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Postreplication Repair and PCNA Modification in *Schizosaccharomyces pombe*

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Ubiquitination of proliferating cell nuclear antigen (PCNA) plays a crucial role in regulating replication past DNA damage in eukaryotes, but the detailed mechanisms appear to vary in different organisms. We have examined the modification of PCNA in *Schizosaccharomyces pombe*. We find that, in response to UV irradiation, PCNA is mono- and poly-ubiquitinated in a manner similar to that in *Saccharomyces cerevisiae*. However in undamaged *Schizosaccharomyces pombe* cells, PCNA is ubiquitinated in S phase, whereas in *S. cerevisiae* it is sumoylated. Furthermore we find that, unlike in *S. cerevisiae*, mutants defective in ubiquitination of PCNA are also sensitive to ionizing radiation, and PCNA is ubiquitinated after exposure of cells to ionizing radiation, in a manner similar to the response to UV-irradiation. We show that PCNA modification and cell cycle checkpoints represent two independent signals in response to DNA damage. Finally, we unexpectedly find that PCNA is ubiquitinated in response to DNA damage when cells are arrested in G2.

INTRODUCTION

Cellular mechanisms have evolved from prokaryotes to eukaryotes to cope with a wide variety of endogenous and exogenous DNA-damaging agents. They include DNA repair processes, cell cycle checkpoints, and DNA damage tolerance pathways that allow S phase progression in the presence of replication-blocking lesions.

Schizosaccharomyces pombe is able to remove UV photoproducts by either classical nucleotide excision repair (NER), or an alternative repair pathway, utilizing the UVDE protein to incise close to the damaged sites (UVER). In addition, DNA damage tolerance or postreplication repair (PRR) pathways are proposed to cope with replication-blocking lesions during S phase, but little work has been done on PRR in *S. pombe*.

PRR in the distantly related *Saccharomyces cerevisiae* has been well characterized genetically (Xiao *et al.*, 2000; Ulrich, 2005) and has been divided into two subpathways: translesion synthesis (TLS) and damage avoidance by template switching. In TLS, when the replicative DNA polymerase is stalled at a DNA lesion, it is replaced with a specialized TLS polymerase (polymerase switching). Depending on which TLS polymerase is recruited, the lesion is replicated in either a relatively error-free mode, for example, using DNA polymerase η (polη) to bypass UV-induced cyclobutane pyrimidine dimers or an error-prone mechanism using polζ and Rev1. Alternatively, a template switch can occur, during which the newly synthesized sister strand is used as the template to bypass the lesion through a recombination-like event. This is thought to be an error-free mechanism.

Two crucial proteins identified by genetic analyses of PRR in *S. cerevisiae* are Rad6 and Rad18, which have, respectively, E2 ubiquitin-conjugating (Jentsch *et al.*, 1987) and E3 ubiquitin ligase activity. The target of their ubiquitinating activity was recently identified as the proliferating cell nuclear antigen (PCNA), a ring-shaped sliding clamp that interacts with many different proteins involved in DNA replication and repair (Maga and Hubscher, 2003). When replication is blocked by DNA damage, the single-stranded DNA binding protein Rad18 (Bailly *et al.*, 1997) is thought to bind to exposed regions of single-stranded DNA and recruit Rad6 to the stalled replication machinery. Together they mono-ubiquitinate PCNA on lysine 164 (Hoege *et al.*, 2002). This modification of PCNA results in the activation of TLS polymerases (Stelter and Ulrich, 2003). A similar process in human cells results in the mono-ubiquitination of PCNA, and this modification increases its affinity for the TLS DNA polymerase, polη (Kannouche *et al.*, 2004; Watanabe *et al.*, 2004; Bienko *et al.*, 2005). This provides an attractive mechanism for switching from replicative to TLS polymerase at the sites of stalled forks. In *S. cerevisiae*, after mono-ubiquitination of PCNA, Rad5, a protein-bridging factor and putative E3 ubiquitin ligase recruits the heterodimer Ubc13/Mms2, an E2 ubiquitin-conjugating enzyme (Hofmann and Pickart, 1999), to the site of damage through its interactions with Rad18 (Ulrich and Jentsch, 2000). Rad5 and Ubc13/Mms2 together poly-ubiquitinate PCNA (Hoege *et al.*, 2002) in a noncanonical lysine-63 ubiquitin chain-linked manner

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(Hofmann and Pickart, 1999). Poly-ubiquitinated PCNA is proposed to control the template-switching event in PRR. To date, poly-ubiquitination of PCNA has not been reported in human fibroblasts (Kannouche *et al.*, 2004; Watanabe *et al.*, 2004).

In *S. cerevisiae*, small ubiquitin-like modifier (SUMO) can also be attached to PCNA at lysines 127 and 164 mediated by Ubc9 and Siz1, which are a SUMO conjugating E2 and a SUMO E3 ligase, respectively (Hoege *et al.*, 2002; Stelter and Ulrich, 2003). This modification occurs in untreated proliferating cells during S phase (Hoege *et al.*, 2002) and has been shown to recruit the Srs2 helicase, which helps prevent inappropriate recombination during S phase (Papouli *et al.*, 2005; Pfander *et al.*, 2005).

In this study we have investigated genetic and molecular aspects of PRR in *S. pombe*. We show that, as in *S. cerevisiae*, *S. pombe* PCNA is both mono- and poly-ubiquitinated after exposure of cells to a variety of DNA damaging agents, and these ubiquitination reactions use the same gene products in the two organisms. However, unlike in *S. cerevisiae*, cycles of PCNA ubiquitination and de-ubiquitination occur during S phases of undamaged cells. We also report that PCNA ubiquitination is not dependent on the DNA-damage checkpoint and that it is induced by ionizing radiation as well as UV. Contrary to currently accepted paradigms, we also find that PCNA is ubiquitinated in response to DNA damage not only in S phase, but also in cells in G2.

MATERIALS AND METHODS

Construction of Plasmids

ubc13 was amplified using PCR from cDNA and cloned into pGEX-4T-3 (Amersham) using BamHI and XhoI to produce an N-terminal GST-fusion. *mms2* was amplified using PCR from cDNA, cloned into PGEM-T Easy (Promega, Southampton, United Kingdom) and then subcloned using PstI/SpeI into pTYB12 (New England Biolabs, Hitchin, United Kingdom). This produces Mms2 fused at its N-terminus to a chitin-binding domain (CBD), linked by the self-cleavable *VMA1* intein sequence, which allows single-step purification of Mms2 bearing three additional amino acids at its N-terminus.

Preparation of Proteins

Recombinant proteins were produced in *Escherichia coli* Rosetta-gami B (RGB) cells (Novagen, Madison, WI). The cells were induced with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and harvested after 16 h of induction at 20°C. Purifications were achieved in a single step by affinity chromatography on the appropriate columns (glutathione sepharose [Amersham, Arlington Heights, IL] or chitin beads [New England Biolabs] for GST-Ubc13 or CBD-intein-Mms2, respectively) according to the manufacturers' recommendations. Purified proteins were dialyzed against 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 10% (vol/vol) glycerol and stored frozen at −80°C.

Ubiquitin Conjugation Assays

Ubiquitin chain synthesis assays contained 5 μM each of recombinant Ubc13 and Mms2 using conditions described previously (Ulrich, 2003). Ubiquitin mutants, no lysine (K0), lysine 63 to arginine (K63R), lysine 63 only (K63 only), and lysine 48 only (K48 only) were purchased from Boston Biochem (Cambridge, MA). Purified *S. cerevisiae* Ubc13 and Mms2 have been described previously (Ulrich, 2003) and were used at the same concentrations as the *S. pombe* proteins.

Strain Constructions

mms2::kanMX6 and *ubc13::natMX6* were transformed into cell strain 501 (*ura4-D18 leu1-32 ade6-704 h*$^-$) as previously described (Bahler *et al.*, 1998; Hentges *et al.*, 2005). The strains were verified by PCR and Southern blot analysis. Standard procedures and media were used for propagation and genetic manipulations (Moreno *et al.*, 1991).

The *pcn1-K164R* mutation was generated as follows: the K164R mutation was introduced into the *pcn1* coding sequence using site-directed mutagenesis. This was then cloned 5′ to the *ura4* gene. The mutant sequence was amplified by PCR using 100-base pair primers corresponding to 80 base pairs of the *pcn1* 5′UTR + 20-base pair *pcn1* coding sequence (5′ primer) and to 80 base pairs of the *pcn1* 3′UTR + 20-base pair *ura4* (3′ primer). The PCR product was then transformed into wild-type cells and integration at the correct locus was confirmed by colony PCR and Southern blotting.

Other strains used in this study are listed in Table 1.

Survival Analysis

For UV irradiation, cells were grown to midlog phase at 30°C in yeast extract peptone (YEP), plated on yeast extract (YE) plates, and irradiated using a 254-nm UV-C lamp with a fluence rate of 0.5 Jm^{-2} s^{-1}. For ionizing radiation (IR) survivals, exponentially growing cells were γ-irradiated in YEP from a ^{137}Cs source (dose rate, 8.5 Gy min^{-1}) before plating on YE plates. Percentage survival was scored as the number of colonies on the YE plates after 4 d of incubation at 30°C, relative to that on unirradiated control plates. Each graph is the average of three or more independent experiments. The 10% survival rate (D10) was estimated using a line of regression against the survival curve using Microsoft Excel (Redmond, WA).

PCNA Modification and Detection

For UV-treatment, a total of 10 A_{595} of midlog phase cells were grown at 30°C in YEP and transferred onto PVDF membranes (Millipore, Bedford, MA; 0.45 μM) using a vacuum pump. The membranes were irradiated with 50 Jm^{-2} UV-C (254 nm), or left as untreated controls. The cells were resuspended in YEP and incubated at 30°C for 30 min. For other treatments, a total of 10 A_{595} of midlog phase cells were grown at 30°C in YEP, and camptothecin (CPT), methyl-methanesulfonate (MMS), or hydroxyurea (HU) was added to a final concentration of 30 μM, 0.9 mM (0.01%), or 50 mM, respectively, and incubation continued for 3 h at 30°C. Cells were pelleted and washed in water, and the total protein was extracted in 20% trichloracetic acid (TCA) using a ribolyser, before being resuspended in Laemmli buffer. The lysates were fractionated by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted using affinity-purified anti-PCNA antibodies, generated in-house against full-length PCNA. To verify that the modified species of PCNA were ubiquitinated, cells were transfected with the vector pMHRep41 expressing ubiquitin N-terminally tagged with two myc-peptide and six histidine epitopes. Transfected cells in midlogarithmic phase were incubated with 50 mM HU for 3 h. Cells, 10^9, were resuspended in 400 μl lysis buffer containing 20 mM Tris-HCl, pH 7.5, 40 mM NaCl, 2 mM $MgCl_2$, 8 U/ml benzonase, protease, and phosphatase inhibitors, and 10% glycerol. Cells were lysed using a ribolyser, NaCl was added to 0.5 M and SDS to 2%, and the lysates were incubated for 15 min at room temperature. After centrifugation at 13,000 rpm for 10 min the extracts were frozen in liquid nitrogen. On thawing, they were diluted 20-fold in phosphate-buffered saline containing 300 mM extra NaCl, protease inhibitors, 0.1% NP40 and incubated with 40 μl nickel-agarose beads at 4°C for 90 min. The beads were washed three times and then boiled in Laemmli buffer before analysis by SDS-PAGE and immunoblotting.

Cell Cycle Synchronization by Elutriation

Approximately 2×10^9 G2 phase cells were sorted from a midlog phase culture in YEP containing 4×10^{10} cells by centrifugal elutriation in a Beckman Model J6 M/E elutriator (Fullerton, CA). The G2 phase cells were then incubated at 30°C, and 2×10^8 cells were removed every 20 min for a total of 240 min. Two samples of 1×10^8 cells were filtered onto PVDF membranes (Millipore) using a vacuum pump. One membrane was UVC-irradiated with 100 Jm^{-2}, and the other was left untreated. Both membranes were transferred to 100 ml YEP and incubated at 30°C for 30 min, the cells were then TCA extracted, and extracts were immunoblotted using anti-PCNA antibodies. For each sample, the septation index was examined using 4,6-diamidino-2-phenylindole (DAPI) and calcofluor staining both before UV-C treatment and after the subsequent 30-min incubation period.

Cell Cycle Synchronization Using a Temperature-sensitive **cdc25** *Mutant*

The temperature-sensitive mutant *cdc25.22* arrests in G2 at the restrictive temperature (36°C). A 250-ml *cdc25.22* culture was grown for 18 h at 25°C until midlog phase and the temperature was raised to 36°C for 3 h. Elongation of the cells was checked microscopically to ensure that they were arrested in G2. Cells, 2×10^8, were either treated with 50 μM 4-nitroquinoline (4NQO) or 50 mM HU for 1 h at 36°C or transferred to a PVDF membrane (Millipore), UV-irradiated with 100 Jm^{-2}, and incubated for 30 min in YEP at 36°C. The cells were kept at 36°C throughout the experiment. After treatment cells were TCA extracted and analyzed by immunoblotting using rabbit anti-PCNA antibodies.

Chk1 *Activation*

Cultures of exponentially growing wild-type, *pcn1-K164R,* and *rad9-T412A* (Furuya *et al.*, 2004) cells, each containing an integrated single copy of HA-tagged *chk1* (Walworth and Bernards, 1996), were split into two; one-half were exposed to 100 Jm^{-2} UV radiation, and the other remaining unirradiated. Cells were then incubated for 30 min in YEP at 30°C. TCA extracts were analyzed by immunoblotting with anti-HA antibodies.

Table 1. *S. pombe* strains used in this study

Strain	Disruption	Reference
pcn1-K164R	*pcn1-K164R:ura4*	This study
rhp18Δ	*rhp18::ura4*	Verkade *et al.* (2001)
rad8Δ	*rad8::ura4*	Doe *et al.* (1993)
rhp54Δ	*rhp54::ura4*	Muris *et al.* (1996)
mms2Δ rhp54Δ	*mms2::kanMX6 rhp54::ura4*	This study
mms2Δ	*mms2::natMX6* or *mms2::kanMX6*	This study
rad3Δ	*rad3::ura4*	Bentley *et al.* (1996)
cds1Δ	*cds1::ura4*	Murakami and Okayama (1995)
mms2Δ cds1Δ	*mms2::kanMX6 cds1::ura4*	This study
Chk1Δ	*chk1::ura4*	Al-Khodairy *et al.* (1994)
mms2Δ chk1Δ	*mms2::kanMX6 chk1::ura4*	This study
tel1Δ	*tel1::ura4*	This study
srs2Δ	*srs2::natMX6*	Gift from J. Murray
rad13Δ	*rad13::ura4*	Carr *et al.* (1993)
rad13Δ uve1Δ	*rad13::ura4 uve1::LEU2*	This study
rhp51Δ	*rhp51::kanMX6*	Gift from S. Lambert
mms2Δ pcn1-K164R	*mms2::kanMX6 pcn1-K164R:ura4*	This study
rhp18Δ pcn1-K164R	*rhp18::ura4 pcn1-K164R:ura4*	This study
rad8Δ pcn1-K164R	*rad8::ura4 pcn1-K164R:ura4*	This study
mms2Δ rhp18Δ	*mms2::kanMX6 rhp18::ura4*	This study
mms2Δ rad8Δ	*mms2::kanMX6 rad8::ura4*	This study
mms2Δ ubc13Δ	*mms2::kanMX6 ubc13::natMX6*	This study
mms2Δ rad3Δ	*mms2::kanMX6 rad3::ura4*	This study
mms2 rad13 uve1	*mms2::kanMX6 rad13::ura4 uve1::LEU2*	This study
rhp51 mms2	*rhp51::kanMX6 mms2::natMX6*	This study
rhp51 rad8	*rhp51::kanMX6 rad8::ura4*	This study
cdc25.22	*cdc25.22*	Nurse *et al.* (1976)
cdc25^{ts} rad13 uve1	*cdc25.22 rad13::ura4 uve1::LEU2*	This study
cdc25^{ts} rad3 tel1	*cdc25.22 rad3::ura4 tel1::ura4*	This study
rhp51 pcn1-K164R	*rhp51::kanMX6 pcn1-K164R:ura4*	This study

All strains are derivatives of the *ura4-D18, leu1–32, ade6–704* genotype.

RESULTS

Ubiquitination Activity of Ubc13/Mms2

In *S. cerevisiae*, after DNA damaging treatments, PCNA is mono-ubiquitinated by the combined actions of Rad6 and Rad18 and poly-ubiquitinated by Mms2-Ubc13 and Rad5. The *S. pombe* orthologues of Rad6 (Rhp6; Reynolds *et al.*, 1990), Rad18 (Rhp18; Verkade *et al.*, 2001) and Rad5 (Rad8; Doe *et al.*, 1993) have been characterized in some detail in previous work from this and other laboratories. *S. pombe* homologues of Ubc13 and Mms2 were recently identified by Brown *et al.* (2002). We cloned these genes and expressed the proteins in *E. coli* to analyze their activities in vitro. *S. cerevisiae* Ubc13/Mms2 heterodimer functions in vitro as a ubiquitin E2-conjugating enzyme forming noncanonical lysine-63 linked ubiquitin chains. We have analyzed recombinant *S. pombe* Ubc13 and Mms2 using a ubiquitin conjugation assay (Figure 1A). When ubiquitin and an E1 enzyme were incubated with recombinant Mms2 and Ubc13, ubiquitin chains were formed (Figure 1A, lanes 2 and 3). These chains were dependent on Mms2 (lanes 10–12) and Ubc13 (lanes 13–15). We also used mutant ubiquitin in which all the lysines were mutated to arginine (lanes 4–6), only K63 was

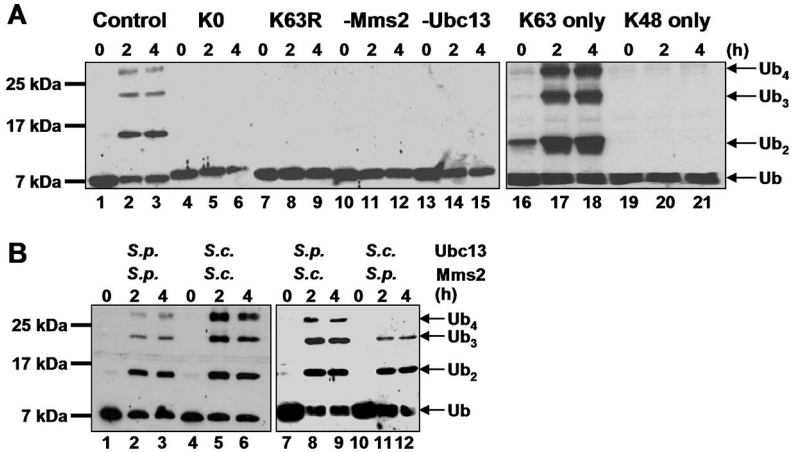

Figure 1. In vitro ubiquitination reactions. (A) Ubiquitination reactions were carried out with E1, recombinant *S. pombe* Ubc13 and Mms2 and either wild-type or lysine mutants of ubiquitin, and reactions were incubated for 0, 2, or 4 h, as indicated. Reaction products were analyzed by immunoblotting with anti-ubiquitin antibody (note that the apparently anomalous migration in lanes 4–6 resulted from a bubble during transfer and was not reproduced in other experiments). (B) Cross-species interactions of Ubc13 and Mms2 from *S. pombe* (*S.p.*) and *S. cerevisiae* (*S.c.*). Reactions carried out as in A. Ub, Ub_2, Ub_3, Ub_4: mono-, di-, tri-, and tetra-ubiquitin.

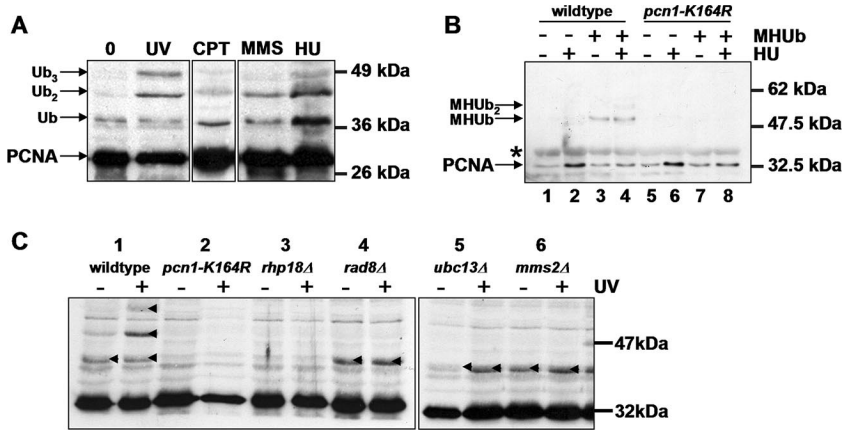

Figure 2. Ubiquitination of PCNA in *S. pombe* in response to DNA damage. (A) Exponentially growing cultures of *S. pombe* were exposed to 50 Jm^{-2} UV and incubated for 30 min or to 30 μM camptothecin (CPT), 0.9 mM MMS, or 50 mM HU for 3 h. The cells were then lysed and analyzed for PCNA modification using anti-PCNA antibody. Ub, Ub_2, Ub_3: mono-, di-, and triubiquitinated PCNA. (B) Cells were transfected with pMHRep41 either as empty vector or expressing Myc_2His_6-tagged ubiquitin and then incubated with or without HU for 3 h. SDS lysates were incubated with Ni-agarose beads, and the bound proteins were analyzed with anti-PCNA antibody. $MHUb_1$, $MHUb_2$: PCNA modified with one or two myc-his–tagged ubiquitins. The asterisk represents a nonspecific cross-reacting band. Note that a small amount of unmodified PCNA (<1% of the total PCNA) bound nonspecifically to the beads in all lanes. (C) The indicated mutants were either untreated (−) or exposed to 50 Jm^{-2} UV-irradiation (+) and incubated for 30 min before harvesting and analysis as in A. Mono-, di-, and triubiquitinated PCNA bands are indicated with arrowheads. The fainter bands are nonspecific cross-reacting species.

mutated (lanes 7–9), all the lysines except K63 were mutated (lanes 16–18), and all but K48 were mutated (lanes 19–21). Only the protein in which K63 was intact (lanes 16–18) supported the formation of polyubiquitin chains, indicating that the polyubiquitination was mediated by K63 linkage.

We also showed that *S. pombe* Ubc13 and Mms2 could function with the orthologues of their partner proteins from *S. cerevisiae* (Figure 1B). Both *S. pombe* Ubc13 together with *S. cerevisiae* Mms2 (lanes 7–9) and *S. pombe* Mms2 together with *S. cerevisiae* Ubc13 (lanes 10–12) were able to catalyze the formation of ubiquitin chains. These data not only demonstrate conservation between *S. cerevisiae* and *S. pombe* but also strongly imply that *S. pombe* Ubc13/Mms2 has a similar mechanism and activity to its *S. cerevisiae* counterpart.

Modification of PCNA after Treatment with DNA-damaging Agents

We next investigated the modification of PCNA in *S. pombe* in response to DNA damage. In untreated asynchronous *S. pombe* cultures we detected both an unmodified and a modified form of PCNA (Figure 2A). The ~8-kDa shift in mobility of the modified band is that expected for monoubiquitinated PCNA. After treatment with a variety of DNA-damaging agents, UV irradiation, CPT, MMS, or HU, more slowly migrating species of PCNA were observed. The mobilities of these bands correspond to those expected of polyubiquitinated PCNA species. To confirm that the slow migrating bands did indeed represent ubiquitinated species of PCNA, we transfected cells with a plasmid expressing ubiquitin N-terminally tagged with two copies of the myc peptide and six histidine residues. We used wild-type cells and *pcn1-K164R* mutants in which lysine 164 of PCNA is mutated to arginine. After treatment with or without HU, cell extracts in SDS buffer were diluted and incubated with nickel-agarose beads. Proteins bound to the beads were analyzed by SDS-PAGE and immunoblotted with anti-PCNA antibody (Figure 2B). Species of reduced mobility relative to unmodified PCNA were observed in wild-type cells transfected with myc-his-ubiquitin (lane 3), and further bands were detected after HU treatment (lane 4). These bands were dependent on K164 (see lanes 7 and 8) and on transfection with the tagged ubiquitin construct (see lanes 1, 2, 5, and 6), confirming that they were indeed PCNA ubiquitinated exclusively on K164 (note that the Myc_2His_6 epitope tag on the ubiquitin significantly reduces the mobility of the ubiquitinated PCNA species). Therefore in *S. pombe*, the mono- and poly-ubiquitination of PCNA on K164 in response to DNA damage appears to be similar to that in *S. cerevisiae*.

We examined the role of different genes on PCNA modification after UV treatment using various deletion strains of the *S. pombe* orthologues of genes known to be involved in PCNA ubiquitination in *S. cerevisiae*. These were (*S. cerevisiae* orthologues in superscript when nomenclature differs) $rhp18^{RAD18}$, $rad8^{RAD5}$, *mms2*, and *ubc13*, as well as the point mutant *pcn1-K164R* (Figure 2C). In the *pcn1-K164R* and $rhp18^{rad18}\Delta$ mutants (lanes 2 and 3), modification of PCNA was totally abolished. In contrast, in $rad8^{rad5}$, *ubc13*, and *mms2* deletion strains, there was a strong band of the size equivalent to mono-ubiquitinated PCNA, whereas potential polyubiquitinated species were no longer detected (lanes 4–6). Similar results were obtained after exposure of the same set of mutant strains to camptothecin (unpublished data).

We carried out epistasis analysis after UV-irradiation of mutants in genes involved in PCNA ubiquitination. We found that *mms2* was epistatic with *ubc13*, $rhp18^{RAD18}$ and $rad8^{RAD5}$, which is consistent with the biochemical data (Figure 3 A–C). Double mutants were no more sensitive than the single mutants. Likewise *pcn1-K164R*, in which PCNA cannot be ubiquitinated, was epistatic with $rhp18^{RAD18}$, $rad8^{RAD5}$, and *mms2* (Figure 3, D–F, summarized in Table 2). This suggests that all these genes operate in the same pathway. We note that the sensitivities of *rad8*Δ, *mms2*Δ, and *ubc13*Δ were lower than those of *rhp18*Δ and *pcn1-K164R*, consistent with the former group being deficient only in polyubiquitination, whereas the latter are deficient in both mono and polyubiquitination of PCNA.

The results described above demonstrate that PCNA modification in response to DNA damage in *S. pombe* is similar to that in *S. cerevisiae* and has the same genetic requirements.

PCNA Modification and Cell Cycle Checkpoints Respond Independently to DNA Damage

We next addressed the question of whether PCNA ubiquitination is dependent on an intact DNA-damage checkpoint and vice versa. We examined the ubiquitination of PCNA in a series of strains deficient in the DNA-damage checkpoint. The $Rad3^{Mec1}$ and Tel1 checkpoint kinases control all DNA-damage checkpoints (Carr, 2002). However PCNA modifi-

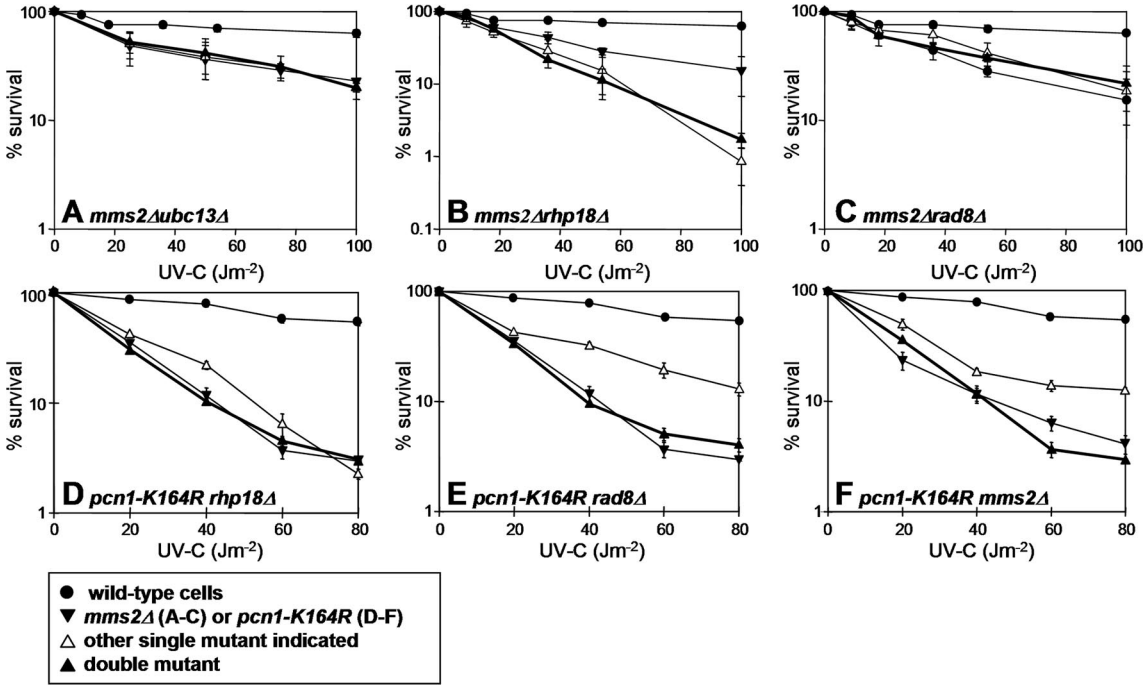

Figure 3. Epistasis analysis. (A–C) UV survival curves of single and double mutants of *mms2*Δ with (A) *ubc13* Δ, (B) *rhp18* Δ, and (C) *rad8* Δ. (D–F) UV survival curves of single and double mutants of *pcn1-K164R* with (D) *rhp18*Δ, (E) *rad8*Δ, and (F) *mms2*Δ. Means ± SEM of three experiments are shown. Double mutants are indicated with a bold line.

cation after UV-irradiation in *rad3*Δ, *tel1*Δ, and *rad3*Δ *tel1*Δ mutants was similar to that in wild-type cells (Figure 4A). Note that the levels of di- and triubiquitination varied between experiments, and we do not consider the apparent increased levels in the double mutant to be significant. The important conclusion is that PCNA modification is not dependent on an intact DNA-damage checkpoint pathway. To determine if the DNA-damage checkpoint is itself dependent on PCNA modification, we have analyzed the phosphorylation of the downstream checkpoint target, Chk1, in the *pcn1-K164R* mutant after UV-irradiation. The phosphorylation of Chk1 in *pcn1-K164R* cells was indistinguishable from that of wild-type cells (Figure 4B, lanes 2 and 4). In contrast, Chk1 phosphorylation was substantially reduced in the known checkpoint mutant *rad9-T412A* (Furuya *et al.*, 2004; lane 6). Thus the DNA-damage checkpoint response is not dependent on PCNA ubiquitination. Consistent with these findings, genetic analysis shows that *pcn1-K164R* is not epistatic with the DNA-damage checkpoint gene, *rad3* (Figure 4C). Similarly *mms2* is not epistatic with *rad3*, *cds1*^{RAD53}, or *chk1* (summarized in Table 2).

These results show that ubiquitination of PCNA and activation of the DNA-damage checkpoint are two independent signaling mechanisms triggered by DNA damage.

Table 2. Sensitivity to UV-irradiation of different single and double mutants

pcn1-K164R	40		
*rhp18*Δ	51	*pcn1-K164R rhp18*Δ	40
*rad8*Δ	130	*pcn1-K164R rad8*Δ	42
*mms2*Δ	137	*pcn1-K164R mms2*Δ	40
*mms2*Δ	137		
rad3D	10	*mms2*Δ *rad3D*	7
*cds1*Δ	>250	*mms2*Δ *cds1*Δ	111
*chk1*Δ	77	*mms2*Δ *chk1*Δ	53
*rhp54*Δ	33	*mms2*Δ *rhp54*Δ	16
*srs2*Δ	214	*mms2*Δ *srs2*Δ	144
*rhp51*Δ	182		
*rad8*Δ	129	*rhp51*Δ *rad8D*	6
*mms2*Δ	137	*rhp51*Δ *mms2*Δ	7

The values represent the doses (Jm^{-2}) needed to reduce survival to 10%.

PRR and Ionizing Radiation

The PRR pathways provide the cell with mechanisms to tolerate lesions in DNA during replication. The biologically important lesions generated in DNA by ionizing radiation are strand breaks, which are thought to inhibit DNA replication by the intra–S phase checkpoint–dependent arrest of initiation, rather than by blocking fork progression. Consistent with this idea, in *S. cerevisiae pol30-K164R*, *mms2*Δ, and *ubc13*Δ are not sensitive to IR (Xiao *et al.*, 1999; Chen *et al.*, 2005). *rad5*Δ and *rad18*Δ mutants are sensitive to IR (Lawrence, 1982; Ahne *et al.*, 1997; Xiao *et al.*, 1999; Chen *et al.*, 2005), but recent data have shown that this can be attributed to functions of these genes which are not involved in ubiquitination of PCNA (Chen *et al.*, 2005).

In striking contrast, we find a novel role in *S. pombe* for genes involved in PCNA ubiquitination after ionizing radiation. In human cells, PCNA is not modified after IR (Kannouche *et al.*, 2004). However, in *S. pombe* we find that PCNA is modified after IR (Figure 5A). The pattern of ubiquitination is very similar to that found for UV-irradiated cells (Figure 2), with mono-ubiquitination dependent on Rhp18 and poly-ubiquitination dependent on Rad8, Ubc13, and Mms2 (Figure 5A). We find that the survival responses of PRR mutants to IR are strikingly similar to their responses to

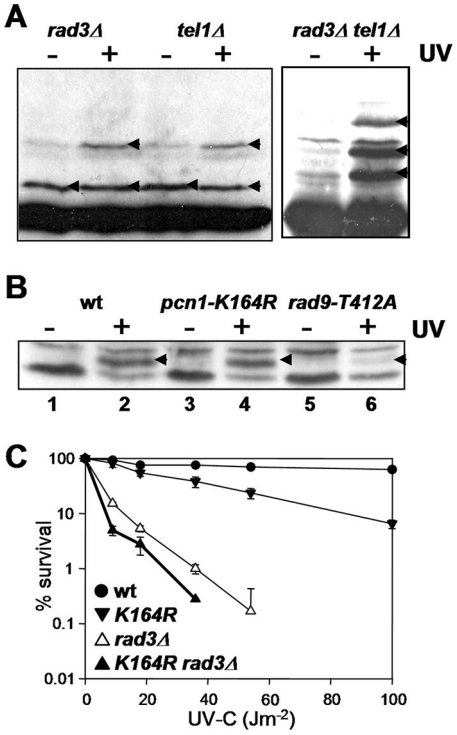

Figure 4. PCNA ubiquitination and cell cycle checkpoints are independent responses. (A) Checkpoint mutants were UV-irradiated (50 Jm^{-2}) and analyzed for PCNA ubiquitination. Arrowheads indicate ubiquitinated species. (B) Chk1 is activated in *pcn1-K164R* after exposure to UV. Cultures of exponentially growing wild-type, *pcn1-K164R* and *rad9-T412A* cells, both containing an integrated single copy of HA-tagged *chk1*, were exposed to 100 Jm^{-2} UV radiation and then incubated for 30 min. Total cell extracts were analyzed by Western blotting with anti-HA antibodies. Arrowheads indicate activated Chk1. (C) UV survival curves of single and double mutants of *pcn1-K164R* with *rad3*Δ.

UV, which is consistent with this observation. *mms2*Δ and *ubc13*Δ are sensitive to IR and are epistatic to each other (Figure 5B) and to *rhp18*Δ and *rad8*Δ (Figure 5, C and D). Furthermore, we found that *pcn1-K164R* is also sensitive to IR and is epistatic to *rhp18*, *rad8*, *mms2*, and *ubc13* (Table 3). We also show that, after IR treatment, *mms2* is not epistatic to genes involved in the checkpoint pathway, *rad3*, *cds1*, and *chk1*, or in recombination repair, $rhp54^{RAD54}$ (Table 3).

rhp51 *and PRR*

It has been proposed in *S. cerevisiae* that poly-ubiquitination of PCNA signals for a recombination-dependent template switch mechanism. If this process involves strand invasion mediated by *RAD51*, double mutants of genes involved in PRR and recombination might be epistatic. In *S. pombe*, double mutants of *rhp51*Δ (the ortholog of *RAD51*) with *rhp18*Δ, *rad8*Δ, *mms2*Δ, or *ubc13*Δ all resulted in slow growth phenotypes. The cells were elongated and had severe growth defects and low plating efficiencies (unpublished data). *rhp51*Δ cells are moderately sensitive to UV-irradiation. Strikingly, the double mutants of *rhp51*Δ with *pcn1*-K164R (Figure 5E) and *rad8*Δ or *mms2*Δ (Table 2) were exquisitely sensitive to UV-irradiation. Synergistic interactions between *UBC13* or *RAD18* and recombination repair genes have also been found recently in *S. cerevisiae* after treatment with 4-NQO (Papouli *et al.*, 2005) and in the ability of *S. cerevisiae* to replicate a plasmid containing closely spaced 6–4 photoproducts on each strand (Zhang and Lawrence, 2005). *rhp51*Δ cells are very sensitive to IR. Nevertheless the double mutants are even more sensitive (Figure 5F and Table 3).

PCNA Is Ubiquitinated during S Phase in Undamaged Cells

In early experiments, we noted that a significant proportion of PCNA was modified by monoubiquitination in undamaged cells (e.g., see Figure 2, A and C, lanes 1). To determine if this modification in undamaged cells was confined to a particular phase of the cell cycle, we synchronized cells in G2 using elutriation and analyzed whether PCNA was modified in *S. pombe* during the cell cycle. Cell cycle progression was monitored by measuring the mitotic and septation indices (Figure 6B). Septation occurs in early S phase in *S. pombe*. We observed bands of mono-, di-, and triubiquitinated PCNA specifically during S phase (Figure 6A). To see if this fluctuation in levels of ubiquitinated PCNA species could be correlated with corresponding levels of the proteins involved in poly-ubiquitination of PCNA, we measured the levels of Rhp18, Mms2, and Ubc13 in the same extracts. No significant changes in the levels of these proteins were detected (unpublished data).

In *S. cerevisiae,* PCNA is sumoylated rather than ubiquitinated during S phase (Hoege *et al.*, 2002). Sumoylated PCNA interacts physically with Srs2, and this recruitment of Srs2 prevents unwanted recombination during S phase. Furthermore, in *S. cerevisiae*, the DNA-damage sensitivity of mutants in the PRR pathway is suppressed by deletion of the *SRS2* gene. In the absence of PRR, recombination-mediated back-up pathways are called into play, but they are inhibited by Srs2. Deletion of *SRS2* enables these pathways to function, resulting in increased resistance to DNA damage (Papouli *et al.*, 2005; Pfander *et al.*, 2005). However we did not detect any sumoylated PCNA in the experiment of Figure 5A, nor did we find any suppression of the UV sensitivity of *S. pombe mms2*Δ by deleting *srs2* (Table 2). Instead, the genes appear to be epistatic.

PCNA Is Ubiquitinated in Response to DNA Damage in G2

The currently accepted model for ubiquitination of PCNA in response to DNA damage is that it is triggered by stalling of the replication machinery at sites of DNA damage. Monoubiquitination is thought to mediate the switch from replicative to translesion polymerases, whereas polyubiquitination channels lesions into an error-free damage avoidance pathway (Hoege *et al.*, 2002; Stelter and Ulrich, 2003). It is implicit in this model that ubiquitination of PCNA in response to damage is an S phase–specific process. To test this prediction, we examined PCNA ubiquitination in synchronized cells at different stages of the cell cycle. At different times after synchronization by elutriation, samples were UV-irradiated and incubated for a further 30 min before harvesting and analysis for PCNA ubiquitination. Cell cycle progression as monitored by septation and mitotic index is presented in Figure 6B. PCNA ubiquitination data are shown in Figure 6, A (unirradiated) and C (irradiated). Modified PCNA species are present in highest amounts in S phase cells (Figure 6, A and C, lanes 3–5 and 10–12), but a strong band corresponding to mono-ubiquitinated PCNA is detected in irradiated cells at all time points. Note in particular that samples 1, 7, and 13 contained no detectable S phase cells either at the time of UV-irradiation or at the time of harvesting. These data suggest that PCNA is, unexpect-

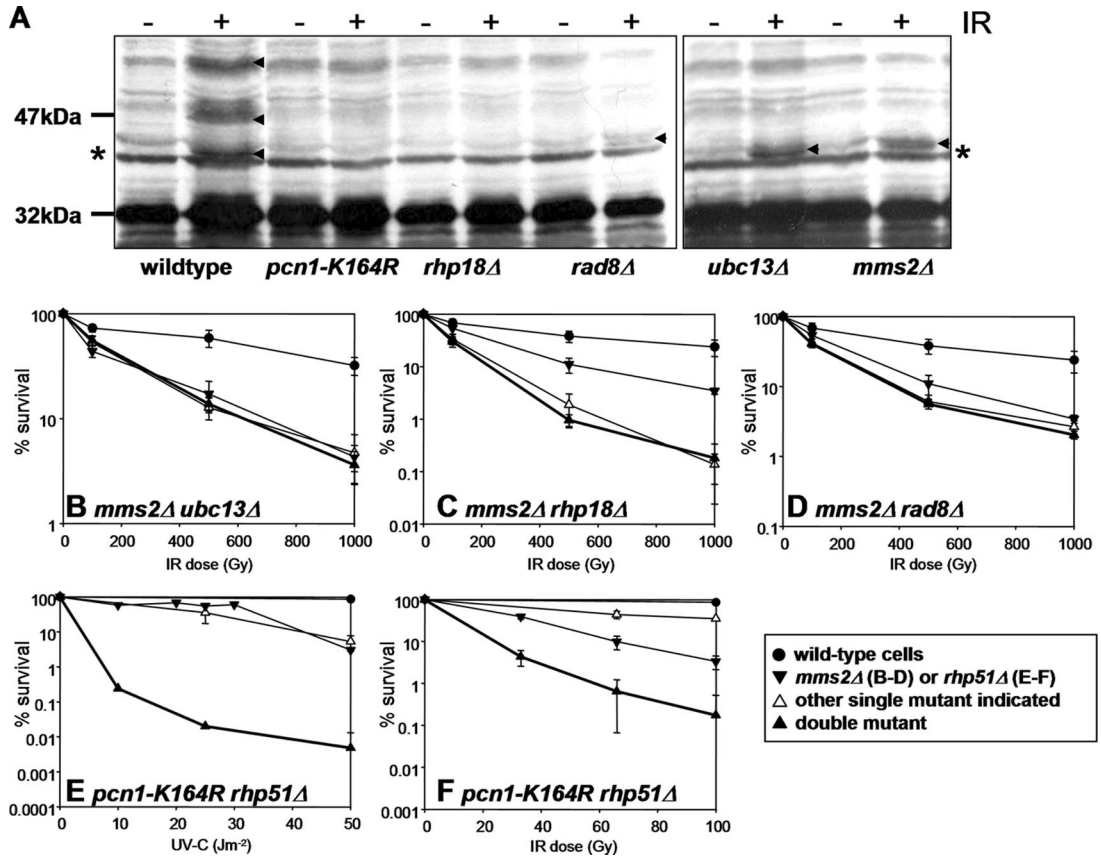

Figure 5. PCNA ubiquitination in response to ionizing radiation. (A) The indicated mutants were exposed to 500 Gy ionizing radiation and incubated for 1 h. PCNA modification was analyzed in whole cell extracts by immunoblotting. Arrowheads indicate ubiquitinated PCNA bands; note that in this experiment there was a strongly cross-reacting nonspecific band in all samples, indicated with an asterisk (*). (B–D) IR survival curves of single and double mutants of *mms2*Δ with (B) *ubc13* Δ, (C) *rhp18* Δ, and (D) *rad8*Δ. (E and F) UV and IR survival curves, respectively, of single and double mutants of *pcn1-K164R* with *rhp51*Δ. Double mutants indicated with bold lines.

edly, ubiquitinated in response to DNA damage even in non-S phase cells.

To confirm these observations, we made use of the temperature-sensitive *cdc25.22* mutant, which is blocked in G2 at the restrictive temperature (Nurse *et al.*, 1976). Cells were held at the restrictive temperature for 3 h, at which time all of the cells were elongated, showing that they were indeed arrested in G2. These cells were exposed to HU, 4NQO, or UV, incubated for a further 30 min, and compared with asynchronous cells subjected to the identical treatments. Results are shown in Figure 7A. In asynchronous cells, all three treatments resulted in ubiquitination of PCNA (lanes 2–4). In the G2-arrested cells, HU treatment, which does not damage DNA but inhibits DNA replication by depleting the deoxyribonucleotide pool, did not result in PCNA ubiquitination (lane 6). In contrast in cells treated with 4NQO (lane 7) or UV (lane 8), clear bands corresponding to mono-, di-, and triubiquitinated PCNA were detected. As expected, no modifications were seen in the *rhp18*Δ or *pcn1-K164R* strains and only mono-ubiquitination in *rad8*Δ and *mms2* Δ strains (unpublished data). These data demonstrate that PCNA is modified in response to DNA damage in G2 cells.

We next tested whether this G2 response was triggered by intermediates in NER or the alternative repair pathway mediated by the UVDE damage–specific endonuclease. We isolated a *rad13*^{rad2}Δ *uvde*Δ *cdc25.22* triple mutant strain, which is defective in both the NER and UVER pathways. We have previously shown that no photoproducts are removed in *rad13*Δ *uvde*Δ strains (Yonemasu *et al.*, 1997). When the triple mutant was held at the restrictive temperature for 3 h, exposed to 4NQO or UV, and incubated for a further 30 min, the ubiquitination of PCNA was similar to that in the repair-proficient strain (Figure 7B). We also created a *rad3*Δ *tel1*Δ *cdc25.22* triple mutant strain that is completely defective in DNA-damage checkpoint signaling. The level of PCNA

Table 3. IR sensitivity of various single and double mutants

pcn1-K164R	593		
*rhp18*Δ	305	*pcn1-K164R rhp18*Δ	298
*rad8*Δ	541	*pcn1-K164R rad8*Δ	540
*mms2*Δ	630	*pcn1-K164R mms2*Δ	621
*mms2*Δ	630		
*rad3*Δ	78	*mms2*Δ *rad3*Δ	60
*cds1*Δ	>402	*mms2*Δ *cds1*Δ	283
*chk1*Δ	78	*mms2*Δ *chk1*Δ	65
*rhp54*Δ	49	*mms2*Δ *rhp54*Δ	41
*rhp51*Δ	71		
*rad8*Δ	541	*rhp51*Δ *rad8*Δ	24
*mms2*Δ	630	*rhp51*Δ *mms2*Δ	29

The values in parentheses represent the doses (Gy) needed to reduce survival to 10%.

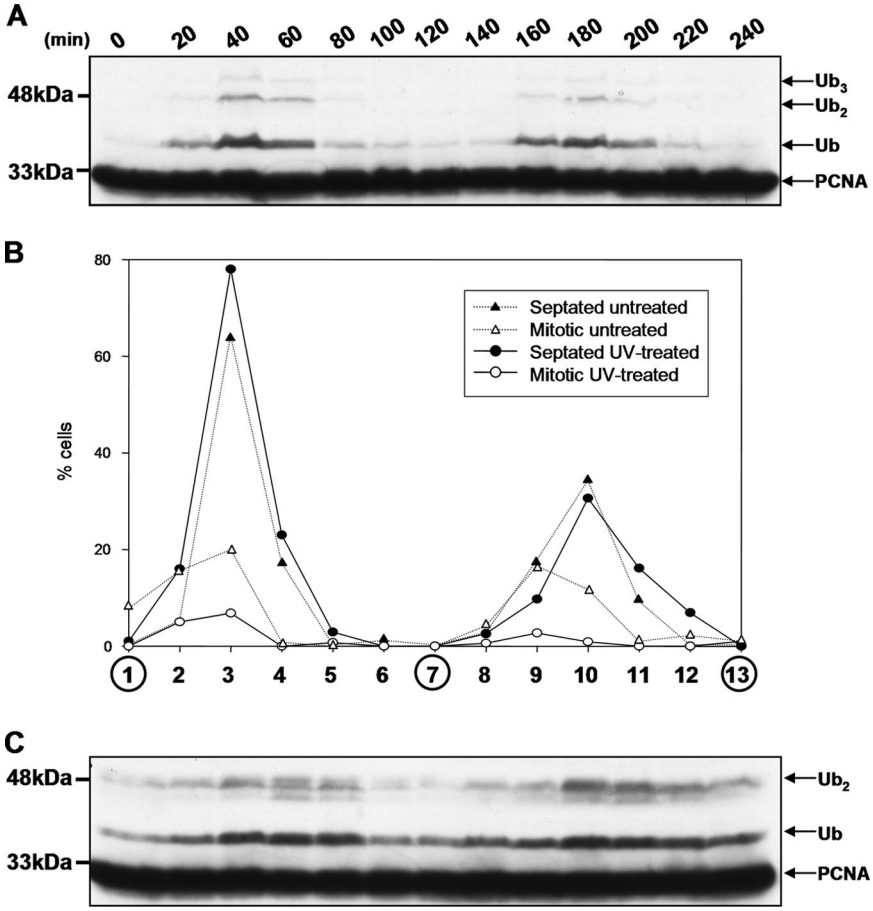

Figure 6. PCNA ubiquitination through the cell cycle. Cells synchronized by elutriation were either mock-irradiated (A) or UV-irradiated (50 Jm^{-2}; C) at different times after reinoculation and then incubated for 30 min. PCNA modification was analyzed by immunoblotting of whole cell extracts. (B) Cell cycle position was analyzed by microscopic measurement of mitotic index and septation index 30 min after UV-irradiation (UV-treated) or mock treatment (untreated). Note that septation occurs in early S phase in *S. pombe*.

ubiquitination at the restrictive temperature was similar to that in the *cdc25.22* single mutant. We conclude that PCNA ubiquitination in G2 is not dependent either on generation of a repair intermediate or on checkpoint activation.

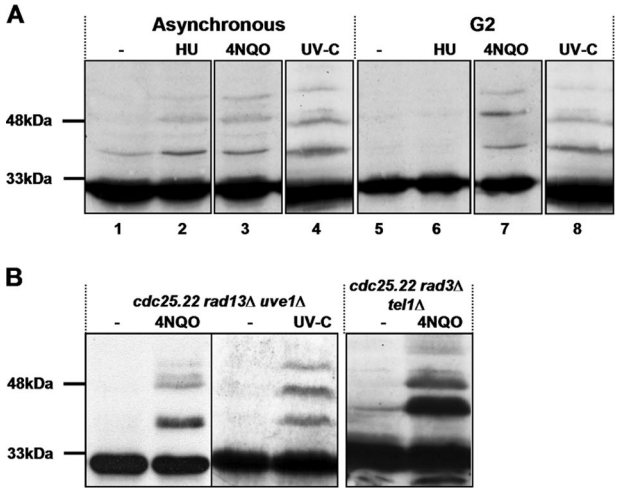

Figure 7. PCNA ubiquitination in G2. (A) *cdc25.22* cells were maintained either at 25°C (asynchronous) or 36°C (G2) for 3 h. Cells were then treated with either 50 mM HU or 50 μM 4NQO for 1 h or UV-irradiated (100 Jm^{-2}) and incubated for 60 min. (B) *cdc25.22 rad13*Δ *uve1*Δ or *cdc25.22 rad3*Δ *tel1*Δ triple mutants were treated at as in A. In all cases PCNA modification was analyzed by immunoblotting of whole cell extracts.

DISCUSSION

In *S. cerevisiae*, PCNA is sumoylated at K127 and K164 during S phase in undamaged cells. After treatment with MMS, PCNA becomes both mono- and poly-ubiquitinated at K164 (Hoege *et al.*, 2002). In transformed human fibroblasts, we have detected mono-ubiquitination of PCNA at K164 after UV-irradiation and a variety of DNA-damaging agents, but we have not detected either poly-ubiquitination or sumoylation (Kannouche *et al.*, 2004 and unpublished observations). In *Xenopus laevis*, PCNA is mono-ubiquitinated and sumoylated during S phase and becomes di-ubiquitinated in response to DNA damage (Leach and Michael, 2005). In *S. pombe* we have now found that we can easily detect both mono- and poly-ubiquitination at K164, but we do not detect sumoylation under the same conditions. There thus appear to be differences in PCNA modifications between organisms. The genetic requirements for mono- and poly-ubiquitination of PCNA in *S. pombe* are similar to those in *S. cerevisiae*, namely that mono-ubiquitination requires $Rhp18^{Rad18}$, whereas poly-ubiquitination requires $Rad8^{Rad5}$, Ubc13, and Mms2. Our data obtained with UV-irradiation of asynchronous cells are consistent with current models for PRR in which blockage of the replication fork at a UV-induced photoproduct results in ubiquitination of PCNA. Mono-ubiquitination has been shown to increase the affinity of (human) PCNA for polη and provides a mech-

anism for switching from replicative to TLS polymerase (Kannouche *et al.*, 2004; Bienko *et al.*, 2005). Genetic analysis in *S. cerevisiae* suggests that poly-ubiquitination channels damage into error-free damage avoidance pathways (Hoege *et al.*, 2002). The importance of PCNA ubiquitination is demonstrated by the UV sensitivities of *pcn1-K164R*, *rhp18*$^{rad18}\Delta$, *rad8*$^{rad5}\Delta$, *ubc13*Δ and *mms2*Δ. *pcn1-K164R* and *rhp18*Δ are deficient in both mono- and poly-ubiquitination and more sensitive than *rad8* $^{rad5}\Delta$, *ubc13*Δ and *mms2*Δ, which are deficient only in poly-ubiquitination. These responses are quite similar to those of the orthologous mutants in *S. cerevisiae* (Xiao *et al.*, 2000; Ulrich, 2005). However there are some minor differences, which relate to the relative importance of sumoylation of PCNA in the two organisms. For example in *S. cerevisiae* *rad18*Δ is considerably more sensitive than *rad30-K164R*, but this excess sensitivity is suppressed in the double mutant (Hoege *et al.*, 2002; Papouli *et al.*, 2005). This is related to sumoylation of PCNA in *S. cerevisiae*, which stimulates Srs2-mediated inhibition of recombination-mediated repair. In the K164R mutant, sumoylation is greatly reduced, so that recombination repair can rescue some of the structures normally repaired via TLS. As mentioned above, sumoylation of PCNA is difficult to detect in *S. pombe*. Furthermore, the C-terminal 138 aa of *S. cerevisiae* Srs2, shown to mediate the interaction with sumoylated PCNA (Pfander *et al.*, 2005), are not conserved in the *S. pombe* ortholog. These observations are consistent with PCNA sumoylation playing only a minor or no role in *S. pombe*. There are at least two possible explanations for this difference between the two organisms. Sumoylation in *S. cerevisiae* recruits Srs2, which suppresses recombination (Papouli *et al.*, 2005; Pfander *et al.*, 2005). Either polyubiquitination in *S. pombe* somehow takes over the function of sumoylation in *S. cerevisiae* or the recombination system in *S. pombe* may be less active during S phase than in *S. cerevisiae*, and it may not be necessary to have a mechanism to suppress it.

After treatment of cells with DNA-damaging agents, cell cycle checkpoints are triggered via the activation of Rad3^{Mec1} and/or Tel1 protein kinases (Carr, 2002). Deletion of either or both of these genes had no effect on PCNA ubiquitination, and conversely checkpoint activation remained intact in the *pcn1-K164R* mutant. These data demonstrate that PCNA ubiquitination and checkpoint activation are independent signaling responses, both of which can be triggered by stalling of replication forks.

We have found both mono- and poly-ubiquitination of PCNA in S phase *S. pombe* cells. It is formally possible that this modification may arise because of spontaneously stalled replication forks. The intensity of the bands in S phase compared with those seen in stressed asynchronous cells would argue that such pausing must be relatively prevalent. Irrespective of the nature of the trigger for ubiquitination during S phase, PCNA modification under such circumstances seems to have limited biological significance because there are no major biological effects of abolishing PCNA modifications in growing cells. The proliferation rates of *pcn1-K164R* mutant cells, as well as those of *rhp18*Δ, *mms2* Δ and *ubc13* Δ, are similar to that of wild-type cells.

Our working hypothesis to account for these data is that in response to single-stranded DNA and/or some other distortion at a stalled replication fork, PCNA is ubiquitinated. If the ubiquitination results from DNA damage blocking the replication machinery, the increased affinity of mono-ubiquitinated PCNA for polη and possibly also for other TLS polymerases will bring about a polymerase switch and facilitate bypass of the lesion (Kannouche and Lehmann, 2004; Kannouche *et al.*, 2004). In the case of blocking of the replication fork by HU or stalling at natural pause sites during unhindered replication, PCNA ubiquitination will be activated. However, TLS polymerases are not able to overcome the block caused by HU-induced deoxyribonucleotide depletion, and there is no reason to suppose that they might be able to overcome natural pause sites. Consequently, cells deleted in the PCNA ubiquitination genes as well as the *pcn1-K164R* mutant proliferate normally, and the *pcn1-K164R* mutant has normal sensitivity to HU (unpublished data).

A further difference between *S. pombe* and *S. cerevisiae* is in the ubiquitination of PCNA after IR treatment. In *S. pombe* PCNA is ubiquitinated after IR treatment, and this ubiquitination makes an important contribution to cell survival, whereas in *S. cerevisiae* PCNA modification does not appear to play a significant role in the response to IR (Xiao *et al.*, 1999; Chen *et al.*, 2005). IR produces single- and double-strand breaks in DNA as well as different types of base lesions. The major effect of strand breaks on DNA replication is to inhibit initiations via the intra-S checkpoint, rather than to block progression of forks. Indeed double-strand breaks induced by EcoRI in the genome of *S. cerevisiae* did not result in ubiquitination of PCNA (Chen *et al.*, 2005). It is possible, therefore, that base lesions, the helical distortion they cause, and/or intermediates in their repair result in modification of PCNA.

We have found a large synergistic interaction between *rhp51* and PRR genes after UV treatment. We interpret these data to suggest that, after UV-induced DNA damage, *S. pombe* requires PCNA polyubiquitination to stabilize a stalled replication fork. If this is inhibited, the replication machinery may collapse and can only be restored by Rhp51-mediated recombination. In the absence of both mechanisms, a UV photoproduct in the DNA is likely to be fatal during S phase.

Our most unexpected finding is that PCNA is ubiquitinated after DNA damage in cells held in G2. This did not appear to be triggered by repair intermediates because it was also seen in cells completely deficient in repair of photoproducts. This suggests that it is either the DNA structure generated by the lesions themselves that triggers ubiquitination of PCNA or some other effect of the damage that is not dependent on DNA replication. One possible candidate might be transcription complexes stalled at sites of damage, though there is no evidence for any involvement of PCNA in transcription. Our finding of ubiquitination of PCNA in G2 after UV and 4NQO treatments may provide an alternative explanation for our observations that PCNA is also ubiquitinated following treatment with IR.

In conclusion, our studies on *S. pombe* have revealed that the role of PCNA ubiquitination in the response to DNA damage might be more complex than has been envisaged previously. Our results have opened up new avenues of research for understanding the functions of PCNA modification.

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