Ubiquitin/SUMO modification of PCNA promotes replication fork progression in *Xenopus laevis* egg extracts

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The homotrimeric DNA replication protein proliferating cell nuclear antigen (PCNA) is regulated by both ubiquitylation and sumoylation. We study the appearance and the impact of these modifications on chromosomal replication in frog egg extracts. *Xenopus laevis* PCNA is modified on lysine 164 by sumoylation, monoubiquitylation, and diubiquitylation. Sumoylation and monoubiquitylation occur during the replication of undamaged DNA, whereas diubiquitylation occurs specifically in response to DNA damage. When lysine 164 modification is prevented, replication fork movement through undamaged DNA slows down and DNA polymerase δ fails to associate with replicating chromatin. When sumoylation alone is prevented, replication occurs normally and neither monoubiquitylation nor sumoylation are required for the replication of simple single-strand DNA templates. Our findings expand the repertoire of functions for PCNA ubiquitylation and sumoylation by elucidating a role for these modifications during the replication of undamaged DNA. Furthermore, they suggest that PCNA monoubiquitylation serves as a molecular gas pedal that controls the speed of replisome movement during S phase.

Introduction

One of the premier events of the cell cycle is the replication of DNA in preparation for cell division. Proliferating cell nuclear antigen (PCNA) was originally characterized as a protein that localized to the nucleus of proliferating cells (Miyachi et al., 1978). It has since been shown to be involved in a large number of DNA metabolic processes, such as transcription, replication, and repair (Shivji et al., 1992; Matsumoto et al., 1994; Waga et al., 1994; Chuang et al., 1997; Jonsson et al., 1998; Tom et al., 2000; Matsumoto, 2001; Hoege et al., 2002; Iida et al., 2002). PCNA is loaded onto replicating DNA by replication factor C and forms a homotrimeric ring that acts as a sliding clamp on DNA (Burgers, 1991; Krishna et al., 1994; Kelman and O'Donnell, 1995; Schurtenberger et al., 1998). Dozens of binding partners have been discovered for PCNA, and it is thought that PCNA serves to tether these proteins to DNA to enhance and localize their function (Kelman and Hurwitz, 1998; Maga and Hubscher, 2003). One example of PCNA enhancing the enzymatic function of a binding partner is the PCNA–DNA polymerase δ interaction. It has been shown that PCNA binds DNA polymerase δ and increases the processivity of the enzyme (Prelich et al., 1987). Because of the wide range of processes PCNA is involved in, it is clear that the binding of proteins to PCNA must be controlled in both a temporal and spatial way. One possible way to regulate the binding of proteins to PCNA is through differential modification of the PCNA trimer. Previous studies have demonstrated that PCNA can be acetylated, phosphorylated, ubiquitylated, and sumoylated (Prosperi et al., 1993, 1994; Hoege et al., 2002; Naryzhny and Lee, 2004).

In yeast, PCNA is monoubiquitylated and polyubiquitylated in response to DNA-damaging agents such as methane methylsulfonate (MMS) and UV radiation (Hoege et al., 2002). The ubiquitylation occurs on lysine 164 and is mediated by the Rad6p ubiquitin E2 in conjunction with the Rad18p single-strand DNA (ssDNA)–binding protein. Through genetic epistasis analysis, a model was proposed in which PCNA ubiquitylation was involved in lesion bypass during S phase to prevent replication forks from arresting at sites of DNA damage (Hoege et al., 2002; Stelter and Ulrich, 2003; Haracska et al., 2004). More specifically, monoubiquitylation was shown to be in the same genetic pathway as DNA polymerase η, a translesion polymerase, and polyubiquitylation was demonstrated to be epistatic to *UBC13*, which is involved in an alternative pathway of postreplication repair (Hoege et al., 2002; Stelter and Ulrich, 2003; Haracska et al., 2004). The polyubiquitylation of PCNA occurs via K63 linkage of ubiquitin monomers, which

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Abbreviations used in this paper: MMS, methane methylsulfonate; PCNA, proliferating cell nuclear antigen; rPCNA, recombinant PCNA; ssDNA, single-strand DNA; SUMO, small ubiquitin-related modifier.

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does not target the substrate for degradation as does the traditional K48-linked polyubiquitin chains.

PCNA is also monoubiquitylated in response to treatment with DNA-damaging agents in mammalian cells (Kannouche et al., 2004; Watanabe et al., 2004). PCNA ubiquitylation in human cells is dependent on the human homologue of *RAD18* and is required for the formation of DNA polymerase η subnuclear foci in response to DNA damage (Watanabe et al., 2004). Kannouche et al. (2004) demonstrated that polymerase η binds preferentially to monoubiquitylated PCNA. These data are consistent with a role for monoubiquitylation of PCNA in translesion synthesis in response to DNA damage. Only monoubiquitylation has been observed in higher eukaryotes (Kannouche et al., 2004; Watanabe et al., 2004).

The role of PCNA sumoylation, which until this study has only been reported in budding yeast, is less clear. Sumoylation of PCNA also occurs on lysine 164 and has been genetically linked to the suppression of *RAD52* function, suggesting that PCNA sumoylation may prevent unwanted and deleterious recombination during DNA replication (Haracska et al., 2004). This hypothesis was further strengthened by the observation that sumoylated PCNA recruits the Srs2p helicase to DNA, which acts to prevent recombination (Papouli et al., 2005; Pfander et al., 2005). There is no clear Srs2p homologue in higher eukaryotes, indicating that this function of sumoylated PCNA may not be conserved.

To gain further insights into the regulation of PCNA function via ubiquitylation and sumoylation in metazoans, we have characterized PCNA modification during DNA replication in *Xenopus laevis* egg extracts. We find that PCNA is both sumoylated and monoubiquitylated during normal S phase. After DNA damage, PCNA is further modified by diubiquitylation via a lysine 63 linkage on ubiquitin. The impact of elimination of PCNA modification on progression through S phase is also examined.

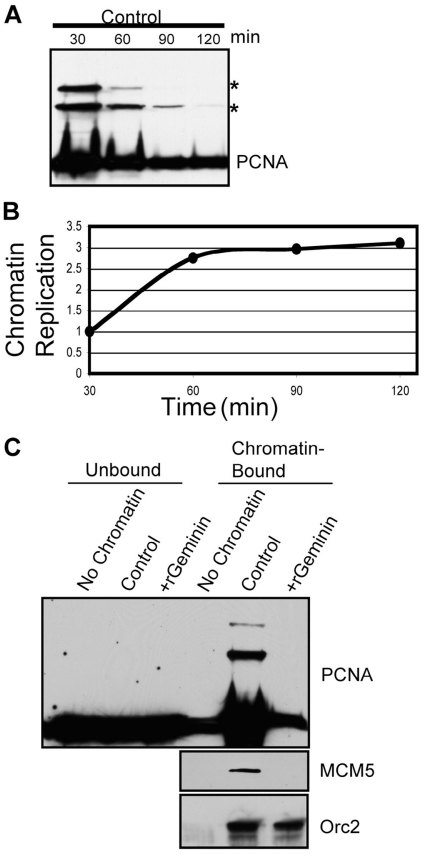

Figure 1. **PCNA undergoes secondary modifications during DNA replication.** (A) Undamaged sperm chromatin was isolated from *X. laevis* egg extract after 30, 60, 90, or 120 min. The associated proteins were analyzed by Western blotting using an antibody recognizing PCNA. (B) Duplicate samples from A contained ^{32}P-dATP and DNA replication was measured as described in Materials and methods. (C) Sperm chromatin was incubated in *X. laevis* egg extract for 30 min with or without 250 μM of recombinant GST-geminin (rGeminin). The chromatin was isolated and resuspended in SDS sample buffer. A sample was also collected from the supernatant fraction to analyze the unbound fraction.

Results

PCNA is monoubiquitylated and sumoylated during DNA replication

X. laevis egg extracts can be used to synchronously replicate sperm chromatin and have proven essential to increasing our understanding of the events that occur during DNA replication. We first wanted to determine if PCNA underwent secondary protein modifications during DNA synthesis in this cell-free system. We prepared a crude extract from *X. laevis* eggs, added sperm chromatin in the presence of an ATP-regeneration system, and incubated it at room temperature. After the indicated amounts of time, the chromatin was isolated from the extracts by sequential centrifugation through two sucrose cushions and resuspended in SDS sample buffer. The samples were separated by PAGE and subjected to Western blotting analysis with an antibody recognizing PCNA. Duplicate samples also contained ^{32}P-dATP. Samples were collected at the indicated times, and DNA replication was measured as described in Materials and methods. As seen in Fig. 1 A, two slower migrating bands are detected at 30 and 60 min, coincident with the majority of nucleotide incorporation (Fig. 1, A and B). To further investigate the dependence of these bands on DNA replication we repeated the chromatin spin down, but in the presence or absence of recombinant GST-tagged geminin. Geminin inhibits the function of the essential replication factor Cdt1 and thereby blocks replication fork assembly and subsequent DNA replication. We also examined the unbound fraction of these chromatin isolations to assess whether or not the modification of PCNA was limited to the chromatin-bound PCNA. Fig. 1 C clearly demonstrates that only chromatin-bound PCNA exhibits slower migrating versions of PCNA. Also, it is clear that inhibition of replication by the addition of GST-tagged geminin prevents the appearance of the slower migrating forms of PCNA.

To determine if these bands corresponded to either ubiquitylated or sumoylated PCNA, we combined freshly prepared crude extract with 0.5 μg/μL of recombinant histidine (His)-tagged ubiquitin, GST-tagged small ubiquitin-related modifier (SUMO) 1, or GST-tagged SUMO2. Sperm chromatin and an ATP-regeneration mix was added to the extract, and the reactions were incubated at room temperature for 40 min.

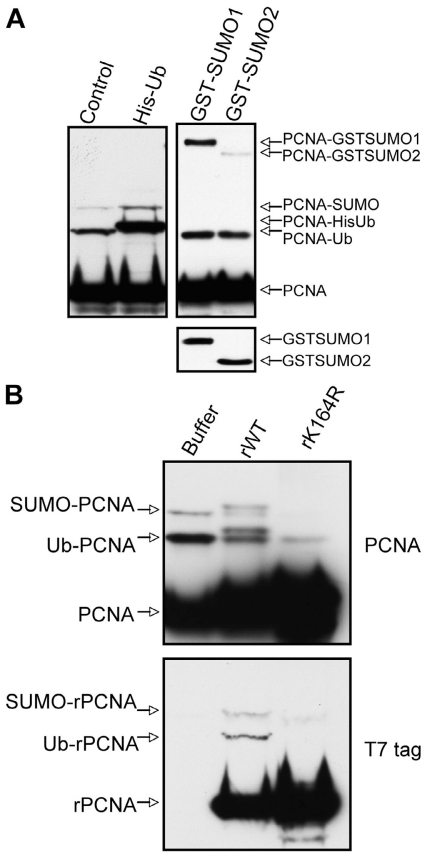

Figure 2. **PCNA is monoubiquitylated and sumoylated during DNA replication.** (A) *X. laevis* egg extract was incubated with sperm chromatin and either buffer, 0.5 μg/μL His-tagged ubiquitin, 0.5 μg/μL GST-SUMO1, or 0.5 μg/μL GST-SUMO2. Chromatin was isolated and analyzed by Western blotting with an antibody recognizing PCNA. The bottom blot shows a Western blot, using an antibody recognizing GST, of the sample before chromatin isolation. (B) *X. laevis* egg extract was incubated with sperm chromatin and either buffer, 200 ng/μL T7-PCNA (rWT), or 200 ng/μL T7-PCNA (rK164R). Chromatin was isolated and analyzed by immunoblotting with an antibody recognizing either PCNA or the T7 tag.

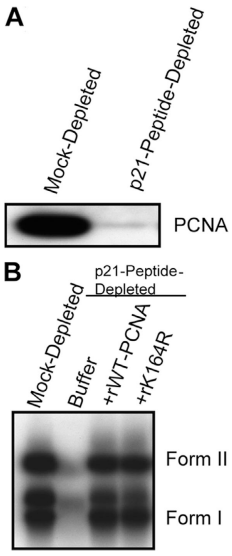

Figure 3. **PCNA modification is not required for M13 replication.** (A) p21-peptide beads were used to deplete PCNA from *X. laevis* egg extract as described in Materials and methods, and the resulting extracts were analyzed by SDS-PAGE and Western blotting using an antibody recognizing PCNA. (B) Either mock- or peptide-depleted extract was incubated with M13 DNA for 30 min. The peptide-depleted extract was supplemented with buffer, 0.2 μg/μL PCNA (wild type), or 0.2 μg/μL PCNA (K164R). The samples were then analyzed for DNA replication as described in Materials and methods.

The chromatin was then purified and the resulting samples analyzed, as in Fig. 1 A. If one of the slower migrating bands was the result of ubiquitin conjugation we would expect that band to undergo an additional shift of ~1 kD as a result of the His-tag on the recombinant ubiquitin. Likewise, if one of the bands was the result of SUMO conjugation we would expect that band to undergo an additional shift of 25 kD, corresponding to the GST tag on the recombinant SUMO. As seen in Fig. 2 A, when His-tagged ubiquitin (His-Ub) is added, the lower of the two bands undergoes an additional shift. When either GST-tagged SUMO1 (GST-SUMO1) or GST-tagged SUMO2 (GST-SUMO2) is added, the upper band undergoes an additional shift. Interestingly, despite adding equal amounts of GST-SUMO1 or GST-SUMO2 we observe that the conjugation of GST-SUMO1 is much more efficient. We conclude that PCNA undergoes both monoubiquitylation and sumoylation during normal DNA replication in *X. laevis* egg extracts.

We wanted to verify that these modifications were dependent on lysine 164, as is the case in yeast and mammalian cells (Hoege et al., 2002). We repeated the aforementioned chromatin spin down experiments, but added recombinant PCNA (rPCNA) with both a T7 and a 6-His tag to a concentration of 0.2 μg/μL. This recombinant protein was either wild type (rWT) or mutated to contain an arginine at position 164 instead of lysine (rK164R). After chromatin isolation, the samples were subjected to SDS-PAGE and Western blotting with either the PCNA antibody or an antibody against the T7 tag to specifically recognize the rPCNA. When these samples are blotted with an antibody recognizing PCNA (Fig. 2 B, top), both sumoylation and ubiquitylation of endogenous and exogenous PCNA can be seen. In the second lane, all of the bands exist as doublets because of the increased size of the recombinant T7-tagged PCNA protein. When a T7 antibody is used it becomes very clear that the mutation of lysine 164 results in the abolishment of PCNA modifications by either SUMO or ubiquitin (Fig. 2 B, bottom). We conclude that both monoubiquitylation and sumoylation occur on lysine 164 of *X. laevis* PCNA. This experiment also shows that PCNA modification is not required for loading PCNA onto chromatin because the mutant PCNA associates with chromatin to the same extent as wild type, if not slightly better.

PCNA modification is not required for ssDNA replication

Previously, it had been shown that PCNA can be removed from *X. laevis* egg extracts using a peptide derived from the p21 protein (Mattock et al., 2001). We used this peptide to deplete PCNA from extracts, as shown in Fig. 3 A. Unfortunately, this extract was unable to replicate sperm chromatin after the addition of recombinant untagged PCNA (not depicted). This is consistent with published data and may be the result of co-depletion of some other factor (Mattock et al., 2001). The PCNA-depleted extract is unable to replicate single-stranded M13 DNA, but the addition of rPCNA restores the activity of the extract, as shown previously. Interestingly, the addition of mutant PCNA (rK164R) also restores the activity of the extract, demonstrating that modification of PCNA is not required for the replication of simple ssDNA templates (Fig. 3 B).

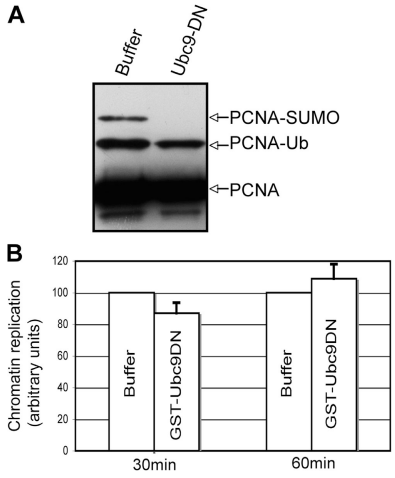

Figure 4. **Sumoylation of PCNA is not required for sperm chromatin replication.** (A) *X. laevis* egg extract was supplemented with either buffer or 5 μg/μL GST-Ubc9 dominant-negative protein (Ubc9-DN). Sperm chromatin was incubated in these extracts for 30 min and isolated. The resulting samples were subjected to SDS-PAGE and Western blotting using an antibody recognizing PCNA. (B) Duplicate samples containing ^{32}P-dATP were used to calculate the amount of DNA replication occurring in these samples after 30 and 60 min as described in Materials and methods. The average of three independent experiments is shown with error bars representing the SEM.

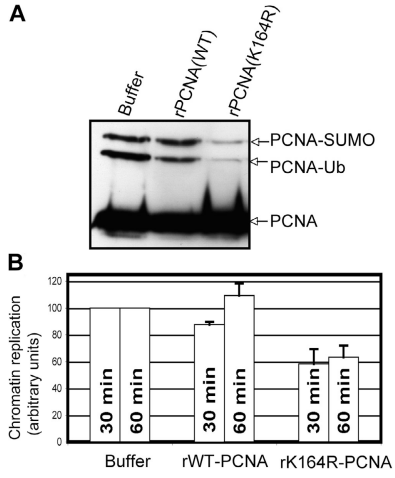

Figure 5. **Ubiquitylation of PCNA facilitates sperm chromatin replication.** (A) Sperm chromatin was incubated in *X. laevis* egg extract supplemented with buffer, 0.2 μg PCNA (wild type), or 0.2 μg PCNA (K164R) for 45 min. The chromatin was then isolated and analyzed by SDS-PAGE followed by Western blotting with an antibody recognizing PCNA. (B) Duplicate samples containing ^{32}P-dATP were used to measure DNA replication after 30 and 60 min as described in Materials and methods. The average of three independent experiments is shown with error bars representing the SEM.

PCNA sumoylation is not required for chromatin replication

We hypothesized that the sumoylation of PCNA during S phase might play a role in chromatin replication. To test this we used a dominant-negative version of Ubc9 (Ubc9-DN), the only known E2 enzyme involved in the conjugation of SUMO to substrate proteins. As shown in Fig. 4 A, the addition of recombinant Ubc9-DN results in the disappearance of SUMO-modified PCNA. We then tested the effect of this dominant-negative protein on DNA replication. We combined *X. laevis* crude extract with 0.8 μg/μL of either buffer or Ubc9-DN. After a short incubation on ice, an ATP regeneration system, ^{32}P-dATP, and sperm chromatin were added. Samples were collected after 30 and 60 min, and DNA replication was measured as described in Materials and methods. Fig. 4 B clearly shows that, despite the ability of Ubc9-DN to prevent sumoylation of PCNA, it has no effect on DNA replication. The Ubc9-DN will inhibit the sumoylation of all proteins in the extract, but because there is no impact on replication we can conclude that sumoylation is not required for DNA replication and, more specifically, that PCNA sumoylation is not required. These data are consistent with a previous paper showing that Ubc9-DN does not affect replication in *X. laevis* egg extracts (Azuma et al., 2003).

PCNA lysine 164 modification is required for proper replication fork progression

Despite repeated attempts using a variety of strategies, we could not selectively eliminate PCNA monoubiquitylation while leaving sumoylation intact. However, we have shown that sumoylation is not important for DNA replication (Fig. 4). To get at a possible function for monoubiquitylation in replication, we determined the effect of eliminating lysine 164 modification on chromosomal replication. To do this we added either untagged wild-type or mutant (K164R) rPCNA protein to extracts and analyzed the effect on both replication and PCNA modification in the extract. As shown in Fig. 5 A, addition of the mutant PCNA results in a decrease in the amount of modified PCNA bound to chromatin. This is presumably attributable to the mutant recombinant protein outcompeting the endogenous PCNA for binding sites on chromatin. In Fig. 5 B, it is clear that the addition of this mutant PCNA inhibits replication at both the 30 and 60 min time points. We conclude that modification of PCNA lysine 164 is important for efficient replication. This is likely the result of the loss of monoubiquitylation of PCNA because the Ubc9-DN protein had no inhibitory effect on replication, but did eliminate sumoylation of lysine 164. It is also possible that PCNA monoubiquitylation and sumoylation act redundantly during replication, as our experiment does not rule this out.

To investigate what step in replication was being affected by the loss of lysine 164 modifications, we used alkaline agarose gels to determine the length of the nascent strands of DNA when either wild-type or mutant PCNA was added to the extract. Replication assays were performed under normal conditions and samples were then treated with a high pH buffer to separate the strands. Samples were run on a gel under basic conditions and dried, and signal was measured with a phosphoimager (Fig. 6 A). Using National Institutes of Health Image software, a line was placed in the center of each lane and the pixel intensity at each point along the line was measured. The pixel intensity at a point is an indication of how many molecules of that length were generated during replication. By comparing the gel to a DNA ladder we were able to plot pixel intensity versus DNA fragment size (Fig. 6 B). As shown in Fig. 6 (A and B), the addition of rPCNA (K164R) to the extracts reduces the length of nascent DNA strands relative to rPCNA wild type. One possible explanation of this result is that in the presence

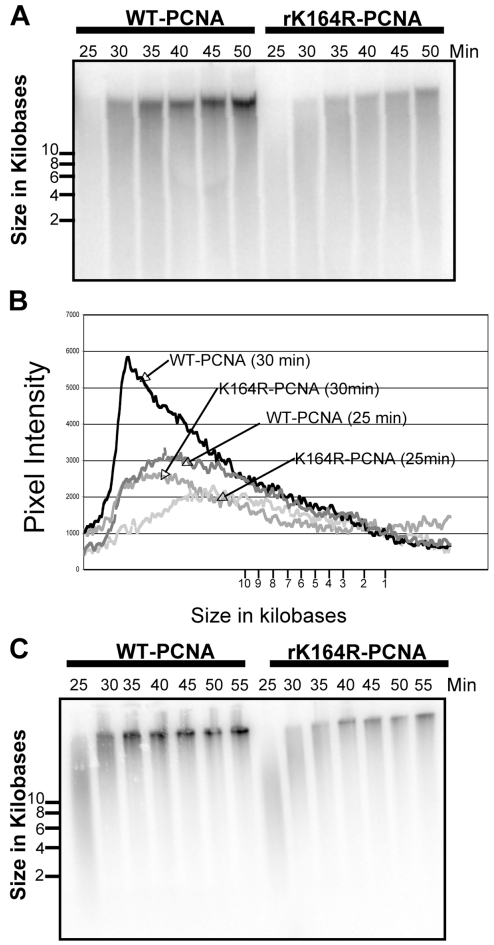

Figure 6. **Loss of PCNA ubiquitylation slows replication fork progression.** (A) Sperm chromatin was incubated in *X. laevis* egg extract containing either 0.2 μg/μL PCNA (wild type) or 0.2 μg/μL PCNA (K164R). Aliquots were removed after 25, 30, 35, 40, 45, and 50 min and prepared for alkaline agarose gel analysis as described in Materials and methods. (B) Fragment lengths were calculated by comparison to a DNA ladder and plotted against pixel intensity obtained with National Institutes of Health Image software. (C) Sperm chromatin was incubated in *X. laevis* egg extract containing either 0.2 μg/μL PCNA (wild type) or 0.2 μg/μL PCNA (K164R). Cold dATP was added after 25 min. Aliquots were removed after 25, 30, 35, 40, 45, and 50 min and prepared for alkaline agarose gel analysis as described in Materials and methods.

of rPCNA (K164R) some replication forks are abandoned, whereas others progress normally. To test this hypothesis we repeated this experiment but added 1 mM of cold dATP after 25 min, which allowed us to watch the progression of only those replication forks that had already initiated DNA synthesis. As seen in Fig. 6 C, it is clear that high molecular mass strands are formed at a slower rate in the presence of K164R, indicating that K164R PCNA does not induce a significant amount of irreversible fork abandonment. We conclude that PCNA modification is required for replication elongation to occur with maximal efficiency on undamaged chromosomes. The fact that modification of lysine 164 is not required for replication of ssDNA, but facilitates chromatin replication, suggests that monoubiquitylation of PCNA may play a role in unwinding the DNA or in relaxing the chromatin structure to allow for proper replication fork progression.

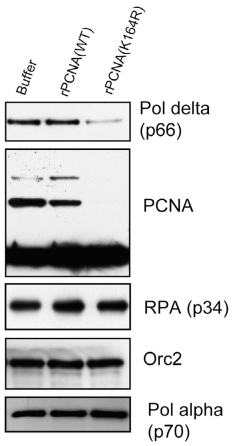

Figure 7. **Loss of PCNA modification interferes with chromatin binding of DNA polymerase δ.** Sperm chromatin was incubated in *X. laevis* egg extract containing either buffer, 0.2 μg/μL PCNA (wild type), or 0.2 μg/μL PCNA (K164R). After 40 min the chromatin was isolated and resuspended in SDS sample buffer. The samples were then analyzed by SDS-PAGE and Western blotting with antibodies recognizing the indicated proteins.

PCNA modification impacts polymerase δ binding to chromatin

In an attempt to determine the cause of the slow fork progression resulting from a knockdown of PCNA lysine 164 modification in the extracts, we examined the binding of polymerase δ to chromatin under these conditions. As can be seen in Fig. 7, the addition of mutant PCNA to the extract resulted in the elimination of lysine 164 modifications, as expected. In extracts containing this mutant, we observed a significant decrease in polymerase δ bound to chromatin when compared with the addition of wild-type PCNA. In contrast, we did not detect any effect of the mutant PCNA on the loading of the prereplication complex component Orc2, the ssDNA-binding protein replication protein A, or DNA polymerase α (p70 subunit) to chromatin. We conclude that lysine 164 modification of PCNA is required for both efficient chromosomal replication and for stable association of polymerase δ with replicating chromatin.

PCNA is polyubiquitylated after DNA damage

The results presented thus far indicate that PCNA is monoubiquitylated and sumoylated during a normal, uninterrupted S phase and that monoubiquitylation is required for efficient replication fork progression. Thus, these findings represent a departure from studies in yeast and human cells where monoubiquitylation is only readily observed after DNA damage (Introduction). Therefore, it was important to determine the status of PCNA in our system after DNA damage. For this we used sperm chromatin that had been damaged by exposure to UV light. This chromatin was incubated in egg extract and isolated, and PCNA was examined by immunoblotting. The inclusion of damaged chromatin resulted in a third slowly migrating band on the PCNA blot, suggesting that PCNA had undergone an additional modification in response to the damaged DNA, and all three slower migrating bands persisted throughout the entire time course of the experiment (Fig. 8 A). This persistence is consistent with the observation that damaged chromatin requires more time to replicate and that, even at 120 min, nucleotide was still being incorporated (Fig. 8 B). To determine if this banding pattern was unique to UV-damaged chromatin, we repeated the chromatin spin down using UV-damaged

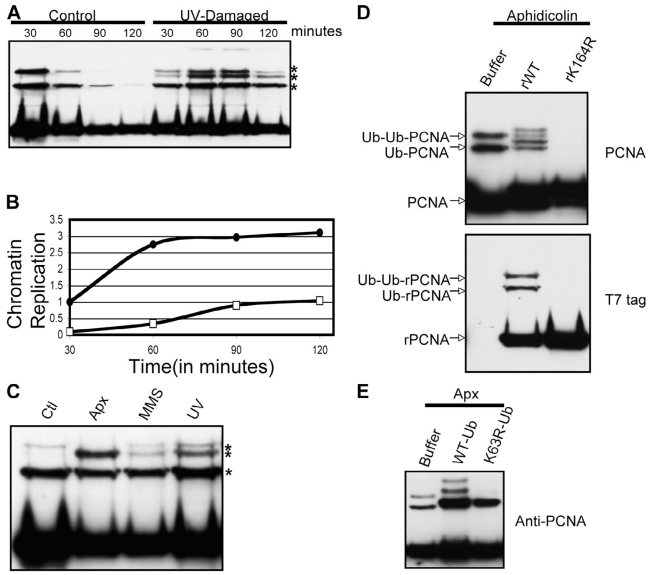

Figure 8. **DNA damage induces polyubiquitylation of PCNA.** (A) UV-damaged sperm chromatin was isolated from *X. laevis* egg extract after 30, 60, 90, or 120 min. The associated proteins were analyzed by Western blotting using an antibody recognizing PCNA. The control portions of this experiment are reproduced from Fig. 1 A to facilitate comparison. (B) Duplicate samples from A contained ^{32}P-dATP and DNA replication was measured as described in Materials and methods. Closed circles represent undamaged chromatin and open squares represent UV-damaged chromatin. (C) Undamaged chromatin, MMS-damaged chromatin, or UV-damaged chromatin was isolated from *X. laevis* egg extract after 45 min. Aphidicolin was added to 100 μg/ml at t = −10 min to the extract incubated with undamaged chromatin. The associated proteins were analyzed by Western blotting using an antibody recognizing PCNA. (D) *X. laevis* egg extract, preincubated with 100 μg/ml aphidicolin, was incubated with sperm chromatin and either buffer, 0.2 μg/μL T7-PCNA (rWT), or 0.2 μg/μL T7-PCNA (rK164R). Chromatin was isolated and analyzed by immunoblotting with an antibody recognizing either PCNA or the T7 tag. (E) *X. laevis* egg extract was preincubated with 100 μg/ml aphidicolin. Undamaged sperm chromatin was incubated in this extract which also contained buffer, 0.5 μg/μL His-tagged ubiquitin, or 0.5 μg/μL His-tagged ubiquitin (K63R). Chromatin was isolated and the bound proteins were analyzed by Western blotting with an antibody recognizing PCNA.

chromatin or MMS-damaged chromatin. We also tested undamaged chromatin in the presence of 100 μg/ml aphidicolin. As seen in Fig. 8 C, MMS-damaged chromatin exhibits the same banding pattern as UV-damaged chromatin. Aphidicolin treatment leads to an increase in the middle band, and there is no detectable PCNA sumoylation. To determine if the broad band induced by aphidicolin treatment merely masked sumoylated PCNA, we repeated the experiment in the presence of GST-SUMO1, which causes a large shift in sumoylated PCNA, but no band appeared, indicating that aphidicolin treatment prevents sumoylation of PCNA (not depicted). Importantly, neither UV, MMS, or aphidicolin treatment resulted in a significant increase in the appearance of the monoubiquitylated form of PCNA.

To determine if this new modification was dependent on lysine 164 of PCNA, we repeated the experiment shown in Fig. 2 B, except that all of the extracts contained 100 μg/ml aphidicolin. As can be seen in Fig. 8 D, the damage- and aphidicolin-induced modification of PCNA, is dependent on lysine 164, suggesting that it may be the result of polyubiquitylation. To determine if the band induced by damaged chromatin or aphidicolin treatment was diubiquitylated PCNA we added undamaged chromatin to an extract containing 100 μg/ml aphidicolin, isolated the chromatin, and analyzed the samples as described for Fig. 2 B. These extracts contained buffer, 0.5 μg/μL of wild-type His-tagged ubiquitin, or 0.5 μg/μL His-tagged ubiquitin containing a lysine to arginine mutation at position 63. Fig. 8 E, clearly shows that the lower band is a monoubiquitylated form of PCNA because nonchain-forming mutants of ubiquitin (K63R) behaved identically to recombinant wild-type ubiquitin. We can conclude that the damage- and aphidicolin-induced modification of PCNA is a result of diubiquitylation of PCNA because the K63R ubiquitin mutant causes a shift in the lower band (because of the His tag) and prevents the induction of the second band. Furthermore, this diubiquitylation appears to represent the "damage mark" on *X. laevis* PCNA, as neither sumoylation nor monoubiquitylation is noticeably increased after DNA damage. Based on these data, we conclude that *X. laevis* PCNA is sumoylated and monoubiquitylated in response to normal DNA replication and that it is diubiquitylated when replication forks stall at sites of DNA damage.

Discussion

PCNA is monoubiquitylated and sumoylated on lysine 164 during normal S phase

In this study, we examined modification of PCNA by monoubiquitylation and sumoylation. Both of these modifications occur exclusively on the highly conserved lysine 164 residue. We found that sumoylation is mediated by Ubc9, but we do not yet know which ubiquitin pathway components are responsible for monoubiquitylation. In yeast, the Rad6p E2 ubiquitylates PCNA. We tested the effect of the addition of a putative dominant-negative *X. laevis* Rad6 protein on PCNA monoubiquitylation and found that monoubiquitylation was not attenuated by the presence of this mutant (unpublished data). Thus, more work will be required to identify the factors that are required for PCNA ubiquitylation. The conditions for modification of PCNA were also determined. We found that both monoubiquitylation and sumoylation require replication fork assembly, as these modifications are lost when the replication inhibitor geminin is included in the extract and the modifications are found exclusively in the chromatin-bound fraction of PCNA (Fig. 1 C). Thus, PCNA is modified on lysine 164 as a function of being loaded onto the replication fork.

In this paper, we showed for the first time that PCNA is sumoylated during DNA replication in metazoans. As is the case in yeast, sumoylation of PCNA is not required for DNA replication in *X. laevis*. Recent work in yeast has yielded a model in which sumoylated PCNA prevents recombination by binding the Srs2 protein, a member of the RecQ family of helicases (Haracska et al., 2004; Papouli et al., 2005; Pfander et al., 2005). Although metazoan genomes contain multiple members of the RecQ helicase family it is unclear which of these is the functional homologue of Srs2p, and our preliminary findings indicate that loss of PCNA sumoylation does not affect association of the Werner's or Bloom's RecQ family helicases with

chromatin in egg extracts (unpublished data). We were unable to detect PCNA sumoylation in either mammalian or *X. laevis* tissue culture cells, which raises the possibility that this modification is specific for embryonic cell cycles in metazoans.

Monoubiquitylation and DNA replication

The most important finding presented in this study is that monoubiquitylation of PCNA is required for proper fork progression and the abolishment of the ubiquitylation of PCNA disturbs polymerase δ association with chromatin. More experiments are required to determine whether the decreased polymerase δ binding is a result of or a cause of the slowed fork progression. It is interesting to note that PCNA modification has already been shown to alter the ability of PCNA to bind DNA polymerases. For example, DNA polymerase η prefers to interact with monoubiquitylated PCNA (Kannouche et al., 2004). One possible explanation of our data is that DNA polymerase δ prefers to bind to monoubiquitylated PCNA in *X. laevis* egg extracts. Because the modification of PCNA is not required for ssDNA synthesis it is reasonable to hypothesize that PCNA ubiquitylation may act in assisting the unwinding of the DNA strands or in the restructuring of local chromatin structure. Consistent with this, we note that replication protein A does not accumulate on chromatin in extracts containing the PCNA K164R mutant as compared with wild type (Fig. 7). This indicates that excess ssDNA is not being generated by the mutant, despite its ability to attenuate DNA replication, and we have observed that the replication slowdown caused by the mutant does not activate a replication checkpoint response (unpublished data). Together these observations indicate that PCNA ubiquitylation can control the rate of fork progression in a manner that couples DNA synthesis to DNA unwinding. The challenge for future studies will be to determine how this coupling occurs.

DNA damage-induced diubiquitylation of PCNA and the interspecies plasticity of PCNA modification during chromosome metabolism

Our data demonstrate that *X. laevis* PCNA is diubiquitylated when replication forks stall. At present, we do not know the function of this modification, and we are currently investigating this important issue. Interestingly, we find that diubiquitylation is the only modification that is affected by DNA damage, as damage does not noticeably alter either sumoylation or monoubiquitylation. Therefore, this finding strengthens the conclusion that in *X. laevis* the sumoylation and monoubuiqitylation of PCNA occurs during normal DNA replication and not in response to low levels of damage that may be present in our sperm chromatin preparations. The finding that DNA damage induces only diubiquitylation of PCNA in *X. laevis* demonstrates a surprisingly high degree of plasticity from species to species in PCNA modifications. For example, in yeast, sumoylation is observed during a normal S phase, whereas both mono- and polyubiquitylation occur after DNA damage. In human cells, neither sumoylation nor polyubiquitylation have been observed under any conditions, and monoubiquitylation is strongly induced by DNA damage. And, as we have shown, in *X. laevis* both sumoylation and monoubiquitylation occur during normal S phase, whereas diubiquitylation is reserved for the DNA damage response. Although some of these differences may be attributable to the different methodologies used to track PCNA modifications, others are likely to reflect specialized functions for the modifications. A major challenge for future studies will be to determine these functions so that this apparent plasticity can be understood.

Materials and methods

Preparation of *X. laevis* egg extracts

Preparation of *X. laevis* egg extracts and preparation of sperm chromatin were performed according to Walter and Newport (1999). To generate damaged chromatin, purified chromatin was exposed to 100 mM MMS or 100 μJoules/m^2 of UV light.

Expression and purification of recombinant proteins

The *X. laevis* complementary DNA encoding PCNA was cloned into both the pET28 and pET3 vectors (Novagen). The *X. laevis* complementary DNA for Ubc9 was cloned into pGEX4T1 (GE Healthcare). For purification of His_6-tagged PCNA, BL21 cells transformed with the appropriate plasmid were induced with 1 mM IPTG for 6 h. The cells were collected by centrifugation at 4,100 rpm for 20 min at 4°C. Pellets were washed twice with 50 mL PBS, and the resulting pellets were resuspended in 15 mL TEN Buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 0.3 M NaCl) and 10 mL PBS. Lysozyme was added to a final concentration of 1 mg/ml, and the suspension was incubated on ice for 15 min. NP-40 was added to a final concentration of 0.2% (vol/vol) and the suspension was incubated for an additional 10 min on ice. The sample was then frozen in liquid nitrogen and stored at −80°C for at least 1 h. After the pellet was thawed, the sample was sonicated three times at 50% power for 30 s each using 1-s pulses. The extract was then centrifuged at 15,000 *g* for 20 min, and the supernatant was transferred to a 50-ml conical tube containing 2 ml of either Ni-NTA beads (QIAGEN) or glutathione beads (GE Healthcare) depending on the protein tag. The suspension was rotated at 4°C for 1 h and then poured into a column. The beads were washed extensively with either Nickel washing solution (20 mM Imidazole, pH 7, 20 mM KP_i, pH 7, and 0.5 M NaCl) or PBS. His-tagged proteins were eluted with 5 ml of Nickel elution solution (0.5 M Imidazole, pH 7, 20 mM KPi, pH 7, and 0.5 M NaCl) and 0.5-mL fractions were collected. GST-tagged proteins were eluted in a similar manner using GST elution solution (10 mM of reduced glutathione and 50 mM Tris-HCl, pH 7.5). Fractions containing protein were pooled and dialyzed with 50 mM MOPS-NaOH, pH 7.5, for 16 h and flash frozen in aliquots.

Purification of untagged PCNA pET3-PCNA was performed as previously described with the phosphocellulose column replaced by a phenyl–Sepharose column (Hubscher et al., 1999).

Chromatin spin downs

50 μL of fresh extract was incubated with sperm chromatin at 2,000 sperm/μL for 40 min, unless otherwise stated. These reactions were mixed every 10 min. 200 μL ELB (0.25 M sucrose, 1 mM DTT, 2.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Hepes-KOH, pH 7.7) was added and the resulting mixture was layered onto a 1-mL sucrose cushion (0.9 M sucrose in 1× ELB salts (2.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Hepes-KOH, pH 7.7). The samples were centrifuged at 11,000 rpm for 2 min at 4°C. All but 100 μL of the sample was aspirated, and an additional 50 μL was removed using a P200 gel-loading tip (VWR). The pellet was then resuspended in 200 μl ELB containing 0.6% Triton X-100, layered on top of another 1-mL sucrose cushion, and centrifuged as before. After aspirating all but 100 μL, an additional 90 μL was removed with a P200 gel-loading tip. The resulting pellet was resuspended in 50 μL SDS sample buffer. The samples were boiled before analysis by Western blotting.

DNA replication

To measure DNA replication, 50 μl of fresh extract was preincubated on ice for 10 min with the treatments indicated in the figure legends. Sperm chromatin or M13 plasmid was then added to the extract to a final concentration of 2,000 sperm/μL or 2 ng/μL, respectively. 5 μCi ^{32}P-dATP was added to the extract and the reactions were incubated at room temperature. At the specified times, 3 μl of the reaction was removed and

combined with 7 μL of stop mix (6 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS, 0.2% Bromphenol blue, and 80 mM Tris, pH 8). Proteinase K was then added to a final concentration of 2 μg/μL and the samples were incubated for 1 h at 37°C. Samples were then electrophoresed on a 0.8% (wt/vol) agarose/Tris-Borate EDTA gel, after which the gel was dried and analyzed by a phosphoimager with ImageQuant software (GE Healthcare).

PCNA depletion

A peptide from p21 (CKRRQTSMTDFYHHSKRRAIAS) was obtained from Invitrogen. The peptide was conjugated to Amino-link beads (Pierce Chemical Co.) following the manufacturer's instructions and at a concentration of 2 mg of peptide per milliliter of beads. For extract depletion, 214 μL of crude *X. laevis* egg extract was added to 100 μl of peptide beads or control beads and incubated for 1 h on ice. The reaction was mixed every 10 min, alternating resuspension with stirring. The mixture was centrifuged at 10,000 rpm at 4°C for 2 min and the supernatant was transferred to a new Eppendorf tube containing 100 μL of peptide beads or control beads. The 1-h incubation was repeated and the resulting supernatant was used to measure replication as described in DNA replication.

Alkaline agarose gels

Replication assays were performed exactly as described above in DNA replication, except that 5 μL of extract was removed and added to 500 μL of ice-cold buffer A (5 mm EDTA, 20 mM Hepes-KOH, pH 7.6, and 50 mM NaCl) containing 0.5 mM spermine and 0.5 mM spermidine. Samples were stored on ice until all time points had been collected. Samples were then centrifuged for 5 min at 14,000 rpm at 4°C. The supernatant was removed and the pellets were resuspended in 50 μL buffer A containing 0.5% SDS and 0.5 mg/ml proteinase K. Samples were incubated at 37°C for 1 h and phenol/chloroform extracted. The aqueous layer was subjected to ethanol precipitation using 40 μg of carrier RNA, and the resulting DNA pellet was resuspended in 20 μL of alkaline loading buffer (300 mM NaOH, 6 mM EDTA, 18% Ficoll, 0.15% Bromcresol blue, and 0.25% xylene cyanol FF). Samples were run on a 0.8% (wt/vol) agarose gel/50 mM NaCl/1 mM EDTA. The gel was equilibrated in alkaline running buffer (1 mM EDTA and 30 mM NaOH) for 1 h before the loading of the samples. The gel was run for 5 h at 3 V/cm. The gel was then dried and analyzed as described for replication assays.

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References

Azuma, Y., A. Arnaoutov, and M. Dasso. 2003. SUMO-2/3 regulates topoisomerase II in mitosis. *J. Cell Biol.* 163:477–487.

Burgers, P.M. 1991. *Saccharomyces cerevisiae* replication factor C. II. Formation and activity of complexes with the proliferating cell nuclear antigen and with DNA polymerases delta and epsilon. *J. Biol. Chem.* 266:22698–22706.

Chuang, L.S., H.I. Ian, T.W. Koh, H.H. Ng, G. Xu, and B.F. Li. 1997. Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science.* 277:1996–2000.

Haracska, L., C.A. Torres-Ramos, R.E. Johnson, S. Prakash, and L. Prakash. 2004. Opposing effects of ubiquitin conjugation and SUMO modification of PCNA on replicational bypass of DNA lesions in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 24:4267–4274.

Hoege, C., B. Pfander, G.L. Moldovan, G. Pyrowolakis, and S. Jentsch. 2002. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature.* 419:135–141.

Hubscher, U., R. Mossi, E. Ferrari, M. Stucki, and Z.O. Jonsson. 1999. Functional analysis of DNA replication accessory proteins. *In* Eukaryotic DNA Replication: A Practical Approach. Sue Cotterill, editor. Oxford University Press. 119–137.

Iida, T., I. Suetake, S. Tajima, H. Morioka, S. Ohta, C. Obuse, and T. Tsurimoto. 2002. PCNA clamp facilitates action of DNA cytosine methyltransferase 1 on hemimethylated DNA. *Genes Cells.* 7:997–1007.

Jonsson, Z.O., R. Hindges, and U. Hubscher. 1998. Regulation of DNA replication and repair proteins through interaction with the front side of proliferating cell nuclear antigen. *EMBO J.* 17:2412–2425.

Kannouche, P.L., J. Wing, and A.R. Lehmann. 2004. Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Mol. Cell.* 14:491–500.

Kelman, Z., and M. O'Donnell. 1995. Structural and functional similarities of prokaryotic and eukaryotic DNA polymerase sliding clamps. *Nucleic Acids Res.* 23:3613–3620.

Kelman, Z., and J. Hurwitz. 1998. Protein-PCNA interactions: a DNA-scanning mechanism? *Trends Biochem. Sci.* 23:236–238.

Krishna, T.S., X.P. Kong, S. Gary, P.M. Burgers, and J. Kuriyan. 1994. Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. *Cell.* 79:1233–1243.

Maga, G., and U. Hubscher. 2003. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *J. Cell Sci.* 116:3051–3060.

Matsumoto, Y. 2001. Molecular mechanism of PCNA-dependent base excision repair. *Prog. Nucleic Acid Res. Mol. Biol.* 68:129–138.

Matsumoto, Y., K. Kim, and D.F. Bogenhagen. 1994. Proliferating cell nuclear antigen-dependent abasic site repair in *Xenopus laevis* oocytes: an alternative pathway of base excision DNA repair. *Mol. Cell. Biol.* 14:6187–6197.

Mattock, H., P. Jares, D.I. Zheleva, D.P. Lane, E. Warbrick, and J.J. Blow. 2001. Use of peptides from p21 (Waf1/Cip1) to investigate PCNA function in *Xenopus* egg extracts. *Exp. Cell Res.* 265:242–251.

Miyachi, K., M.J. Fritzler, and E.M. Tan. 1978. Autoantibody to a nuclear antigen in proliferating cells. *J. Immunol.* 121:2228–2234.

Naryzhny, S.N., and H. Lee. 2004. The post-translational modifications of proliferating cell nuclear antigen: acetylation, not phosphorylation, plays an important role in the regulation of its function. *J. Biol. Chem.* 279:20194–20199.

Papouli, E., S. Chen, A.A. Davies, D. Huttner, L. Krejci, P. Sung, and H.D. Ulrich. 2005. Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p. *Mol. Cell.* 19:123–133.

Pfander, B., G.L. Moldovan, M. Sacher, C. Hoege, and S. Jentsch. 2005. SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. *Nature.* 436:428–433.

Prelich, G., C.K. Tan, M. Kostura, M.B. Mathews, A.G. So, K.M. Downey, and B. Stillman. 1987. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-delta auxiliary protein. *Nature.* 326:517–520.

Prosperi, E., L.A. Stivala, E. Sala, A.I. Scovassi, and L. Bianchi. 1993. Proliferating cell nuclear antigen complex formation induced by ultraviolet irradiation in human quiescent fibroblasts as detected by immunostaining and flow cytometry. *Exp. Cell Res.* 205:320–325.

Prosperi, E., A.I. Scovassi, L.A. Stivala, and L. Bianchi. 1994. Proliferating cell nuclear antigen bound to DNA synthesis sites: phosphorylation and association with cyclin D1 and cyclin A. *Exp. Cell Res.* 215:257–262.

Schurtenberger, P., S.U. Egelhaaf, R. Hindges, G. Maga, Z.O. Jonsson, R.P. May, O. Glatter, and U. Hubscher. 1998. The solution structure of functionally active human proliferating cell nuclear antigen determined by small-angle neutron scattering. *J. Mol. Biol.* 275:123–132.

Shivji, K.K., M.K. Kenny, and R.D. Wood. 1992. Proliferating cell nuclear antigen is required for DNA excision repair. *Cell.* 69:367–374.

Stelter, P., and H.D. Ulrich. 2003. Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature.* 425:188–191.

Tom, S., L.A. Henricksen, and R.A. Bambara. 2000. Mechanism whereby proliferating cell nuclear antigen stimulates flap endonuclease 1. *J. Biol. Chem.* 275:10498–10505.

Waga, S., G. Bauer, and B. Stillman. 1994. Reconstitution of complete SV40 DNA replication with purified replication factors. *J. Biol. Chem.* 269: 10923–10934.

Walter, J., and J. Newport. 1999. The use of *Xenopus laevis* interphase egg extracts to study genomic DNA replication. *In* Eukaryotic DNA Replication: A Practical Approach. Sue Cotterill, editor. Oxford University Press. 201–222.

Watanabe, K., S. Tateishi, M. Kawasuji, T. Tsurimoto, H. Inoue, and M. Yamaizumi. 2004. Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination. *EMBO J.* 23:3886–3896.