# *RAD6*-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO

**Carsten Hoege*, Boris Pfander*, George-Lucian Moldovan*, George Pyrowolakis*† & Stefan Jentsch***

** Department of Molecular Cell Biology, Max Planck Institute of Biochemistry, Am Klopferspitz 18a, 82152 Martinsried, Germany*

**The *RAD6* pathway is central to post-replicative DNA repair in eukaryotic cells; however, the machinery and its regulation remain poorly understood. Two principal elements of this pathway are the ubiquitin-conjugating enzymes RAD6 and the MMS2–UBC13 heterodimer, which are recruited to chromatin by the RING-finger proteins RAD18 and RAD5, respectively. Here we show that UBC9, a small ubiquitin-related modifier (SUMO)-conjugating enzyme, is also affiliated with this pathway and that proliferating cell nuclear antigen (PCNA)—a DNA-polymerase sliding clamp involved in DNA synthesis and repair—is a substrate. PCNA is mono-ubiquitinated through RAD6 and RAD18, modified by lysine-63-linked multi-ubiquitination—which additionally requires MMS2, UBC13 and RAD5—and is conjugated to SUMO by UBC9. All three modifications affect the same lysine residue of PCNA, suggesting that they label PCNA for alternative functions. We demonstrate that these modifications differentially affect resistance to DNA damage, and that damage-induced PCNA ubiquitination is elementary for DNA repair and occurs at the same conserved residue in yeast and humans.**

DNA is highly vulnerable to spontaneous and environmental damage in living cells. If not repaired, DNA damage may cause mutation-induced genetic variation, ageing, carcinogenesis, or cell death. Eukaryotic DNA repair pathways are highly conserved from yeast to humans. Three principal pathways are classified according to genetic relations of yeast (*Saccharomyces cerevisiae*) DNA-repair mutants (for reviews see refs 1–3). The *RAD3* group mediates nucleotide excision repair, the *RAD52* group directs double-strand break repair through homologous recombination, whereas the *RAD6* group functions in post-replication repair. Proteins of the *RAD6* group seem to act on the stalled replication machinery that has encountered a damaged template, thereby accomplishing repair and allowing replication to resume[1,4]. This repair can be achieved by at least two different *RAD6*-dependent mechanisms: one mechanism is considered error-prone as it uses specialized translesion polymerases that insert correct or incorrect nucleotides across a damaged site; the other *RAD6*-dependent mode is error-free because it uses the information of the undamaged sister duplex at the replication fork.

The mechanism by which the *RAD6* pathway controls the different modes of post-replication DNA repair has remained enigmatic. Previous work has shown that modification of proteins by conjugation to ubiquitin is pivotal for *RAD6*-dependent DNA repair. *RAD6* itself encodes a ubiquitin-conjugating enzyme, which attaches ubiquitin to substrate proteins in collaboration with ubiquitin-activating enzyme[5]. Furthermore, two other members of the *RAD6* group, UBC13 and MMS2, form a heterodimeric ubiquitin-conjugating enzyme, which catalyses the formation of non-canonical multi-ubiquitin chains linked via K63 of ubiquitin[6–11]. Both enzymes, RAD6 and UBC13–MMS2, are recruited to chromatin by interaction with the RING-finger-containing, DNA-binding proteins RAD18 and RAD5, respectively[9,12]. We have demonstrated previously that the two distinct ubiquitin-conjugating enzymes associate by means of a RAD18–RAD5 interaction, and we proposed that this assembly might modify proteins by K63-linked multi-ubiquitin chains[9]. In contrast to K48-linked multi-ubiquitination, mono-ubiquitination and modification by K63-linked chains does not generally promote proteasomal degradation, but rather it seems to alter the function of the substrate or to mediate protein–protein interactions[13]. Nevertheless, the key question of how ubiquitination affects DNA repair has not been answered so far, because the proteins relevant to DNA repair that are ubiquitinated by the *RAD6* pathway have remained unidentified.

## Modification of PCNA by SUMO

During a study of the protein modification system that uses the small ubiquitin-related modifier, SUMO, we obtained unexpected insights into the *RAD6* pathway. This type of modification is widespread and affects a large number of cytosolic and nuclear proteins (for reviews see refs 14–16). SUMO, a protein that shares about 18% sequence identity with ubiquitin, has been suggested to mediate protein–protein interaction or to function as a ubiquitin antagonist.

To investigate this modification in yeast we searched for new SUMO substrates. We immuno-purified SUMO–protein conjugates by a two-step protocol and identified the proteins by mass spectrometric analysis of tryptic peptides (Fig. 1a). Peptide masses of one conjugate with a relative molecular mass of 45,000 ($M_r$, 45K) matched those of tryptic fragments predicted for the gene product of *POL30*; that is, the yeast proliferating cell nuclear antigen PCNA. Further analysis verified that a fraction of the protein is modified by SUMO *in vivo* (Fig. 1b). PCNA, a protein of 29K, is loaded onto DNA strands as a trimeric ring where it functions as a clamp and processivity factor for DNA polymerases[17,18]. It is essential for a variety of S-phase functions including replication, replication-linked repair and silencing. These activities are matched by PCNA levels, which rise at S phase[19]. We synchronized cells using α-factor and observed that SUMO conjugation to PCNA is regulated by the cell cycle and that it precedes the rise of PCNA during S phase (Fig. 1c). This finding suggests that SUMO modification of PCNA is important for events that take place during S phase.

## Modification sites

Similarly to ubiquitin, SUMO is conjugated to ε-amino groups of

† Present address: Biocenter, University of Basel, 4056 Basel, Switzerland.

lysine residues of the target protein[14,15]. To identify the acceptor residue(s) for SUMO conjugation, we changed all 18 lysine residues of yeast PCNA individually to arginine. Only mutants at lysine 127 (K127R) and 164 (K164R) specifically altered the pattern of SUMO conjugates in a total yeast lysate (data not shown). Pull-down experiments using wild-type and mutant PCNA verified that yeast PCNA can be modified by SUMO conjugation at both residues and that a K127R K164R PCNA double mutant is not modified by SUMO to detectable levels (Fig. 1d). K127 lies within a postulated SUMO-modification consensus site (ψKxD/E, where ψ is a hydrophobic residue; ref. 20), but appears to be unique for yeast PCNA. In contrast, K164 is a 'non-consensus' SUMO-conjugation site, yet this site is highly conserved in PCNA from yeast to humans. The conserved K164 residue seems to be the prominent site for SUMO conjugation, whereas SUMO conjugation at K127 is stimulated when K164 has been experimentally mutated (Fig. 1d). Both modifications can occur on the same molecule, as doubly modified PCNA species can be detected in western blots of PCNA and SUMO (data not shown).

PCNA is also involved in DNA damage response and has been linked to the *RAD6* pathway of post-replicative DNA repair[21]. When we treated cells with 0.02% of the DNA-damaging agent methyl methanesulphonate (MMS), we observed little change in the level of PCNA–SUMO conjugates (Fig. 2a; band $S^{164}$). However, when we applied a lethal dose of MMS (0.3%), there was marked induction of SUMO conjugation of PCNA, in particular at residue K164 (Fig. 2a, b). Thus, SUMO conjugation of PCNA is not triggered exclusively by S-phase events, but also by severe DNA damage.

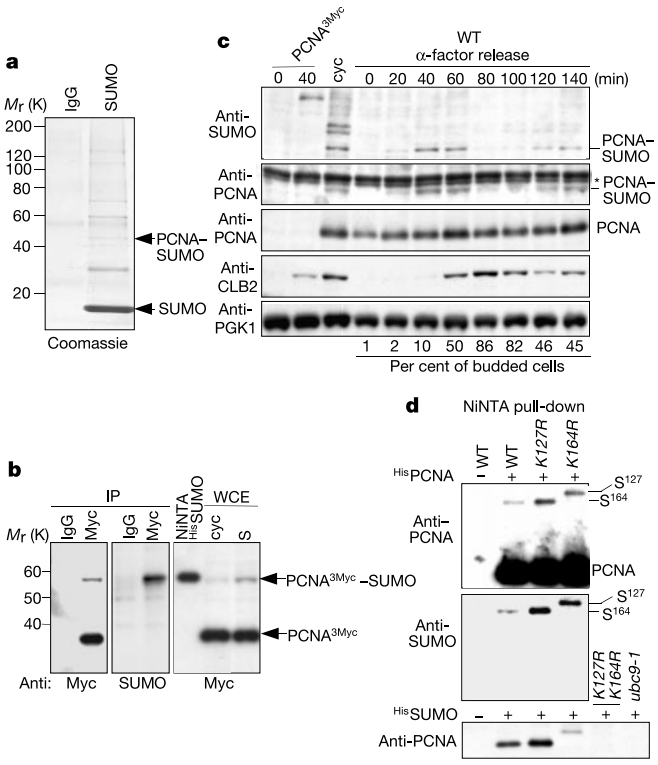


**Figure 1** PCNA is modified by SUMO in *S. cerevisiae*. **a**, Two-step purification of His-tagged SUMO ($^{His}$SUMO) conjugates involving NiNTA pull-down followed by anti-SUMO affinity chromatography. The 45K PCNA–SUMO conjugate was identified by mass spectrometry. **b**, Anti-PCNA (tagged with a triple Myc epitope, $PCNA^{3Myc}$) immunoprecipitations from a *ulp1ts* strain defective in SUMO deconjugation (IP), NiNTA pull-downs from $^{His}$SUMO-expressing cells under denaturing conditions (NiNTA $^{His}$SUMO), and whole-cell extracts (WCE) of cycling wild-type (WT) cells (cyc) or S-phase cells (S). Proteins were detected by western blotting using antibodies as indicated below. **c**, PCNA SUMO modification is upregulated in S phase. Cultures were synchronized by α-factor arrest/release, and analysed by western blotting using the indicated antibodies. The identity of PCNA and PCNA–SUMO bands is demonstrated by the corresponding up-shift when Myc-tagged PCNA ($PCNA^{3Myc}$) was expressed as the sole source of PCNA (left two lanes). Relevant parts of the same gel are shown. Levels of PCNA–SUMO peak at 40 min, whereas those of unmodified PCNA peak at 60 min after release from α-factor arrest. Nonspecific bands are indicated by an asterisk. Cell-cycle stages were monitored by CLB2 cyclin levels, budding index (numbers at bottom), and FACS analysis (not shown). PGK1 served as a loading control. **d**, SUMO modification sites of PCNA. NiNTA pull-down (as above) from cells expressing $^{His}$PCNA (WT), His-tagged PCNA mutants (*K127R* and *K164R*) as the sole source of PCNA, or $^{His}$SUMO. Proteins were identified by western blotting as indicated. SUMO is preferentially attached to K164 ($S^{164}$), whereas modification at K127 ($S^{127}$) is induced when K164 is absent (right lane). PCNA is not modified by SUMO when both lysines are absent (*K127R K164R*) or when a *ubc9-1* strain was used (bottom panel).

## Modification of PCNA by ubiquitin

We also noticed that treatment of cells with 0.02% MMS resulted in the induction of additional PCNA-specific bands, which were also present in *ubc9-1* mutants defective for SUMO conjugation (Fig. 2a–c; bands U1 and U2). From this finding and the sizes of the proteins we speculated that these species might be PCNA–ubiquitin conjugates. Indeed, as verified by pull-down experiments, we found that PCNA is modified by ubiquitination and that species carrying one, two, three and more ubiquitin moieties can be detected (Fig. 2b–d). Notably, mono- and multi-ubiquitination of PCNA does not seem to occur to detectable levels in untreated cells, or during S phase (Figs 1d and 2a–d), but only after treatment with a sublethal dose of DNA-damaging agents (0.02% MMS; Fig. 2a–d). Ubiquitination of PCNA is absent after treatment with 0.3% MMS for several hours, a condition that induces extensive SUMO conjugation at K164 (Fig. 2a, b). By using PCNA mutants that lacked specific lysine residues we noticed that ubiquitin is attached to residue K164 (Fig. 2b, c). Thus, ubiquitination and the principal type of SUMO conjugation target the same conserved lysine residue of PCNA, yet the two distinct modifications are regulated differentially.

## PCNA is a substrate of the *RAD6* pathway

In view of the results indicating that PCNA is ubiquitinated on DNA damage, we speculated that enzymes of the *RAD6* pathway are involved. Indeed, when we monitored PCNA by western blot analysis we noticed that the ubiquitin modifications of PCNA were completely absent in *rad6* deletion mutants, whereas SUMO modification of PCNA was not affected (Fig. 2c). The same specific defect was observed when we used a mutant that lacked RAD18, a DNA-binding protein that recruits RAD6 to chromatin[12]. Previously, we showed that RAD18 can associate with RAD5, which in turn recruits the heterodimeric UBC13–MMS2 ubiquitin-conjugating enzyme to chromatin, and we proposed that RAD6 and RAD18 might collaborate with UBC13–MMS2 and RAD5 in ubiquitination reactions[9]. In fact, ubiquitination of PCNA was affected equally in *ubc13*, *mms2* and *rad5* mutants (Fig. 2c). Only the multi-ubiquitinated PCNA species (two or more ubiquitin moieties) were absent in these mutants, whereas the mono-ubiquitinated PCNA species (band U1) was still present. As the UBC13–MMS2 complex catalyses multi-ubiquitin chains with K63 linkages[7], we also looked for PCNA ubiquitination in a strain[22] that expresses ubiquitin solely as a variant that lacks K63 (*ubiK63R*). Indeed, multi-ubiquitination of PCNA was also absent in this strain, whereas mono-ubiquitination still occurred (Fig. 2c, d). Together, these findings are consistent with a model in which DNA damage induces RAD6- and RAD18-dependent mono-ubiquitination of PCNA at K164, and that UBC13–MMS2 and RAD5 are required to attach additional ubiquitin moieties to the conjugate, thereby forming a K63-linked multi-ubiquitin chain (see Supplementary Information, Fig. 1).

## Physical interactions

PCNA is loaded onto DNA by a clamp loader and requires homo-trimerization. We noticed that a PCNA mutant protein pol30-52 (ref. 23) that is partially defective in trimerization was much less modified *in vivo* (data not shown). One possible interpretation of this finding is that the modifications take place after trimerization and that DNA-associated enzymes are involved. Indeed, when we tested all enzymatic components of the RAD6 ubiquitination system in yeast two-hybrid assays we discovered that only the DNA-binding proteins RAD18 and RAD5 interact with PCNA (Fig. 2e). Importantly, both proteins contain a RING finger, a hallmark of a class of ubiquitin ligases[24]. This observation, together with our finding that RAD18 and RAD5 mediate PCNA ubiquitination, indicates that the two proteins function as DNA-bound ubiquitin ligases for PCNA.

The SUMO-conjugating enzyme UBC9 usually binds substrates directly[16,25]. This is also apparently the case for PCNA, as two-hybrid assays revealed a UBC9–PCNA interaction (Fig. 2f). Of note, UBC9 additionally binds RAD18 and RAD5 (Fig. 2e), and we confirmed these interactions by co-immunoprecipitations (Fig. 2g). This indicates that UBC9 is affiliated with the *RAD6* pathway and that enzymes for ubiquitin and SUMO conjugation may communicate in a regulatory unit. Importantly, the observed physical association, together with our finding that ubiquitination and SUMO conjugation targets the same lysine residue, suggests that the distinct modifications label PCNA for specific functions.

## Functions

To investigate the significance of our finding that PCNA is a substrate of the *RAD6* DNA repair pathway, we determined PCNA mutant phenotypes. Yeast cells that express, from the original gene locus, PCNA variants lacking either the ubiquitination/SUMO-conjugation site K164 (*pol30-K164R*) or the SUMO-conjugation site K127 (*pol30-K127R*) exhibit no discernible growth defects, indicating that the modifications are not essential for replication (Fig. 3a). However, we observed that *pol30-K164R* cells were highly sensitive to ultraviolet light and MMS. This phenotype was specific for *pol30-K164R* cells, as all of the other 17 lysine mutants of PCNA were not hypersensitive (data not shown). Interestingly, when K164 and K127 were absent simultaneously (*pol30-K164R K127R*), cells were less sensitive than the *pol30-K164R* strain. Thus, we conclude that modification at K164 is crucial for DNA repair, whereas SUMO conjugation at K127 seems to inhibit the DNA repair process.

Notably, deletion mutants of *RAD6*, *RAD18* and *RAD5* are more sensitive to ultraviolet light than the *pol30-K164R* mutant (Fig. 3c; see also Supplementary Information, Fig. 2), indicating that the encoded proteins have additional substrates or functions. This is not surprising as RAD6 has several cellular roles[26] and RAD5 is related to SNF2/SWI2 helicases[27]. We found that *pol30-K164R* is epistatic to *mms2* (that is, *mms2* deletion does not confer additional sensitivity to *pol30-K164R*), indicating that K63-linked multi-ubiquitination of PCNA is firmly linked to *MMS2*-dependent DNA repair (Fig. 3c; see also Supplementary Information, Fig. 2). As *ubc13* and *mms2* mutants are specifically defective in error-free repair[6–9], this finding also demonstrates that K63-linked multi-ubiquitination of PCNA is elementary for this branch of *RAD6*-dependent repair. Moreover, the finding that an *mms2 pol30-K164R* double mutant is more

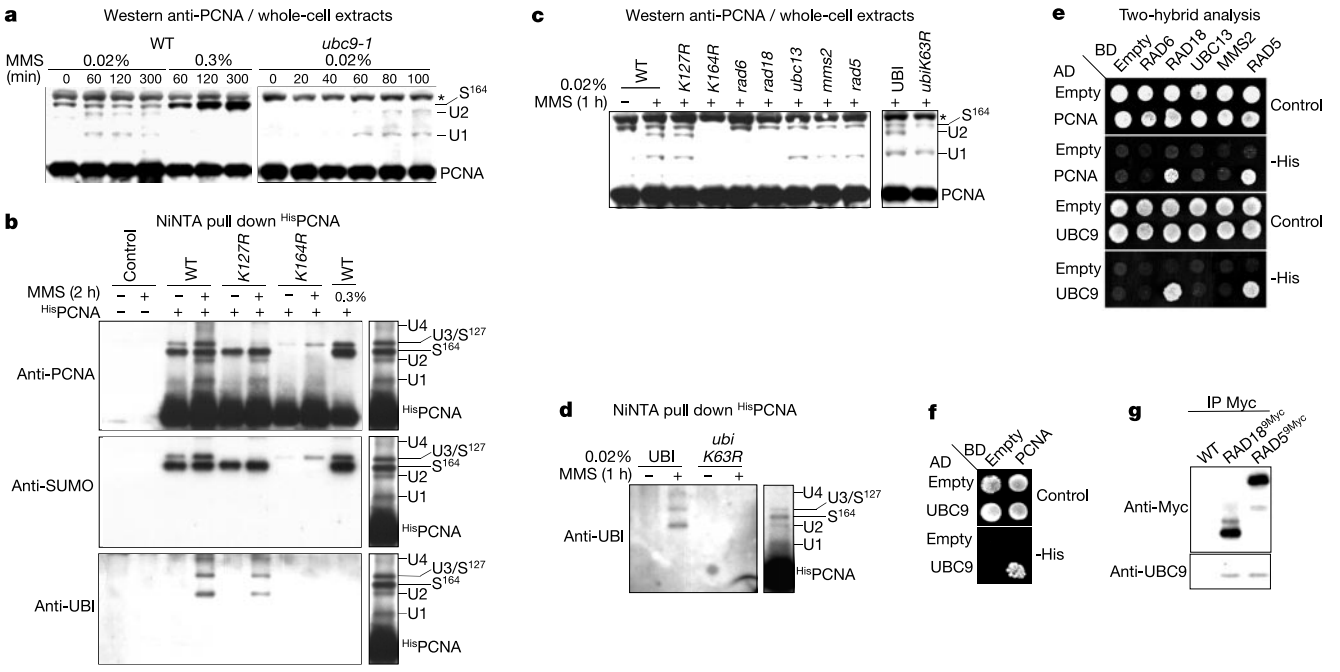


**Figure 2** Regulation of PCNA SUMO modification and ubiquitination by DNA damage and link to the *RAD6* pathway. **a**, PCNA modifications in WT and *ubc9-1* cells after treatment with MMS (0.02% and 0.3%) at 30 °C. Anti-PCNA western blots identify SUMO-modified (S) and ubiquitinated (U) species. Asterisk, nonspecific band co-migrating with forms U3 and $S^{127}$ (see below). **b**, Identification of SUMO-modified and ubiquitinated forms of PCNA after $^{HIS}$PCNA pull-down followed by western blotting. The mono-ubiquitinated form U1 is not recognized by the monoclonal antibody (but see **c** and Supplementary Information, Fig. 1). Strains and conditions are as in Fig. 1d; band assignment is given on the right (U1, mono-ubiquitinated; U2–4, multi-ubiquitinated; $S^{127}$ and $S^{164}$, SUMO-conjugated at K127 and K164, respectively). PCNA mutants (*K127R* and *K164R*) are used to show the lysines involved in conjugation. Forms U3 and $S^{127}$ migrate together in gels. Ubiquitination is specifically induced by MMS. **c**, PCNA ubiquitination depends on the *RAD6* pathway. Ubiquitination is absent in *rad6* and *rad18* mutants. Only mono-ubiquitinated PCNA (U1) is detected in *ubc13*, *mms2* and *rad5* (left panel), as well as in cells that express mutant ubiquitin (*ubiK63R*; right panel). SUMO modification is not affected by these *RAD6* pathway mutants. **d**, Multi-ubiquitin chains on PCNA are linked via K63 of ubiquitin. Pull-downs of $^{His}$PCNA from WT (UBI) and *ubiK63R* mutant cells followed by anti-ubiquitin western blotting. **e**, **f**, Two-hybrid interactions between UBC9, PCNA and proteins from the *RAD6* pathway. Fusion with either activating domain (AD) or DNA-binding domain (BD) of GAL4 are indicated. **g**, Co-immunoprecipitation of UBC9 (*Yiplac211::GAL-UBC9*) with chromosomally expressed Myc-tagged RAD18 ($RAD18^{9myc}$) or RAD5 ($RAD5^{9myc}$) detected by anti-UBC9 western blotting.

sensitive than the *mms2* single mutant suggests that mono-ubiquitination of PCNA at K164 (which can still occur in *mms2* single mutants) supports DNA damage tolerance, although less efficiently than multi-ubiquitination. We also investigated the effect of an *srs2* mutation on *pol30-K164R* mutant cells. *SRS2* encodes a DNA helicase, and loss of function of this enzyme partially suppresses the ultraviolet sensitivities of mutants defective in *RAD6*-dependent, error-free repair, by channelling repair to the *RAD52* pathway[28–31]. We found a detectable suppression of the sensitivity of *pol30-K164R* mutants by the *srs2* mutation (Fig. 3c), confirming that K63-linked multi-ubiquitination of PCNA is linked to the error-free branch of *RAD6*-dependent DNA repair.

Additional double mutant analysis showed that the *pol30-K164R* mutation strongly enhanced the ultraviolet sensitivities of *rad2* and *rad52* mutants (Fig. 3c), suggesting that the PCNA modifications are relevant neither for *RAD2*-dependent excision repair nor for *RAD52*-dependent double-strand break repair. Interestingly, the ultraviolet sensitivities of *rad6*, *rad18* and *rad5* mutants are partially suppressed by the *pol30-K164R* mutation. As wild-type PCNA, but not PCNA-K164R, can be modified by SUMO at K164 in *rad6*, *rad18* and *rad5* single mutants (Fig. 2c), this finding also points towards a DNA repair inhibitory effect of PCNA modification by SUMO at K164 (in addition to K127; see above) in cells deficient in these enzymes.

Importantly, overexpression of wild-type PCNA renders *mms2* and *rad5* mutants—as well as cells that express a ubiquitin variant that is unable to form K63-linked chains (*ubiK63R*)—less sensitive to MMS (Fig. 3d). We conclude from this finding that K63-linked UBC13–MMS2 and RAD5-dependent multi-ubiquitination of PCNA activates the role of PCNA in DNA repair, and that high levels of possibly mono-ubiquitinated PCNA can compensate for a defect in PCNA multi-ubiquitination. Notably, overexpression of *pol30-K164R* does not suppress the sensitivity of these cells but rather seems to be detrimental for repair in these mutant and wild-type cells, indicating that non-ubiquitinated PCNA does not significantly support DNA repair. Indeed, the MMS sensitivities of *rad6* and *rad18* mutants are only barely suppressed by overexpression of wild-type PCNA (which can still be SUMO-conjugated at K164) but are suppressed slightly better by mutant *pol30-K164R*. These findings confirm that ubiquitination of PCNA is elementary for post-replicative DNA repair and indicate that SUMO modification of PCNA at K164 not only prevents PCNA ubiquitination but also inhibits PCNA-dependent DNA repair.

## Human PCNA ubiquitination is linked to DNA damage

PCNA is a highly conserved protein with residues sharing 35% identity between yeast and human orthologues. Because of this similarity we investigated whether human PCNA is also modified.

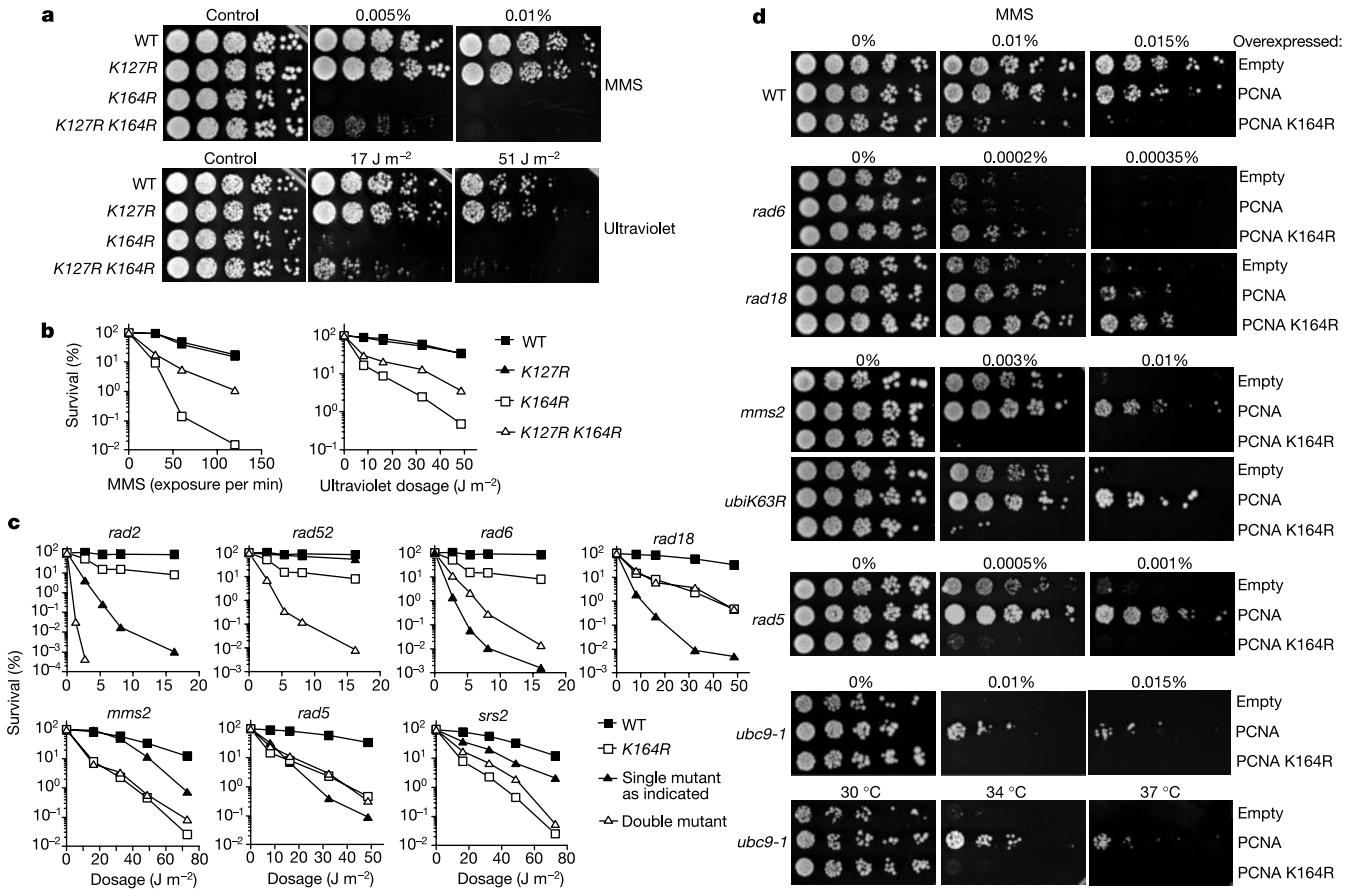


**Figure 3** Role of PCNA modifications in DNA repair. **a**, Sensitivity of cells chromosomally expressing WT and mutant PCNA to MMS and ultraviolet light. Fivefold serial dilutions of an equal number of stationary-phase cells were spotted onto plates. DNA damage was induced by either MMS or ultraviolet light. PCNA mutants lacking K127 or K164, or both lysines, were used. **b**, Quantification of survival rates (as above) determined by plating. Values are presented as the percentage of surviving cells after treatment. **c**, Epistatic analysis of the PCNA mutant lacking residue K164 (*pol30-K164R*) with mutants of DNA repair pathways. Quantification of survival rates after ultraviolet irradiation (as in **b**). Values are from two to four independent experiments with duplicated counts. The genotype to which *pol30-K164R* was crossed is indicated above the panels. **d**, PCNA overexpression from a high-copy plasmid (WT copy was retained in the genome) differentially suppresses MMS sensitivity of *RAD6* pathway mutants. Overexpression of WT PCNA and mutant PCNA (K164R) was compared to control (empty vector). Cells were spotted as above.

We could not detect SUMO modification or ubiquitination of human PCNA in HeLa cells under normal growth conditions. However, when we applied 0.02% MMS to the culture medium, we observed the appearance of an additional PCNA-specific band (Fig. 4a). This protein was verified by western analysis and pull-down experiments to be mono-ubiquitinated PCNA. Notably, the modification site K164 of the *S. cerevisiae* protein is conserved and found at identical positions in PCNA from yeasts, plants and higher eukaryotes, including humans (Fig. 4b). We replaced K164 of human PCNA by an arginine residue and expressed His-tagged forms of mutant and wild-type PCNA in HeLa cells by transfection. We observed that wild-type PCNA, but not mutant PCNA-K164R, was modified by ubiquitination in the presence of MMS. Thus, ubiquitination of PCNA at site K164 is conserved from yeast to mammals and is linked to the DNA damage response.

## Discussion

PCNA is positioned at the crossroads of several replication-linked pathways. It is involved in leading and lagging strand DNA synthesis, cell cycle arrest, replication-linked DNA silencing, mismatch repair, nucleotide- and base-excision repair, and post-replicative error-free and error-prone DNA repair[3,17,18,32]. PCNA directly associates with various DNA polymerases and functions as a sliding clamp, thereby stimulating accurate and processive DNA synthesis. In addition, PCNA seems to function as a platform for accessory factors of replication-linked functions. Previous studies have shown that PCNA is controlled by at least two distinct mechanisms. One important principle seems to be regulated loading/unloading of the trimeric PCNA ring on the DNA template by clamp loader (replication factor C). A second mechanism may involve PCNA inhibition through interaction with repressing molecules, the most prominent being the cell cycle checkpoint protein p21 (refs 17, 18).

We have shown that PCNA is also exquisitely modulated by covalent post-translational modifications involving ubiquitin and the ubiquitin-related protein SUMO. PCNA is a target for SUMO modification, mono-ubiquitination, and K63-linked multi-ubiquitination. We identified two target sites for modification: K164, a conserved modification site that is both modified by SUMO and ubiquitin, and K127, a yeast-specific site that seems to be exclusively modified by SUMO. Both residues are positioned distally from the encircled DNA on the outside rim of the trimeric PCNA ring[33] (Fig. 5). K164 is located in one of the protruding tips of PCNA, whereas K127 lies within a large loop that connects the two domains of a PCNA monomer. This connecting loop mediates interaction with polymerases (refs 34, 35), suggesting that SUMO conjugation at this site might interfere with polymerase binding.

SUMO modification of PCNA is activated during S phase and modifies predominantly the conserved residue K164 and to a lesser extent K127 of yeast PCNA. Importantly, our data indicate that SUMO conjugation inhibits the role of PCNA in repair and suggests a function in conjunction with normal DNA replication. Modification of PCNA by SUMO is also markedly induced by a lethal concentration of MMS (Fig. 2a), but whether this upregulation is linked to DNA repair or to polymerase stalling is currently unclear. We also noticed that modification of PCNA by SUMO is mediated by SIZ1, a SUMO ligase of the SIZ/PIAS family[36] (see Supplementary Information, Fig. 3). This finding demonstrates that modification of PCNA by SUMO does not support DNA repair because *siz1* mutants are not sensitive to DNA-damaging agents[36]. A possible clue with respect to the function of SUMO-modified PCNA comes from our finding that not only the MMS sensitivity of the *ubc9-1* mutant, but also its growth defect at high temperatures can be suppressed partially by overexpression of wild-type PCNA, but not the K164R mutant (Fig. 3d). This finding emphasizes the crucial importance of PCNA for the SUMO pathway even in the absence of DNA-damaging agents, again pointing to an important role of SUMO-modified PCNA in normal S phase.

Ubiquitination of PCNA is clearly linked to DNA damage and strictly depends on the *RAD6* pathway of DNA repair. It specifically targets the conserved K164 residue of yeast and human PCNA. Moreover, we show that PCNA ubiquitination is elementary for *RAD6*-dependent DNA repair. Previous data have indicated a complex orchestration of *RAD6*-dependent functions and revealed

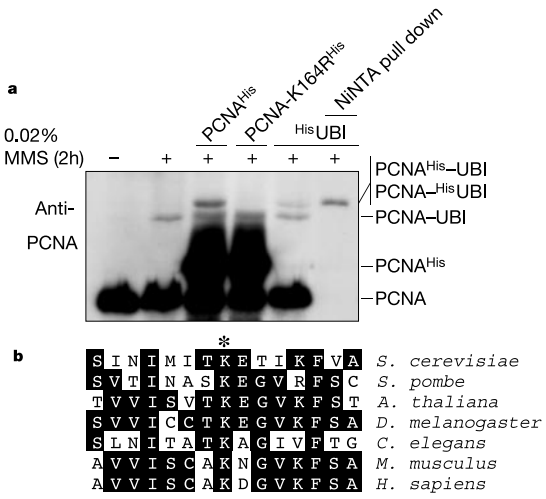


**Figure 4** Human PCNA is ubiquitinated at the conserved K164 residue on DNA damage. **a**, Western blot against PCNA from HeLa cells. Cells were either untransfected (two left lanes) or transfected with constructs expressing His-tagged versions of human WT PCNA (PCNA$^{His}$) or K164R mutant PCNA (PCNA-K164R$^{His}$). Cultures were treated with 0.02% MMS for 2 h as indicated. Anti-PCNA antibodies detect endogenous (PCNA) and introduced His-tagged PCNA (PCNA$^{His}$). For the last two lanes, a construct expressing His-tagged UBI was transfected, and samples were loaded on a gel before and after NiNTA pull-down. **b**, K164 is conserved in PCNA from yeast to humans. Shown is an alignment between residues 157 and 171 of yeast PCNA. K164 is marked by an asterisk.

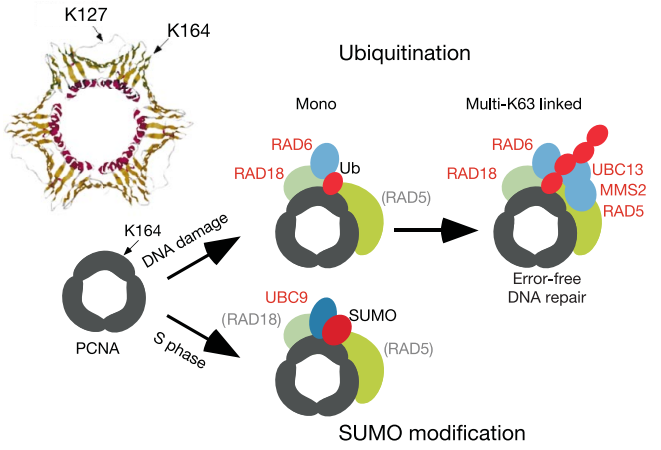


**Figure 5** Model for ubiquitination and SUMO modification of PCNA. The structure of yeast PCNA is derived from ref. 33 (top left). K127 and K164 modification sites are indicated. DNA damage induces mono-ubiquitination of PCNA at K164, which is catalysed by ubiquitin-activating enzyme UBA1 (not shown), RAD6 and RAD18. DNA damage induces nuclear import of UBC13 and MMS2, which are recruited to chromatin by RAD5. The enzyme assembly catalyses K63-linked multi-ubiquitination of PCNA. In the absence of DNA-damaging agents, PCNA is modified by SUMO during S phase, involving SUMO-activating enzymes UBA2/AOS1 (not shown), UBC9, and the SUMO ligase SIZ1 (not shown). UBC9 interacts directly with PCNA and, perhaps transiently, also with RAD18 and RAD5. Enzymes required for the modification reactions are labelled in red; associated proteins are grey and in parentheses. Ubiquitin- and SUMO-conjugating enzymes are in blue; ubiquitin ligases are in green.

distinct pathways for error-free and error-prone modes of repair[4]. These pathways may be subdivided into distinct error-free branches and an error-prone mode that uses the translesion polymerases REV1/Polζ (encoded by *REV3* and *REV7*). Another RAD6/RAD18-dependent translesion polymerase is Polη (encoded by *RAD30*)[8,37–39]. Our findings provide a new conceptual framework for the function of the *RAD6* pathway as it suggests that the stalled replication machinery may be switched to different modes of repair through distinct PCNA modifications.

We provide strong evidence that multi-ubiquitination of PCNA at K164 is pivotal for the error-free branch of *RAD6*-dependent DNA repair. We also show that two ubiquitin-conjugating enzymes, RAD6 and UBC13–MMS2, and two RING-finger ubiquitin ligases, RAD18 and RAD5, are involved in PCNA ubiquitination. Importantly, our findings suggest a mechanism by which these enzymes collaborate in a sequential manner (Fig. 5). Triggered by DNA damage, RAD6 appears to initially mono-ubiquitinate PCNA at residue K164. This reaction additionally requires RAD18, which recruits RAD6 to DNA-bound PCNA. DNA damage also induces nuclear translocation of UBC13 and MMS2, which results in an association of the heterodimeric ubiquitin-conjugating enzyme with chromatin-bound RAD5 (ref. 9). Moreover, through RAD5–RAD18 interaction, UBC13–MMS2 is brought into contact with RAD6. In a second enzymatic reaction, this assembly of two ubiquitin-conjugating enzymes and two ubiquitin ligases (together with ubiquitin-activating enzyme) seems to catalyse the conjugation of additional ubiquitin molecules onto the ubiquitin moiety of mono-ubiquitinated PCNA, thereby forming K63-linked multi-ubiquitin chains. These chains, unlike K48-linked chains, do not promote proteasomal degradation, and, indeed, we found no evidence for accelerated turnover of ubiquitinated PCNA (data not shown). In contrast, our data indicate that K63-linked multi-ubiquitination activates PCNA for being engaged in error-free repair. Post-replicative, error-free repair is thought to involve a transient template switch to the undamaged sister chromatid[3]. An attractive speculation is, therefore, that the ubiquitin chains on PCNA may stimulate interaction of the stalled replication machinery with proteins associated with the undamaged sister duplex. Interestingly, recent studies have indicated that K63-linked multi-ubiquitination involving UBC13–MMS2 can also activate certain protein kinases[40,41]. Moreover, K63-linked multi-ubiquitination has a function in ribosome activity[42] and endocytosis[43]. It will be important to identify the parallels between these roles in order to understand the mechanism through which K63-linked multi-ubiquitin chains function.

Mono-ubiquitination also seems to be relevant for DNA repair (Fig. 3c). Because error-free repair requires K63-linked multi-ubiquitination[7,9,44], mono-ubiquitination of PCNA is expected to mediate another, possibly error-prone, mode of repair. Perhaps mono-ubiquitinated PCNA binds specific repair factors, such as polymerases involved in translesion synthesis. Mono-ubiquitination of PCNA seems to be prevalent in human HeLa cells, yet multi-ubiquitinated forms can be detected at lower levels (G.-L.M. and S.J., unpublished data). Importantly, mammals have homologues of UBC9, SIZ1, RAD6, RAD18, UBC13, MMS2 and possibly RAD5. Given this conservation, it seems probable that the mechanisms and functions are highly conserved, and, indeed, genetic studies have confirmed that human RAD18 and MMS2 are crucial for DNA repair[45,46].

This study also revealed an affiliation of the SUMO-modification system with the ubiquitin-conjugating machinery. We have demonstrated that SUMO modification and ubiquitination of PCNA are physically and functionally linked. Importantly, ubiquitination and SUMO modification target the same K164 residue of PCNA, demonstrating that the two modifications are mutually exclusive. SUMO-conjugating enzyme UBC9 not only interacts with PCNA but also with the ubiquitin ligases RAD18 and RAD5; however, this interaction is not required for SUMO modification of PCNA (Fig. 2c), but it might be crucial for regulation. The most plausible model is that the ubiquitin/SUMO-conjugating enzymes are part of a regulatory switchboard, which directs PCNA for alternative functions. Several previous investigations have provided evidence for a link between SUMO modification and ubiquitination[15]. In cases where these two processes target the same lysine residue, studies suggest that SUMO might function as an antagonist for K48-linked multi-ubiquitination, thereby inhibiting proteasomal degradation of the substrate[47]. Our data suggest that SUMO modification can also antagonize the non-proteolytic role of ubiquitin. Importantly, SUMO modification of PCNA at K164 does not merely block ubiquitination at this site, but it seems to direct the substrate for other functions. As both SUMO modification and ubiquitination are reversible processes[14,15], a sequential cycle of SUMO modification/ubiquitination for substrates can be envisioned. It seems attractive to speculate that the ubiquitin/SUMO switch discovered here might be a prevalent regulatory mechanism. □

## Methods

### Yeast techniques and cloning

The strains that we used (grown at 30 °C, if not stated otherwise) are isogenic to strains described in ref. 9, and have the genotypes as described in the text and figures. Yeast His-tagged SUMO was expressed from the *ADH* promoter and the integrative vector *YIplac211*. His-tagged UBI was expressed from a 2μ plasmid with *CUP1* promoter by the addition of 100 μM $CuSO_4$. Yeast PCNA (*POL30*) was cloned into the 2μ-based plasmid *YEplac195* for overexpression studies or the *YCplac22* centromeric plasmid to create point mutations. Seven His codons were placed between the promoter and open reading frame (ORF) of *POL30* by a polymerase chain reaction (PCR)-based technique to create His-tagged PCNA in *YCplac22*. The shuffle strain *pol30::kanMX4 YCplac33* PCNA was used to create strains that express variants of PCNA or His-tagged PCNA as the only source of PCNA by selection on 5-fluoro-orotic acid. For Fig. 2d we expressed His-tagged PCNA in wild-type (UBI) or *ubiK63R* strain background. PCNA lysine mutants were integrated by homologous recombination into the *POL30* locus. Chromosomally tagged strains and mutants, if not already described[9,48], were constructed by a PCR-based strategy[49]. The *ubc9-1* mutant strain (Y0233) contains an additional *bar1* knockout. For two-hybrid analysis, complete ORFs of *POL30* and *UBC9* were cloned into pGAD vectors. Assay and other *RAD6* pathway clones were described previously[9]. Details of strains and plasmids will be provided on request.

### Protein techniques and antibodies

Protein conjugates with His-tagged SUMO from 6 litres of cells with an absorbance at 600 nm of 1 ($A_{600=1}$) of the *ulp1ts* strain[50] (grown at 23 °C) were purified by NiNTA chromatography under denaturing conditions, diluted 20-fold with buffer as described[20], and immunopurified by yeast SUMO (SMT3)-specific antibodies or nonspecific immunoglobulin-γ (IgG) antibodies crosslinked to magnetic protein A beads (Dynal). Bound proteins were eluted with 1% SDS. Coomassie-stained bands were excised and analysed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Protana). Four peptides of the 45K band matched yeast PCNA (34% coverage). An antibody against yeast PCNA was raised against bacterially expressed His-tagged PCNA from pET28c (Novagen) and affinity purified against PCNA–glutathione *S*-transferase (GST) expressed from pGEX4 (Amersham Pharmacia). Antibodies against human PCNA (PC10, Abcam), PGK1 (Molecular Probes), ubiquitin, Myc-epitope, UBC9 and CLB2 (all Santa Cruz) were used. Yeast protein extracts were prepared as described[49]. Analytical denaturing NiNTA chromatography/pull-down was done from 0.2 litre of logarithmic cells ($A_{600=1}$) as described[20], but at a small scale. For immunoprecipitation, yeast cells were grown to late logarithmic phase and lysed with glass beads in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and protease inhibitors. Cell extracts were incubated with detergent (0.2% Triton X-100, 0.1% SDS) and precleared by centrifugation. Proteins were precipitated with Myc-antibody and protein A Sepharose (Amersham Pharmacia). After extensive washing, proteins were eluted with sample buffer containing 8 M urea.

### Sensitivity and overexpression assays

For qualitative analysis of sensitivity to MMS or ultraviolet light, stationary-phase cells were either spotted on YPD plates containing MMS or irradiated with ultraviolet light. For quantification, fixed amounts of logarithmic cells were either plated onto YPD plates after incubation with 0.1% MMS for the indicated time, or irradiated with different dosages of ultraviolet light after plating. Colony-forming units were counted after 2–3 days of growth in the dark. Values are averages from two to four experiments. Overexpression analysis was done by spotting serial dilutions onto SC-URA plates containing indicated MMS concentrations.

### Cell cycle analysis

For cell-cycle synchronization, logarithmic cells grown at 23 °C were arrested in G1 by 10 μM α-factor for 2 h, washed, and resuspended in fresh medium. Aliquots were taken at 20-min intervals and analysed by western blotting. Additionally, 200 cells for each time

  

point were analysed by microscopic inspection for bud formation.

### Mammalian cell techniques

Vector for His-tagged PCNA was *pcDNA3.1*/GeneStorm from Invitrogen, and *pMT107* expressing His-tagged UBI was described previously[51]. Cells were transfected with Lipofectamine plus reagent (Invitrogen). NiNTA pull-downs were performed as described[52] but cells were boiled in lysis buffer (100 mM Tris-HCl, pH 7.5, 1% SDS) and lysates were diluted tenfold in PBS before pull-down.

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### Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to S.J. (e-mail: Jentsch@biochem.mpg.de).