were irradiated with UV-C (10 J/m<sup>2</sup>), and incubated for 0, 2 or 24 hours to allow RNA synthesis recovery. RNA was labeled for 1 hour in medium supplemented with 1 mM EU (Click-iT® RNA Alexa Fluor® 488 Imaging Kit, Life Technologies) according to the manufacturer's instructions. Nuclei were stained by DAPI (Sigma). Imaging was performed on a BD PathwayTM 855 Bioimager, using BD AttoVisionTM (BD Biosciences). RNA synthesis recovery was determined by measuring the mean Alexa 488 intensity of all nuclei per well and analyzed using Cell Profiler software.

#### **RNA** interference

For siRNA-mediated depletion of proteins using single siRNAs, two sequential transfections with 40 nM siRNA (Dharmacon) were performed using Lipofectamine® RNAiMAX (Invitrogen) in Opti-MEMTM (Gibco) containing 10% FBS. The following siRNAs were used:

5'-CGUACGCGGAAUACUUCGA-3' (Luciferase);

5'- UCACCAAGAUAAAGCGGAAUU-3' (Zimp7-1);

5'-GCUUUGACCUGGAGUCGUAUU-3' (Zimp7-2);

5'-UCUACAAGACCCUGAUAAUUU-3' (Zimp7-3);

5'-ACUCUGACUAUGAGGAGAUUU-3' (Zimp7-4).

To deplete Zimp7 by means of shRNA, lentivirus was produced from pLKO.1 plasmids that contained the shRNA of interest and the pMD2.G, pMDLg-RRE and pRSV-REV plasmids (Addgene). Cells were infected cells using Polybrene® (Sigma). The following shRNAs were used:

5'-ACCGGACACTCGAGCACTTTTTGAATTC-3' (Control);

5'-CCGGCGGTGATGGTTCATTCGCATACTCGAGTATGCGAATGAACCATCACCGTTTTTG-3' (Zimp7-1); 5'-CCGGGACCTCCCTACGAACAACAATCTCGAATTGTTGTTCGTAGGGAGGTCTTTTTG-3' (Zimp7-2).

#### Whole cell extract preparation

For detection of overall protein levels, whole cell extracts were prepared by lysis in 5 µl lysis buffer (30 mM Tris pH 7.5, 150 mM NaCl, 2 mM MgCl2, 0.5 % Triton X-100, 500 U/mL Benzonase® nuclease, protease inhibitor cocktail (Roche)) per 100.000 cells during 10 min at room temperature. Equal volumes of Laemmli-SDS sample buffer were added and the samples were heated at 95 °C for 10 minutes prior to Western blot analysis.

#### GFP pulldowns

For isolation of protein complexes, cells were lysed in IP buffer (30 mM Tris pH 7.5, 150 mM NaCl, 2 mM MgCl2, 0.5 % Triton X-100, 2.5 mM EGTA, 5 mM NaF, 5 mM β-glycerol phosphate, 5 mM NaPy, 10 mM NEM, 70 mM chloroacetamide, protease inhibitor cocktail (Roche)) supplemented with 250 U/mL benzonase® nuclease during 1-1.5 hours. Samples were centrifuged at 13.000 rpm.

For pulldown of proteins under denaturing conditions, cells were lysed in 200 µl denaturing IP buffer (20 mM Tris pH 7.5, 50 mM NaCl, 0.5% NP-40, 1% sodium deoxycholate (DOC), 1% SDS, 5 mM MgCl2, 1 mM PMSF, protease inhibitor cocktail (Roche)) supplemented with 500 U/mL benzonase® nuclease during 30 minutes, with

forced resuspension of the pellet by pipetting every 10 min. 800 µl wash buffer (20 mM Tris pH 7.5, 50 mM NaCl, 0.5% NP-40, 0.5% DOC, 1 mM EDTA, 1 mM PMSF, protease inhibitor cocktail (Roche)) was added before centrifugation of the samples at 13.000 rpm.

GFP-tagged proteins were precipitated from the supernatant using GFP-Trap®\_A beads (Chromotek) and eluted by boiling of the beads in Laemmli-SDS sample buffer.

#### Western blotting

Proteins were separated in 4-12% Bis-Tris NuPAGE® gels (Invitrogen) or 4-12% Bis-Tris CriterionTM gels (BIO-RAD) in MOPS buffer (Life Technologies). Separated proteins were blotted onto PVDF membranes (Millipore), which were incubated with the following primary antibodies: mouse α-RNAPIIo (Abcam, ab5408); rabbit α-RNAPIIo (Abcam, ab5095); mouse α-RNAPIIo/a (Santa Cruz Biotechnology, sc-17798); rabbit α-CSB (Santa Cruz Biotechnology, sc-25370); goat α-DDB1 (Abcam, ab9194); mouse α-Tubulin (Sigma, T6199); mouse α-GFP (Roche, #11814460001); rabbit α-GFP (Abcam, ab290); rabbit α-p89 (Santa Cruz Biotechnology, sc-19); mouse α-RPA70 (Calbiochem, NA13); mouse α-XPA (Invitrogen, MA5-13835); rabbit α-PCNA (Abcam, ab15497); rabbit α-H3 (Abcam, ab1791); rabbit α-PIAS3 (Cell Signaling, #9042); mouse α-SUMO-2/3 (Abcam, ab81371). Rabbit α-Zimp7 antibodies were kindly provided by Z.J. Sun. Protein bands were visualized using the Odyssey® Imaging System (LI-COR) after incubation with CFTM dye labelled secondary antibodies (Sigma) and analyzed using the Odyssey® Imaging System software (LI-COR).

#### **UV-C** irradiation

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

#### Multiphoton laser micro-irradiation

Cells were grown on 18 mm coverslips, the medium was replaced by CO2- independent Leibovitz's L15 medium supplemented with 10% FBS (Bodinco BV) and penicillin/streptomycin (Sigma), and coverslips were placed in a Chamlide CMB magnetic chamber in an environmental chamber set to 37 °C coupled to a Leica SP5 confocal microscope. UV-type laser damage was generated using a titanium-sapphire laser (I = 800 nm, pulse length = 200 fs, repetition rate = 76 MHz). 1-15 minutes after damage induction, coverslips were incubated in CSK buffer (300 mM sucrose, 100 mM NaCl, 3 mM MgCl2) containing 0.25% Triton X-100 (Sigma) during 2 minutes on ice and fixed in 2% formaldehyde during 20 minutes. Upon fixation, coverslips were incubated in 5% NP-40 (Sigma) during 5 minutes, washed with PBS, blocked in PBS containing 5 g/L BSA and 1.5 g/L glycine during 30 minutes and incubated overnight with mouse  $\alpha$ -DDB2 (MyBioSource, MBS120183) primary antibody. Subsequently, coverslips were incubated goat  $\alpha$ -mouse Alexa Fluor® 555 (Thermo Scientific) secondary antibody for visualization of DDB2 and DAPI (Sigma) for nuclear staining. Images were acquired on a Zeiss AxioImager D2 widefield fluorescence microscope equipped with 40x, 63x and 100x PLAN APO (1.4 NA) oil-immersion objectives (Zeiss) and an HXP 120 metal-halide lamp used for excitation. Images were recorded using ZEN 2012 software.

#### Treatment with hydroxyurea

Cells were seeded at low density. The next day, different concentrations of hydroxyurea (Sigma) were added and cells were incubated in HU-containing medium for 24 hours. Subsequently, plates were washed twice with PBS and fresh medium without HU was added. Clonogenic survival was determined after 8 days.

#### Proliferation assay upon treatment with MMS

Different concentrations of methyl methanesulfonate (MMS, Sigma) were added to attached cells in 6-well plates. 24 hours after addition of MMS, plates were washed twice with PBS and fresh medium without MMS was added. After 72 hours, cells were trypsinized and counted using a Coulter Counter (Beckman Coulter) to determine proliferation.

#### **IR** irradiation

lonizing radiation (IR) damage was inflicted by a YXIon X-ray generator (YXIon International) at 200 kV, 12 mA and a dose rate of 4 Gy/min.

#### **Clonogenic survivals**

Cells were seeded at low density and UV or IR irradiated at different doses or treated with different concentrations of hydroxyurea. After 8-14 days of incubation, cells were washed with 0.9% NaCl and stained with methylene blue. Colonies of >20 cells were scored.

#### Production of recombinant proteins

DNA fragments encoding amino acids 419-920 of Zimp7 WT or CD were cloned into pGEX-6p-3. Upon transformation of Rosetta E.coli with these constructs, GST-Zimp7 WT or CD expression was induced by addition of 0.5 mM IPTG, 1 mM MgCl2 and 0.05% glucose. After 5.5 hours cells were washed in cold PBS and lysates were prepared by resuspension in cold PBS containing 0.5 M NaCl, 1 mM PMSF and protease inhibitor cocktail (Roche) and sonification using a Misonix Sonicator 3000. Lysates were cleared by centrifugation and the soluble fraction was collected and incubated with GST-Sepharose beads (Amersham) during 2 hours at 4 °C. Beads were washed twice with cold PBS containing 0.5 mM NaCl, 1mM PMSF and protease inhibitors (without EDTA), followed by three washes with a washing buffer containing 50 mM Tris-HCl (pH 7.5) and 0.5 mM NaCl. Proteins were eluted once with 20 mM glutathione in washing buffer.

Purified SAE1 and UBC9 were obtained as previously described<sup>49</sup>.

#### In vitro SUMOylation

In vitro SUMOylation reactions contained 0.3  $\mu$ g SUMO E1 (except control samples), 0.05-0.2  $\mu$ g SUMO E2 and 0.3  $\mu$ g purified fragments of human ZIMP7 protein (see above). Each reaction contained 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 3.5 U/ml creatine kinase, 10 mM creatine phosphatase, 0.6 U/ $\mu$ l inorganic phosphatase and 0.5  $\mu$ g either SUMO1 or SUMO2/3. Reactions were carried out for 3 hours at 37 °C before quenching with 1⁄4 volume of 4x NuPAGE loading dye and flash-freezing in liquid nitrogen. SUMOylated proteins were detected by Western blotting.

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