Rad18 Regulates DNA Polymerase κ and Is Required for Recovery from S-Phase Checkpoint-Mediated Arrest

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We have investigated mechanisms that recruit the translesion synthesis (TLS) DNA polymerase Polκ to stalled replication forks. The DNA polymerase processivity factor PCNA is monoubiquitinated and interacts with Polκ in cells treated with the bulky adduct-forming genotoxin benzo[a]pyrene dihydrodiol epoxide (BPDE). A monoubiquitination-defective mutant form of PCNA fails to interact with Polκ. Small interfering RNA-mediated downregulation of the E3 ligase Rad18 inhibits BPDE-induced PCNA ubiquitination and association between PCNA and Polκ. Conversely, overexpressed Rad18 induces PCNA ubiquitination and association between PCNA and Polκ in a DNA damage-independent manner. Therefore, association of Polκ with PCNA is regulated by Rad18-mediated PCNA ubiquitination. Cells from Rad18−/− transgenic mice show defective recovery from BPDE-induced S-phase checkpoints. In Rad18−/− cells, BPDE induces elevated and persistent activation of checkpoint kinases, indicating persistently stalled forks due to defective TLS. Rad18-deficient cells show reduced viability after BPDE challenge compared with wild-type cells (but survival after hydroxyurea or ionizing radiation treatment is unaffected by Rad18 deficiency). Inhibition of RPA/ATR/Chk1-mediated S-phase checkpoint signaling partially inhibited BPDE-induced PCNA ubiquitination and prevented interactions between PCNA and Polκ. Taken together, our results indicate that ATR/Chk1 signaling is required for Rad18-mediated PCNA monoubiquitination. Recruitment of Polκ to ubiquitinated PCNA enables lesion bypass and eliminates stalled forks, thereby attenuating the S-phase checkpoint.

Polycyclic aromatic hydrocarbons (PAHs) are abundant and ubiquitous environmental pollutants with well-documented mutagenic and carcinogenic properties (4). The biological effects of the PAH benzo[a]pyrene (B[a]P) have been studied extensively in vivo and in vitro. B[a]P and many related PAHs are subject to intracellular cytochrome P450-mediated metabolism (11). Cytochrome P450-dependent oxidation of B[a]P generates the reactive species and ultimate carcinogen B[a]P dihydrodiol epoxide (BPDE). BPDE can react covalently with exocyclic deoxyguanosine (majority, ~90%) and deoxyadenosine (minority, ~10%) residues in genomic DNA to generate bulky adducts (18). The DNA adducts resulting from covalently bound BPDE are believed to account for the mutagenic and carcinogenic properties of B[a]P (19). Potentially, error-prone repair or replication of BPDE-adducted DNA can result in mutations. Propagation of cells containing BPDE-induced mutations in oncogenes or tumor suppressor genes can contribute to multistep carcinogenesis.

Because of the potential threat to genomic stability posed by DNA adducts (as well as other forms of DNA damage), cells have evolved elaborate mechanisms to detect and repair damaged DNA. Cell cycle checkpoints are signal transduction pathways that respond to damaged DNA by inhibiting cell cycle progression (45). The cell cycle delays elicited by checkpoint signaling enable integration of cell cycle progression with DNA repair. Consequently, checkpoints are important for preserving the integrity of the genome. Cells can acquire DNA damage throughout the cell cycle. Therefore, DNA damage-inducible checkpoint mechanisms exist that arrest cells in G1, S, and G2/M phases. Individuals with congenital defects in checkpoint genes (such as ATM, ATR, p53, and CHEK2) are prone to cancer, highlighting the importance of checkpoint signaling pathways as important tumor-suppressive mechanisms.

Cell cycle responses to DNA damage acquired during S phase are highly conserved in eukaryotes. During a normal S phase, DNA synthesis initiates at multiple loci (termed origins of replication) that are activated (a process known as firing) in a temporally ordered manner (17). When ongoing replication forks encounter DNA lesions, a signal is generated that prevents initiation of DNA synthesis from unfired origins. The inhibition of DNA synthesis due to delayed firing of late origins is termed the S-phase checkpoint (or the intra-S-phase checkpoint).

Bulky adducts such as those induced by BPDE and UV elicit an S-phase checkpoint pathway involving the proximal checkpoint components ATR (an ATM/RAD3-related protein kinase) and the heterotrimeric Rad9-Rad1-Hus1 (9-1-1) complex (20, 21, 44). ATR and 9-1-1 are recruited separately to damaged DNA (47, 48). Activation of ATR involves its recruitment to RPA-coated single-stranded DNA (ssDNA), which is generated by the uncoupling of replicative helicases from fork progression, via the ATR-interacting protein ATRIP (also termed Rad26) (47, 48). After recruitment to damaged DNA,
the concerted actions of ATR and 9-1-1 activate the checkpoint kinase Chk1, which mediates the inhibition of late-firing origins in response to DNA damage. The mechanism by which Chk1 inhibits DNA synthesis at late origins might involve degradation of the Cdc25A protein phosphatase (38) and inhibition of the Dbf4-Cdc7 protein kinase complex (14, 15) which is required for initiation of DNA replication at individual origins throughout S phase. ATM, Nbs1, and Chk2 are dispensable for the BPDE-induced checkpoint pathway (44) and probably also for the UV-induced checkpoint. In addition to inhibiting initiation of late origins, S-phase checkpoint signaling is important for stabilizing stalled replication forks via an unknown mechanism(s) (16, 31, 40).

Replicative DNA polymerases are generally unable to carry out accurate or efficient DNA synthesis when they encounter bulky adducts or other lesions. However, specialized DNA polymerases can be used to replicate past lesions in a process termed translesion synthesis (TLS). Replicative bypass of DNA lesions is an inherently error-prone process due to the low fidelity of TLS polymerases. Thus, error-prone TLS is considered to be one of the causes of mutagenesis and carcinogenesis due to DNA lesions.

TLS DNA polymerases in mammalian cells include Polk, Polβ, Polɛ, Rev1 (the Y family polymerases), and Polξ (a B family polymerase comprising the catalytic Rev3 subunit and the noncatalytic Rev7 protein). Polβ was the first mammalian TLS polymerase identified (25, 32). Polβ is encoded by the XPV gene, which is defective in xeroderma pigmentosum variant patients. The role of mammalian Polη (and that of its Saccharomyces cerevisiae homolog encoded by the Rad30 gene) in TLS has been studied extensively. Polη is unique among eukaryotic DNA polymerases in its ability to replicate templates containing cis-syn thymine-thymine dimers (the species generated by UV radiation).

Studies in vitro with yeast indicate that Polη promotes error-free DNA translesion synthesis in a manner which is stimulated by PCNA and regulated by the Rad6/Rad18 epistasis pathway (22, 39). Rad6 is an E2 ubiquitin (Ub)-conjugating enzyme (also termed UBC2) that forms a tight complex with the RING-containing E3 ligase Rad18. The Rad18-Rad6 complex binds ssDNA and has ssDNA-stimulated ATPase activity (2). Recent reports have demonstrated that in yeast and mammalian cells Rad18 is important for monoubiquitination of PCNA in response to DNA damage (22, 28, 39, 43). Moreover, Polη interacts preferentially with monoubiquitinated PCNA. It has also been shown that Rad18 and Polη interact via their C-terminal motifs and that this interaction is important for guiding Polη to PCNA (43). Therefore, according to current models, Rad18/Rad6-mediated monoubiquitination of PCNA constitutes a molecular switch that recruits Polη to stalled replication forks (41).

In contrast to Polη, which bypasses BPDE-adducted templates very poorly, Polκ is able to bypass benzo[a]pyrene-adducted guanine, efficiently inserting the correct C opposite the bulky lesion (36). Polκ-deficient mutant mouse embryonic stem (ES) cells are highly sensitive to BaP-induced mutagenesis and genotoxicity (35), further suggesting a role for Polκ in cellular responses to BaP-adducted DNA. BPDE, as well as hydroxyurea (HU) and UV irradiation, can elicit recruitment of Polκ to nuclear foci (5, 6, 34), possibly suggesting a general role for Polκ in responses to genotoxins and replication stress.

Recently we showed that Polκ is specifically recruited to PCNA-containing foci in response to BPDE treatment concomitant with activation of the S-phase checkpoint (6). Furthermore, we showed that, in contrast to wild-type (WT) cells, Polκ− mouse embryonic fibroblasts (MEFs) arrested irreversibly in S phase after BPDE treatment. In BPDE-treated, Polκ-deficient cells, failure to recover from the S-phase checkpoint was associated with persistent activation of ATM, Chk1, and Chk2 kinases, and phosphorylation of the double-strand breakage (DSB) marker γH2AX. Taken together, those results suggested that Polκ-mediated replicative bypass of BPDE adducts contributes to attenuation of DNA damage signaling and recovery from the S-phase checkpoint.

The specific mechanisms that recruit Polκ to the replication machinery in mammalian cells have not been identified. Because of the important role of Polκ in the BPDE-induced S-phase checkpoint, we have investigated the mechanisms that recruit Polκ to stalled replication forks and promote TLS. Experiments presented here demonstrate an important role for Rad18 in regulating PCNA monoubiquitination, Polκ recruitment, and S-phase checkpoint recovery. Importantly, we show that PCNA ubiquitination and associations between PCNA and Polκ are regulated by checkpoint signaling.

MATERIALS AND METHODS

Adenovirus construction and infection. Adenovirus construction and infections were performed as described previously (20). cDNAs encoding hemagglutinin (HA)-Rad18 and HA-PCNA WT and PCNA K164A were subcloned into pAC-CMV to generate pAC-HA-Rad18 and pAC-PCNA WT and pAC-PCNA K164A, respectively. The resulting shuttle vectors were cotransfected into 293T cells with the pJM17 plasmid to generate recombinant adenovirus as described previously. AdGFP-Polκ, AdYFP-Polη, and AdChk1KR were described previously (6). H1299 cells were routinely infected with 5 × 10^6 PFU/ml adenovirus. As controls for adenoviral infection, cells received AdCon (empty adenovirus vector) or AdGFP.

Cell culture. Human lung carcinoma H1299 cells, Rad18+/− and Rad18−/− MEFs, and TERT-immortalized xeroderma pigmentosum variant (XPV) CRL1162 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, streptomycin sulfate (100 μg/ml), and penicillin (100 U/ml).

ET163 cells from ataxia-telangiectasia patients and a matched cell line designated YZS, which is complemented with the ATM cDNA (46), were cultured in Dulbecco modified Eagle medium supplemented with 15% fetal bovine serum, streptomycin sulfate (100 μg/ml), and penicillin (100 U/ml).

Genotoxic treatments. BPDE (National Cancer Institute carcinogen repository) was dissolved in anhydrous dimethyl sulfoxide and added directly to the growth medium as a 1,000× stock to give a final concentration of 100 or 600 nM. For HU treatment, HU was dissolved in water and added directly to the growth medium as a 1,000× stock to give a final concentration of 1 μM. For UV treatment, the growth medium was removed from the cells and replaced with phosphate-buffered saline (PBS). The plates were transferred to a UV cross-linker (Stratagenes) and then irradiated. The UV dose delivered to the cells was confirmed with a UV radiometer (UVP, Inc.). The cells were then refed with complete growth medium and returned to the incubator. For ionizing radiation (IR) treatment, cells were placed in PBS, irradiated with a cesium source, refed with complete growth medium, and then returned to the incubator. In some experiments, cells were incubated in medium containing 5 mM caffeine (Sigma) or 150 μM UCN-01 for 1 h before genotoxic treatment.

RNA interference (RNAi). Cells were plated into six-well culture dishes. At 24 h later, when cells were 50% confluent, the cultures were placed in P3-free medium (2 ml per well). For each transfection, 6.25 μl of 20 μM stocks of Cy3 (control) small interfering RNA (siRNA), siRPA, or siRAD18 or 10 μl of siATR (Dharmacon smart pool) was diluted into 0.25 ml of Opti-MEM. After 5 min, the siRNA–Opti-MEM solution was mixed with 0.25 ml of Opti-MEM containing 5
μl of Lipofectamine 2000. Twenty minutes later, the resulting mixture was added to culture medium. After incubation overnight, the transfection medium was removed and replaced with standard culture medium. All tubes, tips, and solutions used for RNAi experiments were certified RNase free.

**Clonogenic survival assays.** Cells were grown to 70 to 80% confluence and then transferred into 5 ml of Pi-free MEM solution was mixed with 1.5 ml of Opti-MEM containing 20 μl of Lipofectamine 2000. Twenty minutes later, the resulting mixture was added to 12 ml of culture medium. After incubation for 6 h, the transfection medium was removed and replaced with standard culture medium. At 24 h after transfection, cells were treated with 600 nM BPDE for 4 h prior to harvest. Cells were washed twice with PBS and then separated into soluble and insoluble fractions with cytoskeleton (CSK) buffer (23). The insoluble fraction was treated with 1,000 U/ml DNase (Roche 10776 785 001) for 30 min at room temperature and then clarified by centrifugation at 10,000 × g for 5 min. The supernatants (containing solubilized chromatin) were normalized for protein content. H1299 cells were plated in 10-cm culture dishes and infected with adenovirus as described above. Genotoxin treatments were performed at ~70% confluence. To isolate chromatin fractions for IP, cells were rinsed twice with PBS and then extracted with 5 ml of CSK buffer containing 100 mM HEPES (pH 7.4), 0.1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 200 mM NaF, 80 mM β-glycerophosphate, and 1 mM protease inhibitor mixture (Roche Applied Science). In some experiments, whole nuclei were prepared with CSK buffer as described previously (6). Total cell extracts or nuclear protein samples were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with the following antibodies: rabbit anti-Chk1 (FL-476; Santa Cruz Biotechnology), rabbit anti-Chk2 (H-300; Santa Cruz Biotechnology), mouse monoclonal anti-PCNA (catalog no. sc-56; Santa Cruz Biotechnology), rabbit anti-phospho-Chk1 Ser-345 (catalog number 2341; Cell Signaling), rabbit anti-phospho-Chk2 Thr-68 (catalog number 2661; Cell Signaling), polyclonal HA tag antibody (ab9110; abcam Inc.), and polyclonal PCNA antibody (FL-261, catalog no. sc-7907; Santa Cruz Biotechnology).

**Comunmunoprecipitation (co-IP) of PCNA and TLS DNA polymerases.** H1299 cells were plated in 10-cm culture dishes and infected with adenovirus as described above. Genotoxin treatments were performed at ~70% confluence. To isolate chromatin fractions for IP, cells were rinsed twice with PBS and then extracted with 5 ml of CSK buffer for 10 to 15 min. The CSK-extracted cells were treated with 1% Triton X-100 and 1% formaldehyde in PBS (4.5 M) for 10 min. Then, 0.5 ml of a 1 M glycine solution in PBS was added for 5 min to quench the cross-linking reaction. The cross-linked nuclei were rinsed with PBS and then lysed in 500 μl of IP lysis buffer (freshly supplemented with protease and phosphatase inhibitors). Lysates were scraped from the plates and transferred into 1.5-ml Microfuge tubes. Samples were sonicated for two 8-s pulses at 30% of the maximum output. Pulses were separated by a 10-s interval on ice to prevent excessive heating. Lysates were clarified by centrifugation at 10,000 × g for 5 min. Supernatants were removed and normalized for protein concentration (approximately 600 μg of protein in 1 ml was used for each IP). PCNA was immunoprecipitated overnight at 4°C with 5 μl of monoclonal PCNA antibody (Santa Cruz). A 25-μl volume of protein A/G beads was added to each sample for 4 h. The beads were recovered by brief centrifugation and washed three times with 1 ml of IP lysis buffer (15 min per wash). The washed immune complexes were boiled in protein loading buffer for 5 min to reverse the cross-links prior to separation by SDS-PAGE.

In some experiments, cells were transfected with siRNA duplexes prior to analysis of PCNA-TLS polymerase associations. For these experiments, cultures were grown to 70 to 80% confluence and then transferred into 5 ml of P<sub>f</sub>ree medium. For siRNA transfections, 30 μl of Cy3 control siRNA, siRPA, or siAng10 were transfected with 600 nM Lipofectamine 2000. Following a 25-min incubation at room temperature, the transfection mixture was added to the cells. After 24 h, when the cultures were fully confluent, the cells were split 1:2 into fresh 10-cm plates. These were infected with adenovirus vectors (AdGFP-Pool, AdChk1) for 24 h prior to genotoxin treatment. IPs were performed as described above.

**Co-IP of Rad18 and TLS polymerases.** H1299 cells growing in 10-cm plates were infected at 40 to 50% confluence with the various combinations of adenovirus for 36 h. Infected cells were treated with 600 nM BPDE for 6 h prior to harvest. Isolation of chromatin fractions for IP was performed essentially as described by Watanabe et al. (43). In brief, cells were washed twice with PBS and cross-linked with 9 ml of 1% formaldehyde for 10 min at room temperature. One milliliter of 1 M glycine was then added for 5 min at room temperature to quench the cross-linking reaction. Cells were washed twice in PBS and then fractionated into soluble and insoluble fractions with CSK buffer. The insoluble fraction was sonicated three times for 10 s at 30% of the maximum output and then clarified by centrifugation at 10,000 × g for 5 min. The supernatant (containing solubilized chromatin) and the soluble fraction from the initial cell lysis were normalized for protein content. GFP-Pool and YFP-Pool were immunoprecipitated overnight at 4°C with 1 ml of IP lysis buffer (Pharmaco smart pool) was added to 1.25 ml of Opti-MEM. After 5 min, this was mixed with 1.25 ml of Opti-MEM containing 30 μl of Lipofectamine 2000. Following a 25-min incubation at room temperature, the transfection mixture was added to the cells. After 24 h, when the cultures were fully confluent, the cells were split 1:2 into fresh 10-cm plates. These were infected with adenovirus vectors (AdGFP-Pool, AdChk1) for 24 h prior to genotoxin treatment. IPs were performed as described above.
fraction was also analyzed by SDS-PAGE. After transfer to nitrocellulose, immunoblotting was performed with GFP antibody (Molecular Probes A11122) at 2 μg/ml and HA-antibody (Santa Cruz sc-805) at 0.2 μg/ml.

**RESULTS**

**PCNA is monoubiquitinated in response to BPDE.** We previously showed that ectopically expressed GFP-Polκ redistributes and colocalizes with replication forks in BPDE-treated cells (6). The Lehman and Yamaizumi laboratories reported that PCNA undergoes monoubiquitination and recruits the TLS enzyme Polη to stalled forks in genotoxin-treated cells (28, 43). In contrast to Polη, mechanisms of Polκ regulation are not well understood. Therefore, we asked if similar PCNA-dependent mechanisms recruit Polκ and Polη to stalled replication forks.

First, we determined whether BPDE treatment induced PCNA monoubiquitination concomitant with the S-phase checkpoint. H1299 cells were chosen for these experiments. We and others have studied DNA replication extensively in this cell line (6, 20, 42). Importantly, we have demonstrated that the BPDE-induced S-phase checkpoint is intact and that GFP-Polκ is recruited to sites of ongoing DNA replication in H1299 cells. H1299 cells were treated with 100 or 600 nM BPDE. At different times after BPDE treatment, nuclear fractions were prepared from BPDE-treated cells and assayed for monoubiquitinated PCNA, which is detected as a distinct band of ~40 kDa, in contrast to unmodified PCNA, which is ~32 kDa on immunoblots.

The inhibition of DNA synthesis induced by 100 nM BPDE results mainly from inhibition of initiation of replication unfired origins (12, 29). In a previous report, we have shown that 100 nM BPDE elicits a transient inhibition of DNA synthesis that is maximal 1 to 3 h after BPDE treatment (20). As shown in Fig. 1A, 100 nM BPDE induced a modest (1.6-fold) increase in the levels of an anti-PCNA immunoreactive band of 40 kDa, which corresponds to the correct size for monoubiquitinated PCNA. The amount of putative monoubiquitinated PCNA declined to basal levels by 3 to 5 h after BPDE treatment, concomitant with recovery from the S-phase checkpoint. Therefore, the kinetics of PCNA modification induced by 100 nM BPDE were similar to the kinetics of inhibition of DNA synthesis. In contrast to the transient inhibition of DNA synthesis elicited by 100 nM BPDE, 600 nM BPDE results in a persistent inhibition of DNA synthesis due to inhibition of both initiation and elongation steps of DNA synthesis (12, 29).

Inhibition of elongation represents a physical stalling of replication forks and is not a regulated checkpoint response. PCNA was also posttranslationally modified in a manner consistent with its monoubiquitination in cells treated with 600 nM BPDE, concomitant with inhibition of elongation. However, both the duration and the levels of PCNA modification were increased in cells treated with the higher dose of BPDE (Fig. 1A, bottom).

Based on the apparent molecular weight of the posttranslationally modified PCNA induced by BPDE, and because of previous studies showing that PCNA is monoubiquitinated in response to DNA damage, it appeared likely that BPDE induced a monoubiquitinated form of PCNA. However, PCNA may be subject to other posttranslational modifications that affect its mobility (22). Therefore, we performed experiments to test specifically if PCNA is ubiquitinated after BPDE treatment.

We expressed a hexahistidine- and Myc-tagged form of Ub (designated H₆M-Ub) in AdCon (empty adenovirus vector)- or AdRad18 (encoding Rad18, a PCNA E3 ligase)-infected H1299 cells by transient transfection. The resulting H₆M-Ub-expressing cells were given BPDE or left untreated as controls. H₆M-Ub-containing proteins were purified from chromatin fractions with metal affinity resin as described in Materials and Methods. The resulting H₆M-Ub-conjugated proteins were resolved by SDS-PAGE and probed with an anti-PCNA antibody. As shown in Fig. 1B, both BPDE and Rad18 induced a 45-kDa PCNA species that was enriched by metal affinity resin as described in Materials and Methods. The resulting H₆M-Ub-conjugated proteins were resolved by SDS-PAGE and probed with an anti-PCNA antibody. As shown in Fig. 1B, both BPDE and Rad18 induced a 45-kDa PCNA species that was enriched by metal affinity resin as described in Materials and Methods. The resulting H₆M-Ub-conjugated proteins were resolved by SDS-PAGE and probed with an anti-PCNA antibody.

Taken together, these results show that PCNA is ubiquitinated concomitant with the BPDE-induced inhibition of DNA synthesis caused by BPDE doses that inhibit late origin firing (100 nM BPDE) or concentrations that elicit blocks in elongation (600 nM BPDE). The temporal pattern of BPDE-induced PCNA ubiquitination shown here correlates well with the pattern of GFP-Polκ focus formation that we described in a previous study (6). These data are consistent with a putative role for PCNA ubiquitination in Polκ regulation.
GFP-Polκ associates with PCNA in BPDE-treated cells. Our previous study demonstrated colocalization of GFP-Polκ with replication forks (as identified by bromodeoxyuridine-labeled regions) in BPDE-treated cells (6). To test specifically if Polκ colocalizes with PCNA, we treated GFP-Polκ-expressing H1299 cells with BPDE, fixed the resulting cells, and probed them with anti-PCNA antibodies. We detected bound anti-PCNA antisera with Cy3-coupled secondary antibodies. Patterns of PCNA and GFP fluorescence were visualized by deconvolution microscopy. A representative nucleus showing the distribution of BPDE-induced GFP-Polκ foci (green) and PCNA (red) staining is shown in Fig. 2A. As shown in Fig. 2A, there was extensive (although not complete) overlap between GFP and PCNA fluorescence, indicating colocalization of Polκ and PCNA.

We performed co-IP experiments to determine if Polκ was present in a complex with PCNA after BPDE treatment. For comparison, we also performed parallel assays to measure association of Polη with PCNA under the same experimental conditions. H1299 cells were infected with adenovirus encoding GFP-Polκ, YFP-Polη, or GFP as controls. The resulting cells were treated with 600 nM BPDE for 6 h (or left untreated) and then lysed to prepare chromatin extracts. The resulting chromatin extracts were analyzed directly for PCNA and Polκ or Polη expression by immunoblotting or solubilized and immunoprecipitated with PCNA antibodies prior to blotting with PCNA or GFP antibodies.

As shown in Fig. 2B, BPDE treatment had no effect on the levels of GFP-Polκ, YFP-Polη, or PCNA in chromatin extracts. However, as expected, PCNA ubiquitination was induced by BPDE. Anti-PCNA immunoprecipitates from the chromatin fractions were resolved by SDS-PAGE and then immunoblotted with anti-GFP (to detect immunoprecipitated YFP-Polη and GFP-Polκ) or anti-PCNA (to confirm IP of PCNA).

As shown in Fig. 2B, the amount of GFP-Polκ that com- munoprecipitated with PCNA was increased approximately sixfold by BPDE treatment. Interestingly, although YFP-Polη and GFP-Polκ were expressed at similar levels, there was a relatively high basal level of association between Polη and PCNA (the association between Polη and PCNA was also increased by BPDE treatment). In our previous study, we noted that YFP-Polη, but not GFP-Polκ, forms large numbers of foci in H1299 cells in the absence of genotoxin treatment (6). Therefore, Polκ shows a DNA damage-induced association with PCNA concomitant with its redistribution to nuclear foci. In contrast, Polη is associated with PCNA and subnuclear foci in H1299 cells that do not receive genotoxin. In these experiments, we noticed that overexpression of YFP-Polη (but not of GFP-Polκ) resulted in increased monoubiquitination of PCNA, even in the absence of DNA damage (Fig. 2B, PCNA immunoblot, right side). YFP-Polη-induced PCNA ubiquitination likely accounts for the high basal association of PCNA with YFP-Polη in H1299 cells.

Previously it was shown that Polη is required for genotoxin-induced association of Polκ with nuclear foci (27). To test if Polη is similarly required to recruit Polκ to PCNA, XPV cells (which lack functional Polη) were infected with adenovirus encoding GFP-Polκ. The resulting cells were treated with 600 nM BPDE for 4 h (or left untreated) and then lysed to prepare chromatin extracts. The resulting chromatin extracts were analyzed directly for PCNA and Polκ expression by immunoblotting or solubilized and immunoprecipitated with PCNA antibodies prior to blotting with PCNA or GFP antibodies.

As shown in Fig. 3A, BPDE treatment had no effect on the levels of chromatin-associated GFP-Polκ in XPV cells. We consistently observed an increase in levels of chromatin-bound PCNA in XPV cells after BPDE treatment. Nevertheless, as expected, BPDE induced PCNA ubiquitination in XPV cells. Moreover, as also shown in Fig. 3A, BPDE induced association of GFP-Polκ with PCNA, similar to results of experiments with Polη-expressing H1299 cells (Fig. 2). Therefore, Polκ recruitment to PCNA is BPDE inducible in the absence of Polη.

To further investigate if Polκ and Polη are regulated coordinately in response to DNA damage, we investigated the relative subcellular distribution of these polymerases in control and BPDE-treated cells. H1299 cells were cotransfected with HA-Polκ and YFP-Polη expression plasmids. Two days after transfection, cells were treated with 600 nM BPDE for 6 h and then solubilized with CSK buffer to remove soluble proteins.
cells by adenoviral infection. The resulting cultures were treated with 600 nM BPDE or coinfected with AdRad18. Chromatin extracts were prepared, resolved by SDS-PAGE, transferred to nitrocellulose, and then probed with HA antibodies. As expected, HA-PCNA-WT but not HA-PCNA-K164A underwent a mono-ubiquitination-induced mobility shift in response to BPDE treatment or Rad18 overexpression (Fig. 4A).

H1299 cells were coinfected with adenoviral vectors encoding GFP-Polk and WT or K164A PCNA. The resulting cultures were treated with BPDE and lysed to prepare nuclear fractions. Solubilized chromatin extracts were immunoprecipitated with anti-HA antisera to recover ectopically expressed WT or mutant PCNA. The resulting immune complexes were separated by SDS-PAGE, blotted, and then probed with anti-GFP antisera to detect GFP-Polk. Similar to the experiment in Fig. 2, in which we detected association between GFP-Polk and endogenous PCNA, HA-tagged WT PCNA associated with GFP-Polk in a BPDE-inducible fashion (Fig. 4B). In contrast, in the parallel experiment performed with HA-tagged K164A PCNA, we detected no basal or DNA damage-induced co-IP of GFP-Polk. Taken together, our results indicate that BPDE-induced association between Polκ and PCNA requires ubiquitination on K164 of PCNA. This is similar to the mechanism proposed for recruitment of Polη to PCNA (28). However, as shown in our experiments in Fig. 2B, there are clear differences in the levels of PCNA-associated Polη and Polκ without BPDE treatment which are proportional to the level of monoubiquitinated PCNA.

Rad18 status influences association between PCNA and Polκ. The results shown in Fig. 1 to 3 suggested a role for PCNA monoubiquitination in Polκ regulation. Studies with yeast and mammalian cells have shown that PCNA monoubiquitination in response to genotoxins requires the E3 ligase Rad18 (22, 28, 39, 43). Therefore, we used siRNA strategies to test the role of Rad18 in BPDE-induced PCNA ubiquitination and Polκ regulation.

We tested the effectiveness of Rad18 siRNA on levels of ectopically expressed HA-tagged Rad18 protein. H1299 cells were infected with Ad-HARad18 adenovirus. The resulting cultures were transfected with siRNA against Rad18 or control Cy3 RNA oligonucleotides. Protein extracts from these cultures were separated by SDS-PAGE, blotted to nitrocellulose, and then probed with anti-HA antibodies. As shown in Fig. 4C, HA-Rad18 was readily detected in extracts from cells transfected with control RNA oligonucleotides. However, expression of HA-Rad18 was ablated by >90% in Rad18 siRNA-transfected cells. Therefore, our Rad18 siRNA oligonucleotides were effective for silencing Rad18 expression.

We tested the effects of Rad18 siRNA on BPDE-induced monoubiquitination of PCNA and PCNA-Polκ interactions. H1299 cells were infected with AdGFP-Polk and then transfected with control or Rad18 siRNA oligonucleotides. The resulting cells were treated with BPDE or left untreated as controls. After lysis, chromatin extracts from the cells were immunoprecipitated with anti-PCNA antibodies and analyzed for PCNA modification and coimmunoprecipitated GFP-Polk.

As shown in Fig. 4D, BPDE induced PCNA monoubiquitination and association between PCNA and GFP-Polk in Cy3-transfected (control) cells. However, both the BPDE-induced increase in monoubiquitinated PCNA and PCNA-bound GFP-
Polk were ablated in Rad18 siRNA-transfected cells. These data suggest that Rad18 is required for BPDE-induced associations between Polk and PCNA.

We also determined the effect of Rad18 overexpression on PCNA ubiquitination and PCNA-Polk interactions. Adenovirus vectors were used to express GFP-Polk individually or in combination with HA-tagged Rad18. Rad18-overexpressing cells had very high basal levels of monoubiquitinated PCNA, equivalent to levels of monoubiquitination induced by BPDE under our standard experimental conditions (Fig. 4D). The high basal levels of PCNA ubiquitination in AdRad18-infected cells were further increased after BPDE treatment. Interestingly, in HA-Rad18-expressing cells there was also a high-level association between PCNA and GFP-Polk. Therefore, overexpressed Rad18 induces PCNA ubiquitination and associations between PCNA and GFP-Polk in the absence of DNA damage. These results demonstrate an important role for Rad18 in Polk regulation.

**Interactions between Rad18 and Polk.** Recently, Watanabe et al. showed that Rad18 directly interacts with Polη and that the interaction is required for Polη to form nuclear foci after DNA damage (43). We performed reciprocal co-IP experiments to investigate if similar associations exist between Rad18 and Polk. As a positive control for these experiments, we observed association between Rad18 and Polk. Chromatin fractions from cells expressing HA-Rad18 and YFP-Polη or GFP-Polk were fixed and sheared by sonication as described by Watanabe et al. (43). Solubilized chromatin fractions were immunoprecipitated with anti-GFP antisera, and the resulting immune complexes were analyzed for associated HA-Rad18 by SDS-PAGE and Western blotting.

As expected, Rad18 was present in anti-GFP immunoprecipitates from YFP-Polη-expressing cells (Fig. 5A, lower right part). Interestingly, anti-GFP immunoprecipitates from GFP-Polk- and YFP-Polη-expressing cells contained similar amounts of Rad18, indicating that the two polymerases associated with Rad18 to similar extents. It should be noted, however, that only a small fraction of the cellular HA-Rad18 associated with Polk and Polη in these experiments. Moreover, the association between Polk (or Polη) and Rad18 was only evident in immunoprecipitated chromatin fractions derived from formaldehyde-fixed nuclei. Even when we massively overexpressed GFP-Polk or YFP-Polη and HA-Rad18, we were unable to detect association between soluble pools of polymerase and Rad18 or associations between unfixed chromatin-bound proteins (L.R.B. and C.V., data not shown). Therefore, it is likely that the associations between TLS polymerases and Rad18 that we detected in our chromatin IP assays are indirect and perhaps mediated by PCNA and/or DNA. Alternatively, putative direct associations that exist between Polk or Polη and Rad18 might be weak, transient, and dynamic or poorly preserved in vivo under the experimental conditions used in this study.

**FIG. 4. DNA damage-induced association between GFP-Polk and PCNA requires Rad18-dependent PCNA monoubiquitination.** (A) H1299 cells were infected with AdHA-PCNA-WT or AdHA-PCNA-K164A individually or in combination with AdHA-Rad18. At 48 h postinfection, some cultures were treated with 600 nM BPDE. Cells were harvested 6 h after BPDE treatment. Chromatin fractions were collected and analyzed for expression of HA-tagged PCNA. (B) H1299 cells were coinfected with AdGFP-Polk and AdHA-PCNA-WT or AdHA-PCNA-K164A. Twenty-four hours after infection, cells were given 600 nM BPDE or 10 J/m² of UVC or left untreated as controls. Chromatin extracts from the cells were immunoprecipitated with anti-HA antibody. The resulting immune complexes were analyzed for associated HA-Rad18 by SDS-PAGE and Western blotting.

As a positive control for these experiments, we observed association between Rad18 and Polk. Chromatin fractions from cells expressing HA-Rad18 and YFP-Polη or GFP-Polk were fixed and sheared by sonication as described by Watanabe et al. (43). Solubilized chromatin fractions were immunoprecipitated with anti-GFP antisera, and the resulting immune complexes were analyzed for associated HA-Rad18 by SDS-PAGE and Western blotting.
Rad18 and Polκ interact (directly or indirectly) within intact cells. These data are consistent with a role for Rad18 in Polκ-mediated TLS.

**Rad18 deficiency perturbs recovery from BPDE-induced S-phase checkpoint.** The experiments described above suggested an important role for Rad18 in PCNA modification and Polκ recruitment after BPDE treatment. Previously, we reported that Polκ deficiency results in defective recovery from the BPDE-induced S-phase checkpoint. Since Rad18 is important for Polκ regulation, we expected that Rad18−/− and Polκ−/− cells would have similar checkpoint recovery defects. To test this prediction, Rad18−/− and WT MEFs (derived from Rad18 knockout mice and WT animals, respectively) were analyzed for S-phase checkpoint responses to BPDE. Exponentially growing cultures of Rad18+/+ and Rad18−/− cells were treated with 100 nM BPDE. Then, at different time points after BPDE treatment, we determined rates of DNA synthesis by using [3H]thymidine incorporation assays.

As shown in Fig. 6A, 2 h after BPDE treatment, DNA synthesis was reduced by 40% in Rad18+/+ cells. However, 4 h post-BPDE treatment, rates of DNA synthesis recovered to control levels. In Rad18−/− cells, BPDE inhibited DNA synthesis with kinetics similar to those of WT MEFs, but DNA synthesis failed to recover to control levels within the time frame of this experiment. These data suggest a requirement for Rad18 in recovery from the BPDE-induced S-phase checkpoint. We performed similar experiments to determine the role of Rad18 in recovery from the UV-induced checkpoint. As shown in Fig. 6B, recovery from UV-induced S-phase arrest was also defective in Rad18−/− cells.

We considered the possibility that Rad18−/− cells might have a general defect in recovery from S-phase arrest. Therefore, we examined recovery from S-phase arrest induced by other agents, including IR (which induces DNA DSBs) and HU (which depletes deoxyribonucleoside triphosphate pools required for DNA synthesis). Figure 6C and D show the rates of DNA synthesis in Rad18+/+ and Rad18−/− cells at different times after treatment with IR and HU, respectively. As shown in these experiments, the kinetics of inhibition of DNA synthesis after HU or IR treatment, and the kinetics with which DNA synthesis resumed, were indistinguishable between Rad18+/+ and Rad18−/− cells. Therefore, the defective recovery of Rad18−/− cells from the S-phase checkpoint is relatively specific for BPDE- and UV-induced lesions. We have observed no loss of viability (as measured by trypan blue staining) in BPDE-treated or UV-irradiated Rad18+/+ or Rad18−/− cells during the ~8-h time period of the DNA synthesis measurements (X.B. and C.V., data not shown). Therefore, defective checkpoint recovery of Rad18−/− cells (as measured by our DNA synthesis assays) is not simply a consequence of increased mortality after genotoxic treatment.

It was formally possible that defects other than Rad18 deletion resulted in the checkpoint recovery defects we observed in Rad18-null MEFs. To address this possibility, we performed transient-expression experiments to reconstitute Rad18 in Rad18−/− MEFs. As shown in Fig. 6E, ectopic expression of HA-Rad18 corrected the defective checkpoint recovery of Rad18−/− cells. Taken together, our data show that Rad18 is required for recovery from the BPDE-induced S-phase checkpoint.
Checkpoint signaling in Rad18−/− cells. S-phase checkpoint signaling is thought to result when replicative enzymes encounter DNA lesions, thereby uncoupling the activities of replicative helicases from fork progression (9). Our results suggested that replication forks in Rad18-null cells fail to carry out TLS of BPDE-adducted DNA and remain stalled. Therefore, we predicted that checkpoint signaling would be elevated in Rad18−/− cells relative to WT cells (which accumulate fewer blocked replication forks). To test this prediction, we treated cultures of Rad18+/+ or Rad18−/− cells with 100 nM BPDE. At different time points after BPDE treatment, the cells were lysed and the resulting protein extracts were analyzed for activation of checkpoint kinases. Chk1 is activated by replication blocks, and we previously showed that Chk1 mediates BPDE-induced S-phase arrest (20). We determined the activation status of Chk1 by using phosphospecific antisera against serine
Stalled replication forks are prone to breakage if left unprotected (10). If Rad18/Pok-mediated TLS contributes to protection or elimination of stalled forks, we expected that defective TLS of BPDE-adducted DNA might result in increased formation of DNA DSBs. In contrast to bulky adducts and replication blocks that principally elicit ATR/Chk1 signaling, DSBs are considered to activate ATM/Chk2 signaling pathways (3). We used the protein extracts from BPDE-treated Rad18\(^{+/+}\) and Rad18\(^{−/−}\) cells to monitor active Chk2 (which is readily detected with phosphospecific antisera against phospho-T384). As shown in Fig. 7B, Chk2 phosphorylation was strongly induced by BPDE treatment in Rad18\(^{−/−}\) cells (but not in Rad18\(^{+/+}\) cultures). We obtained similar results in experiments with UV as a genotoxin (Fig. 7C). At different times after genotoxin treatment, cells were trypsinized and counted and then replated at a density of 1,000 cells/10-cm plate. The resulting plates were returned to the incubator and given fresh growth medium every 3 days. After 7 days, cells were fixed and stained with Giemsa. Giemsa-stained colonies containing >50 cells were scored.

![FIG. 7. Checkpoint signaling in Rad18\(^{−/−}\) MEFs. Rad18\(^{+/+}\) or Rad18\(^{−/−}\) MEFs were treated with 100 nM BPDE (A and B) or 10 J/m\(^2\) UV (C). At different times after genotoxin treatment, cells were lysed and the resulting protein extracts were analyzed for activation of checkpoint kinases with phosphospecific antibodies against Chk1 (A and B) or Chk2 (C). NS indicates nonspecific bands recognized by the antibodies that served as loading controls.](http://mcb.asm.org/)
Thr68-phosphorylated Chk2 (soluble fraction) levels were prepared and analyzed directly for PCNA (chromatin fraction) and untreated as controls. After 4 h, soluble and chromatin fractions were immunoblotted (IB) with anti-PCNA or anti-GFP antibodies. Nuclear fractions were immunoprecipitated with anti-PCNA, resulting cultures were given 600 nM BPDE, and 6 h later the cells were lysed. Nuclear fractions were immunoprecipitated with anti-PCNA, and the resulting immune complexes were resolved by SDS-PAGE and immunoblotted (IB) with anti-PCNA or anti-GFP antibodies. (A) H1299 cells were transfected with siRNA oligonucleotide duplexes against ATR and RPA or with control Cy3 RNA oligonucleotides. Some cultures were infected with AdChk1KR or AdCon. The resulting cultures were analyzed for ATR, RPA, or Chk1 expression with appropriate antibodies. (B) H1299 cells expressing GFP-Polx were transfected with siRNA oligonucleotides against ATR, RPA, and Rad18 or with Cy3 (control) oligonucleotides. Alternatively, some cultures were infected with AdChk1KR. The resulting cultures were given 600 nM BPDE, and 6 h later the cells were lysed. Nuclear fractions were immunoprecipitated with anti-PCNA, and the resulting immune complexes were resolved by SDS-PAGE and immunoblotted (IB) with anti-PCNA or anti-GFP antibodies. (C) ET163 and YZ5 cells were treated with 600 nM BPDE or left untreated as controls. After 4 h, soluble and chromatin fractions were prepared and analyzed directly for PCNA (chromatin fraction) and Thr68-phosphorylated Chk2 (soluble fraction) levels.

BPDE (or left untreated as controls). PCNA was immunoprecipitated from solubilized chromatin extracts, and PCNA ubiquitination and levels of PCNA-associated GFP-Polx in the immune complexes were determined by immunoblotting (Fig. 9B). As expected, BPDE induced PCNA ubiquitination and association between Polx and PCNA in Cy3 control siRNA-transfected cultures. However, in cells transfected with siATR and siRPA duplexes, both PCNA ubiquitination and association between PCNA and Polx were reduced by approximately 60%.

Since Chk1 is an important effector of ATR and 9-1-1 in the S-phase checkpoint, we asked if Chk1 was involved in Polk regulation. We previously generated a kinase-inactive dominant-negative mutant form of Chk1 that we expressed with an adenovirus vector. We and others have shown that this reagent can be used to inhibit S and G2 checkpoints mediated by Chk1 signaling (20, 21). Therefore, we tested the effect of dominant-negative Chk1 on the BPDE-induced association between PCNA and Polk. As shown in Fig. 9B, expression of dominant-negative Chk1 reduced the levels of monoubiquitinated PCNA and the amount of PCNA-associated Polk in BPDE-treated cells by 50% relative to those in control cultures. Therefore, Chk1 signaling contributes to PCNA modification and Polk regulation.

A recent publication by Jazayeri et al. demonstrated that ATR activation by DSBs is regulated by ATM in a cell cycle-dependent manner (24). Therefore, it was of interest to determine if ATM is required for BPDE-induced (and ATR/Chk1-dependent) PCNA ubiquitination. To test the role of ATM in BPDE-induced PCNA ubiquitination, we compared the effect of BPDE treatment on PCNA ubiquitination in ET163 fibroblasts from AT patients and in matched cells designated YZ5 that express reconstituted ATM (46). As shown in Fig. 9C, BPDE-induced PCNA ubiquitination was evident in both ET163 and YZ5 cells and therefore is ATM independent. As expected, DNA damage-induced Chk2 phosphorylation (which is known to be largely ATM dependent) was only observed in YZ5 cells. Taken together, these data suggest that efficient PCNA ubiquitination requires ATR and Chk1, but not the ATM pathway.

**DISCUSSION**

Our previous work demonstrated an important role for Polk in recovery from the BPDE-induced S-phase checkpoint (6). Those studies suggested that Polk is recruited to replication forks stalled by BPDE-adducted DNA and that subsequent Polk-mediated lesion bypass allows attenuation of the S-phase checkpoint. Here we have investigated the mechanism(s) that recruits Polk to stalled replication forks. We show that PCNA is ubiquitinated in a Rad18-dependent manner after acquisition of DNA damage and that Polk associates specifically with monoubiquitinated PCNA. Further, consistent with a role for Rad18-mediated PCNA monoubiquitination in Polk regulation, Rad18−/− and Polk−/− cells have similar defects in recovery from the BPDE-mediated S-phase checkpoint.

Although we have shown that Polk forms a complex with monoubiquitinated PCNA, we have not demonstrated that this interaction is direct. Therefore, it is possible that the interaction of Polk with monoubiquitinated PCNA is mediated by another TLS polymerase and/or other factors. For example,

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**FIG. 9. Roles of RPA, ATR, and Chk1 in BPDE-induced PCNA ubiquitination and Polk recruitment.** (A) H1299 cells were transfected with siRNA oligonucleotide duplexes against ATR and RPA or with control Cy3 RNA oligonucleotides. Some cultures were infected with AdChk1KR or AdCon. The resulting cultures were analyzed for ATR, RPA, or Chk1 expression with appropriate antibodies. (B) H1299 cells expressing GFP-Polx were transfected with siRNA oligonucleotides against ATR, RPA, and Rad18 or with Cy3 (control) oligonucleotides. Alternatively, some cultures were infected with AdChk1KR. The resulting cultures were given 600 nM BPDE, and 6 h later the cells were lysed. Nuclear fractions were immunoprecipitated with anti-PCNA, and the resulting immune complexes were resolved by SDS-PAGE and immunoblotted (IB) with anti-PCNA or anti-GFP antibodies. (C) ET163 and YZ5 cells were treated with 600 nM BPDE or left untreated as controls. After 4 h, soluble and chromatin fractions were prepared and analyzed directly for PCNA (chromatin fraction) and Thr68-phosphorylated Chk2 (soluble fraction) levels.
Polη and Polκ interact directly and Polη is required for genotoxin-induced redistribution of Polκ to replication foci (27). In contrast to Polκ, we have shown that the recruitment of Polκ to ubiquitinated PCNA does not require Polη. Our finding that the association of Polκ with PCNA is Polη independent is consistent with previous reports that XP cells do not show increased sensitivity to BPDE (8, 13) or defects in recovery from the BPDE-induced S-phase checkpoint (6).

Although Polη is not required for BPDE-induced association of Polκ with monoubiquitinated PCNA, it is possible that an alternative TLS polymerase helps recruit Polκ to the replication fork. However, a recent study showed that Y family polymerases, including Polκ, contain novel Ub-binding motifs (7), and it is also likely that Polκ and monoubiquitinated PCNA interact directly.

Okada et al. previously found additive effects of Rad18 and Polκ deletion in chicken DT40 cells (37), arguing against a dependence of Polκ on Rad18 gene function in this experimental system. This contrasts with our finding that Polκ is regulated in a Rad18-dependent manner in mammalian cells. It is possible that Polκ is regulated via Rad18-independent mechanisms in avian cells. Alternatively, the Rad18 independence of Polκ regulation may be an idiosyncrasy of DT40 cells. Recombination activity is very high in DT40, as indicated by the very high efficiency of gene targeting in these cells. It is possible that DNA damage tolerance in DT40 depends more heavily on a DNA recombination pathway than on TLS compared with other systems. Another possibility is that the Rad18 mutant described by Okada and colleagues is not a complete genetic null. Regardless of the reason for the Rad18 independence of Polκ regulation in DT40 cells, our data demonstrate a role for Rad18 in Polκ regulation in mammalian cell lines. As discussed above, the recent finding that Polκ contains a Ub-binding motif (7) provides a plausible molecular basis for direct recruitment of Polκ to ubiquitinated PCNA and supports a role for Rad18 in Polκ regulation.

Interestingly, our data indicate that ATR/Chk1-mediated S-phase checkpoint signaling may contribute to PCNA ubiquitination and Polκ recruitment in response to DNA damage. Taken together, our results suggest the model in Fig. 10, whereby replication blocks initiate ATR/Chk1 signaling. Chk1 activity inhibits late origin firing and stabilizes stalled replication forks. Potentially, stabilization of a stalled fork could facilitate PCNA ubiquitination and recruitment of TLS polymerases indirectly. Alternatively, Chk1 signaling could directly stimulate PCNA ubiquitination, perhaps via Rad18 activation. Subsequently, Polκ-mediated lesion bypass recovers the stalled replication fork, thereby attenuating checkpoint signaling and enabling resumption of DNA synthesis. In the absence of lesion bypass, stalled forks collapse to generate DSBs that elicit ATM/Chk2 signaling. A model in which ATR/Chk1 signaling is a prerequisite for TLS is not necessarily implied since overexpression of Rad18 can elicit DNA damage-independent PCNA ubiquitination. Therefore, checkpoint signaling is likely to modulate TLS but is probably not an absolute requirement for PCNA ubiquitination and Polκ recruitment.

Other workers have also suggested a role for checkpoint proteins in Polκ regulation. Kai and Wang showed that the 9-1-1 complex recruits DinB (Schizosaccharomyces pombe Polκ) by direct interactions with Hus1p (26). In unpublished studies, we have also tested a putative role for 9-1-1 in Polκ regulation in mammalian cells. By co-IP or immunofluorescence microscopy, we were unable to demonstrate biochemical associations or colocalization between Polκ and 9-1-1 proteins in mammalian cells, even when using overexpressed Polκ and 9-1-1 components (B.X.H. and C.V., unpublished results). However, because efficient Polκ recruitment in mammalian cells requires Chk1 signaling, and since BPDE-induced Chk1 activation requires 9-1-1 (44), there is an indirect requirement for Hus1 in Polκ regulation in mammalian cells. Therefore, mammalian cells and S. pombe require 9-1-1 for efficient Polκ recruitment. The regulation of Polκ (and possibly other TLS enzymes) by checkpoints is likely to be conserved in eukaryotes.

We do not know the precise mechanism by which Chk1 signaling contributes to PCNA ubiquitination and Polκ recruitment. Checkpoint signaling could stimulate PCNA ubiquitination and interactions with TLS enzymes via phosphorylation of Rad18 and/or Polκ. Alternatively, the critical role of checkpoint signaling in Polκ recruitment might be indirect via stabilization of the stalled replication fork. The major role of the S-phase checkpoint is considered to be stabilizing the replication fork and preventing the collapse of stalled forks via an unknown mechanism(s) (16, 31, 40). Since checkpoint signaling is important for Polκ recruitment, it is interesting to speculate that stalled replication forks are stabilized in part via
recruitment of TLS enzymes. Clarification of these issues will require a more detailed analysis of mechanisms that stimulate PCNA ubiquitination.

The mechanism by which Rad18-mediated monoubiquitination of PCNA occurs in response to DNA damage is not clear. Potentially, PCNA ubiquitination could result from an increase in Rad18 activity, from decreased deubiquitination of PCNA, or from a combination of both mechanisms. Experimentally increasing Rad18 activity inside cells by adenovirus-mediated overexpression or from a combination of both mechanisms. Experimentally increasing Rad18 activity inside cells by adenovirus-mediated overexpression or from a combination of both mechanisms.

Additionally, there exists a precedent for regulated deubiquitination as a branch of the DNA damage-signaling pathway. D’Andrea and colleagues showed that the deubiquitinating (DUB) enzyme USP1 is involved in removal of monoubiquitin from the FANCD2 protein and that USP1 is downregulated by ubiquitination. The mechanism by which Rad18-mediated monoubiquitination of PCNA occurs in response to DNA damage is not clear. Potentially, PCNA ubiquitination could result from an increase in Rad18 activity, from decreased deubiquitination of PCNA, or from a combination of both mechanisms. Experimentally increasing Rad18 activity inside cells by adenovirus-mediated overexpression or from a combination of both mechanisms.

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