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ATR-mediated phosphorylation of DNA polymerase η is needed for efficient recovery from UV damage

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DNA polymerase η (polη) belongs to the Y-family of DNA polymerases and facilitates translesion synthesis past UV damage. We show that, after UV irradiation, polη becomes phosphorylated at Ser601 by the ataxia-telangiectasia mutated and Rad3-related (ATR) kinase. DNA damage–induced phosphorylation of polη depends on its physical interaction with Rad18 but is independent of PCNA monoubiquitination. It requires the ubiquitin-binding domain of polη but not its PCNA-interacting motif. ATR-dependent phosphorylation of polη is necessary to restore normal survival and postreplication repair after ultraviolet irradiation in xeroderma pigmentosum variant fibroblasts, and is involved in the checkpoint response to UV damage. Taken together, our results provide evidence for a link between DNA damage–induced checkpoint activation and translesion synthesis in mammalian cells.

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Introduction

Cellular DNA sustains many types of DNA damage, much of which is removed by excision-repair pathways. Most unrepaired lesions block the replication machinery. Cells have therefore developed damage tolerance mechanisms either to avoid the damage during replication or to replicate past the lesion (Friedberg, 2005). Translesion DNA synthesis (TLS), the major process with which mammalian cells overcome replication blocks (Lehmann, 2005), is performed by a class of specialized DNA polymerases. These enzymes possess a spacious active site and are able to accommodate a variety of DNA lesions that block the high fidelity replicative polymerases (Prakash et al., 2005). Most TLS polymerases belong to the Y-family, which includes Polη, Polκ, Polι, and Rev1 (Ohmori et al., 2001). Polη is the best characterized of these enzymes and is required for accurate replicative bypass of cyclobutane pyrimidine dimers induced by UV radiation (McCulloch et al., 2004). In humans, loss of Polη activity results in the variant form of xeroderma pigmentosum (XPV; Johnson et al., 1999; Masutani et al., 1999).

A crucial step during TLS is the polymerase switch, in which the stalled replicative polymerase is replaced by a specialized TLS polymerase. This process has been linked to DNA damage–induced PCNA monoubiquitination (Hoege et al., 2002; Stelter and Ulrich, 2003; Kannouche et al., 2004). Monoubiquitination of PCNA occurs at lysine 164 and is performed by the E2 ubiquitin-conjugating enzyme Rad6 and the E3 ubiquitin ligase Rad18 (Hoege et al., 2002; Stelter and Ulrich, 2003; Watanabe et al., 2004). Monoubiquitinated PCNA has an increased affinity for polη, which helps to recruit polη to stalled replication forks (Kannouche et al., 2004; Watanabe et al., 2004). All TLS polymerases contain ubiquitin-binding domains located close to their C termini, which are responsible for mediating interactions with monoubiquitinated PCNA (Bienko et al., 2005; Plosky et al., 2006).

In this study we show that, in human cells, polη becomes phosphorylated by ATR at Ser601 after UV irradiation. Phosphorylation requires physical interaction of polη with Rad18 but is independent of PCNA monoubiquitination. We show that UV-induced phosphorylation of polη is required for normal survival and postreplication repair and is involved in checkpoint control.

Results and discussion

Polη is phosphorylated after UV irradiation

We recently showed that a proportion of polη exists in a monoubiquitinated form in human fibroblasts and this was lost when

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Abbreviations used in this paper: ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia mutated and Rad3-related; polη, polymerase η; PRR, post-replication repair; TLS, translesion DNA synthesis; XPV, xeroderma pigmentosum.

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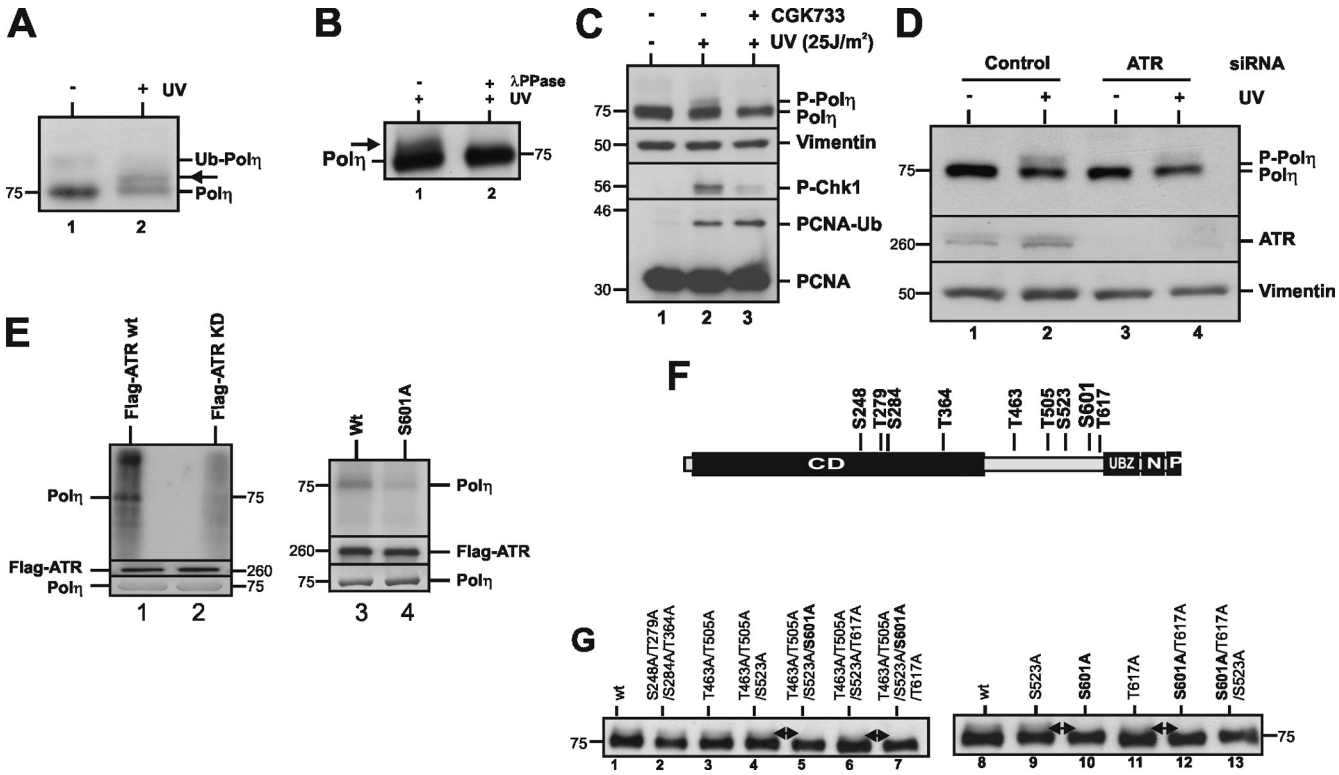

Figure 1. **Polη is phosphorylated at Ser601 after UV irradiation.** (A) Anti-polη Western blot analysis of cell lysates from either unirradiated or UV-irradiated (25 J/m^2) MRC5 cells, incubated for 6 h. The band of ubiquitinated polη (only seen in unirradiated cells) is indicated and the band of interest (only in irradiated cells) is denoted with an arrow. (B) polη was immunoprecipitated from UV-irradiated MRC5 cells (25 J/m^2), incubated for 6 h, and immunoprecipitates were split in half and treated with or without λPPase. (C) MRC5 cells were treated with and without an ATM/ATR inhibitor (10 µM CGK733). Cells were UV irradiated (25 J/m^2) and cell lysates were analyzed 6 h after irradiation. (D) MRC5 cells were transfected with control (lanes 1 and 2) or ATR siRNA (lanes 3 and 4), UV irradiated 48 h later (25 J/m^2), and incubated for a further 6 h. (E) In vitro kinase assay with wild-type Flag-ATR (lanes 1, 3, 4) and a kinase-dead Flag-ATR (lane 2) immunoprecipitated from HEK 293 cells. The substrate was recombinant wild-type His$_6$-polη (lanes 1–3) or S601A mutant (lane 4). Bottom panels show Western blot of immunoprecipitated wild-type and kinase-dead Flag-ATR and Coomassie staining of recombinant polη. (F) Schematic of polη. CD, catalytic domain; UBZ, ubiquitin-binding zinc-finger motif; N, nuclear localization sequence; P, PIP box PCNA-binding motif. Potential ATR phosphorylation sites (S/TQ) are indicated. (G) MRC5 cells were transfected with different polη mutant constructs. 24 h after transfection cells were UV irradiated (25 J/m^2), and 6 h later cell lysates were prepared and analyzed. Double-headed arrows indicate loss of phosphorylated polη to the right and presence to the left.

cells were exposed to DNA-damaging treatments (Bienko et al., 2005, 2010; see also Fig. 1 A, top band, lane 1). In UV-irradiated MRC5 human fibroblasts, we noticed a hint of another subpopulation of polη with a very slightly reduced mobility (but with higher mobility than ubiquitinated polη). By using longer gels and running times, we were able to visualize the slower-migrating form (Fig. 1 A, arrow), which was not detectable in unirradiated cells (Fig. 1 A). It sometimes migrated as a band that was clearly discernible from unmodified protein, but in other experiments produced a less defined signal migrating just above unmodified polη.

When we immunoprecipitated polη from UV-irradiated MRC5 cells and treated immunoprecipitates with λ-phosphatase, the mobility shift was abolished (Fig. 1 B), indicating that the shifted band represented a phosphorylated form of polη (P-polη). Major regulators of the DNA damage response are the protein kinases ataxia-telangiectasia mutated (ATM) and ATR. When we treated MRC5 cells with an inhibitor of ATM/ATR kinases (CGK733, Calbiochem; Alao and Sunnerhagen, 2009), there was a significant reduction of P-polη (Fig. 1 C, top, compare lanes 2 and 3). There was also a strong reduction in UV-induced P-polη in MRC5 cells treated with ATR siRNA (Fig. 1 D, top, compare lanes 2 and 4), showing that the phosphorylation of polη is ATR dependent. We found no effect of knocking down Chk1, ATM, or Chk2 (unpublished data). These results show that polη is phosphorylated in an ATR-dependent manner after UV irradiation, but this is not dependent on the downstream checkpoint kinases.

Polη is directly phosphorylated by ATR on Ser601

We next expressed Flag-tagged wild-type ATR (ATR-wt) and a kinase-dead version (ATR-KD) in HEK-293 cells. ATR was immunoprecipitated with α-Flag antibody, and kinase assays were performed using His$_6$-polη protein purified from insect cells as a substrate. ATR-wt phosphorylated His-polη, whereas no phosphorylation could be detected with ATR-KD (Fig. 1 E). ATR kinase preferentially phosphorylates SQ/TQ sites (Traven and Heierhorst, 2005). We mutated each of the nine SQ/TQ sites in Polη (Fig. 1 F) to AQ either individually or in combinations. Mutant versions of polη were transfected into MRC5 cells, which were then UV irradiated. Fig. 1 G shows that the phosphorylated polη species was abolished in those samples in which Ser601 was mutated (Fig. 1 G, lanes 5, 7, 10, 12, and 13, compare lanes on the

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right of each double arrow with those on the left). This strongly suggests that the phosphorylation occurs on Ser601. We therefore repeated the ATR kinase assay, using polη that was either wild type or mutated at Ser601. In vitro phosphorylation was substantially reduced with the mutant construct (Fig. 1 E, lanes 3 and 4), consistent with Ser601 being the major direct substrate of ATR kinase. Ser601 is in a poorly conserved region of polη, but is itself conserved in mammals. Although it is not followed by gln in mouse polη, there is an SQ sequence a few residues downstream, which may be the corresponding phosphorylation site.

In a previous report, Chen et al. (2008) showed increased phosphorylation after UV irradiation in polη immunoprecipitates from EGFP-polη–transfected XP30RO cells. The authors also showed that XPV cells expressing polη-T617A were more sensitive to UV irradiation than cells expressing wild-type polη, and proposed, without further evidence, that Thr617 was the site of phosphorylation. We have confirmed the UV sensitivity of XP30RO cells expressing mutant polη-T617A (unpublished data). However, although we cannot exclude Thr617 as a phosphorylation site, our results of Fig. 1 G provide no support for this proposal.

Damage-dependent phosphorylation of the Y-family member Rev1 in *Saccharomyces cerevisiae* has been reported by two groups (Sabbioneda et al., 2007; Pagès et al., 2009). This phosphorylation was mediated by the ATR orthologue Mec1, but no phosphorylation of the Polη orthologue Rad30 was detected.

Phospho-specific antibody

We next generated a phospho-specific antibody that recognizes the epitope MDLAHNS(*)QSMHAS (the asterisk denotes phosphorylation), corresponding to the sequence spanning Ser601. The antibody recognizes a band with molecular weight similar to that of polη (~75 kD) in UV-irradiated MRC5 but not in polη-deficient XP30RO cells (Fig. 2 A, compare lanes 2 and 4). In XP30RO cells transfected with wild-type or S601A mutant polη, the band was detected in the wild-type but not in the mutant transfectants (Fig. 2 A, compare lanes 6 and 8). It disappeared on incubation of immunoprecipitates with λ-phosphatase (Fig. 2 B, compare lanes 3 and 4). These results confirm that the antibody specifically recognizes polη phosphorylated on Ser601.

With this antibody, we were able to detect P-polη in cells treated with the replication inhibitor hydroxyurea, to a lesser extent with the DNA cross-linker cisplatin and the topo-isomerase I inhibitor camptothecin, but not in cells exposed to ionizing radiation (Fig. S1). Although ionizing radiation results in activation of ATR several hours after irradiation, this does not appear to be sufficient to phosphorylate polη at several times after radiation treatment (3, 6, and 24 h after 10 Gy irradiation; unpublished data). We confirmed that P-polη was reduced in cells treated with ATR siRNA (Fig. 2 C, top).

The response to UV was dose and time dependent (Fig. 2, D and E). In nuclear fractionation experiments, P-polη was detectable in the chromatin fraction (Fig. 2 F, lane 4), with little in the soluble fraction (lane 3). The time-course of appearance of P-polη in the chromatin fraction roughly paralleled that of total polη (Fig. 2 G), and the distribution of (unmodified) polη between the soluble and chromatin fractions was similar for wild type (Fig. 2 H, lanes 1–4) and the S601A mutant (lanes 5–8). We conclude that phosphorylation occurs only in the chromatin fraction but it does not affect the distribution of polη between the different nuclear fractions.

Rad18 and the UBZ domain of polη are necessary for Ser601 phosphorylation

Polη is localized in replication foci during S phase and the number of cells with polη foci increases after UV irradiation (Kannouche et al., 2001). Rad18 is required for polη focus formation and Rad18 and polη interact constitutively through sequences in their C terminus (Watanabe et al., 2004). P-Polη levels were strongly reduced in cells treated with Rad18 siRNA (Fig. 3 A, top, compare lanes 2 and 4).

Rad18 targets polη to stalled replication forks and is an E3 ubiquitin ligase for monoubiquitination of PCNA. We transfected MRC5 cells with two different dominant-negative constructs, expressing Rad18 either lacking the polη-binding domain (Rad18 DC2) or mutated in the RING finger domain (Rad18–C28F; Watanabe et al., 2004). There was a strong reduction in P-polη in cells transfected with Rad18 DC2 (Fig. 3 B, lanes 2 and 4), suggesting that recruitment of polη to the chromatin by physical interaction with Rad18 is necessary for its phosphorylation. When cells were transfected with Rad18 C28F, although PCNA ubiquitination was reduced as expected (Fig. 3 C, bottom), there was no change in P-polη, indicating that the E3 ubiquitin ligase activity of Rad18 is dispensable for P-polη phosphorylation. This suggests that PCNA monoubiquitination is not necessary for polη phosphorylation. We confirmed this using an MRC5 cell line expressing His_6-PCNA mutated at lysine 164, which cannot be ubiquitinated after UV irradiation (Niimi et al., 2008). There was no difference from wild type in P-polη levels in these cell lines (Fig. S2 A, top, lanes 2 and 4).

Three motifs that are important for function of polη are the nuclear localization sequence, the UBZ ubiquitin-binding motif, and the PIP box PCNA interaction motif (see Fig. 1 F). These are all involved in direct interaction with ubiquitinated PCNA (Bienko et al., 2005, 2010). Fig. 3 D shows that polη with mutations in the PIP box (FF708-709AA) had P-polη levels similar to the wild-type protein after UV irradiation (compare lanes 2 and 4). Acharya et al. (2008) have suggested that there is a second PIP box in polη at aa 443–444 (PIP1). We have compared phosphorylation in cells expressing wild-type polη with that in cells expressing polη mutated in either or both PIP boxes. In no case was phosphorylation significantly affected (Fig. 3 E). In striking contrast, a mutation in the UBZ domain (D652A), which prevents binding to ubiquitin, resulted in a marked decrease in P-polη (Fig. 3 D, lane 6).

In earlier work, we identified a ubiquitinated form of polη that disappeared after UV irradiation and other types of DNA damage (Bienko et al., 2010). The presence of ubiquitinated polη and its disappearance after UV irradiation were similar for wild-type and S601A mutant (Fig. S2 B). Likewise,

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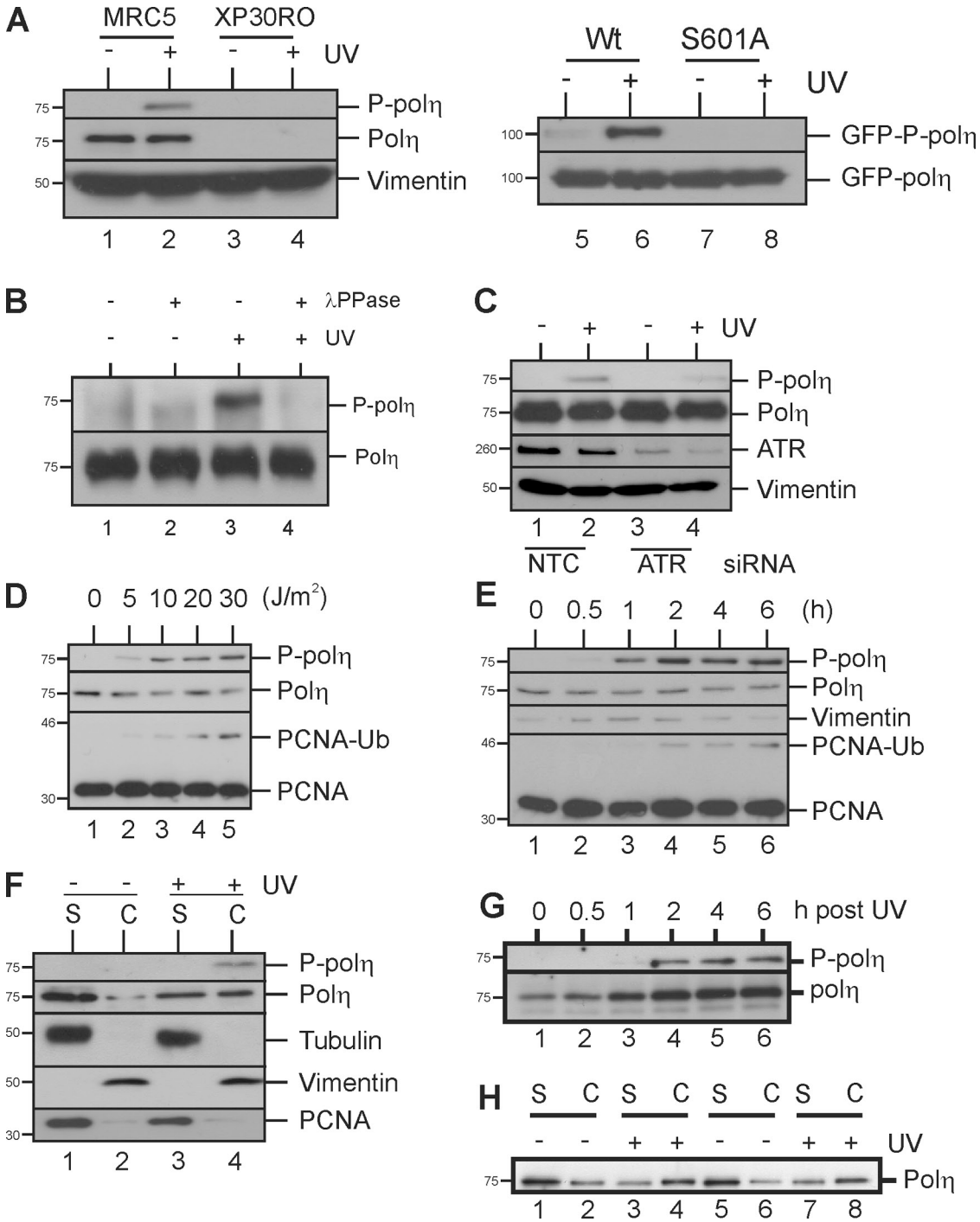

Figure 2. **Use of phospho-specific antibody to characterize polη phosphorylation.** (A) Analysis of lysates from cells that were either unirradiated or UV irradiated (25 J/m^2) and incubated for 6 h. MRC5 (lanes 1 and 2), XP30RO (lanes 3 and 4), or MRC5 cells transfected with eGFP-polη (lanes 5 and 6) or with eGFP-polη-S601A (lanes 7 and 8). (B) polη immunoprecipitates from irradiated (25 J/m^2) and unirradiated MRC5 cells, incubated for 6 h, were either treated or untreated with λPPase. (C) MRC5 cells were depleted of ATR and treated as described in Fig. 1 D. (D) MRC5 cells were either unirradiated or UV irradiated and incubated for 6 h. (E) MRC5 cells either unirradiated or irradiated (20 J/m^2) were incubated for the indicated times. (F) MRC5 cells either unirradiated or UV irradiated (25 J/m^2) were incubated for 6 h and then extracted with Triton X-100. Triton-soluble fractions (S, lanes 1 and 3) and insoluble chromatin fractions (C, lanes 2 and 4) were subjected to Western blot analysis. Tubulin and vimentin were used as cytoplasmic and nuclear marker, respectively. (G) UV-irradiated cells were incubated for different times after UV irradiation and extracted with Triton X-100. Western blots of chromatin fractions were probed with antibody to the phosphorylated form and to total polη. (H) The experiment of Fig. 1 F was repeated but using cells expressing either wild-type polη (lanes 1–4) or S601A mutant (lanes 5–8) and blots probed with antibody to total polη.

phosphorylation of polη was similar in wild-type and 4K/R mutant cells (Bienko et al., 2010) that cannot be ubiquitinated (unpublished data).

Together these results show that chromatin recruitment of polη by physical interaction with Rad18 and an intact UBZ motif in polη are necessary for DNA damage–induced

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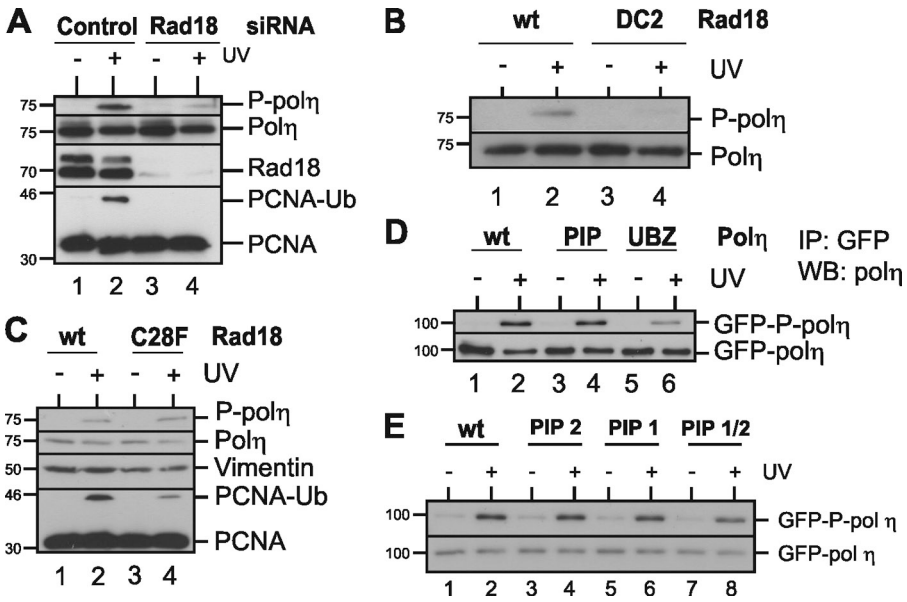

Figure 3. **Polη phosphorylation depends on interaction with Rad18.** (A) MRC5 cells were incubated with control (lanes 1 and 2) or Rad18 siRNA (lanes 3 and 4). Cells were either unirradiated or UV irradiated (20 J/m^2) and incubated for 6 h. (B) MRC5 cells were transfected with a wild-type Rad18 construct (lanes 1 and 2) or Rad18 DC2 (lanes 3 and 4) together with GFP as a transfection marker. Sorted cells were either unirradiated or UV irradiated and incubated for 6 h. (C) As in B, but using Rad18 C28F (lanes 3 and 4). (D) MRC5 cells were transfected with wild-type EGFP-polη (lanes 1 and 2), PIP box mutant (lanes 3 and 4), or UBZ mutant (lanes 5 and 6). Cells were either unirradiated or UV irradiated (20 J/m^2), incubated for 6 h, and polη proteins were immunoprecipitated with a GFP antibody, followed by immunoblotting with anti-polη and the P-polη antibody. (E) As in D, but using cells expressing polη that was wild-type, mutated at PIP1 (aa 443-444), PIP2 (aa 707,708), or both.

phosphorylation. Interestingly, interaction with PCNA, and PCNA monoubiquitination are dispensable for this modification.

Phosphorylation of polη impacts on DNA damage tolerance

XP30RO cell lines stably expressing wild-type EGFP-polη, EGFP-polη-S601A, and a phosphomimic mutant (S601D) were compared in a UV clonogenic survival assay. To ensure that we always used populations expressing the same level of GFP-polη, the cell lines were sorted in every experiment shown in Figs. 4 and 5. The S601A mutant displayed lower survival when compared with XP30RO cells complemented with wild-type EGFP-polη, whereas the phosphomimic mutant showed levels of survival similar to the wild-type protein (Fig. 4 A). Survival analysis of XPV cells entails the use of caffeine in the post-irradiation incubation medium. We are aware that caffeine is an inhibitor of ATR. However, at the low concentrations of caffeine used in our experiments, ATR-mediated Chk1 activation was unaffected (Fig. S2 C) and there was substantial polη phosphorylation (Fig. S2 D).

We examined polη-containing replication foci formation after UV irradiation but observed no differences in foci formation in cells expressing wild-type EGFP-polη and EGFP-polη-S601A proteins (Fig. S3).

Post-replication repair (PRR) activity was assayed by pulse-labeling UV-irradiated cells with ^{3}H-thymidine to label newly synthesized strands of DNA and then incubating further without radioactive precursor. The size distribution of the labeled DNA strands was measured on alkaline sucrose gradients. The deficiency in this process in XPV cells (Lehmann et al., 1975) could be rescued by wild-type EGFP-polη. In agreement with our survival data, we found a small decrease in the rescue of PRR in cells expressing the S601A mutant protein (Fig. 4 B). Though modest, this decrease was reproducible in three independent experiments (Fig. 4 B, inset).

The modest defects in survival and PRR of the S601A mutant are reminiscent of results that we reported recently with PIP, NLS, and UBZ mutants of polη (Bienko et al., 2010). We also showed that the effects of mutations in two of these motifs were much more dramatic than the single mutations (see also Schmutz et al., 2010). Accordingly, we examined the effect of the S601A mutation together with mutations in one of these motifs. As seen in Fig. 4 C, the survivals of PIP and UBZ mutant alone are similar to the S601A mutant. The UBZ/S601A double mutant is only slightly more sensitive than the UBZ mutant alone, but the PIP/S601A mutant protein shows a dramatically reduced survival, similar to that of XP30RO cells. Similarly, the deficiency in PRR is slightly enhanced in the UBZ/S601A double mutant, but substantially increased in the PIP/S601A double mutant (Fig. 4 D).

We interpret these data to indicate that binding of polη to PCNA (via the PIP box) and to an as-yet unidentified protein by P-polη cooperate to independently assist in ensuring efficient bypass of UV photoproducts. This is consistent with our finding that ubiquitination of PCNA and phosphorylation of polη are not interdependent (Fig. 3). We can thus add phosphorylation on Ser601 to the other three C-terminal motifs that together contribute to efficient PRR and survival.

Effects on cell cycle progression

We have analyzed the cell cycle profile and checkpoint activation of XP30RO cells expressing wild-type polη and polη-S601A, as well as the UBZ and PIP box mutants after UV irradiation. All cells lines showed similar cell cycle distributions before UV irradiation (Fig. 5 A). However, 24 h after UV irradiation there was a small increase in cells blocked in G2 in cells expressing wild-type polη, whereas cells expressing no protein were completely blocked in S phase as previously reported (Stary et al., 2003). The other mutants showed some blockage in S and S/G2 phase, the severity of the block being UBZ > S601A > PIP mutant. Phosphorylation of polη is therefore required for correct cell cycle control after UV damage.

We have also examined Chk1 phosphorylation (P-Chk1), a downstream target of ATR signaling. Under the conditions

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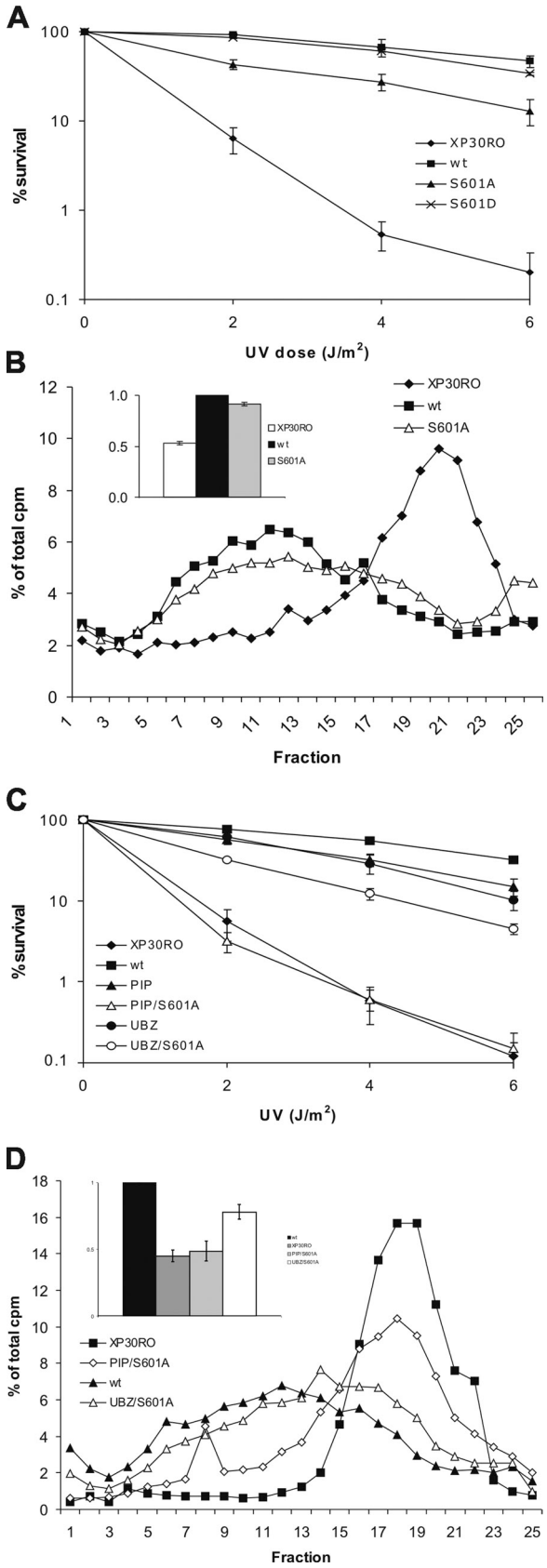

Figure 4. **Polη phosphorylation is necessary for efficient bypass of UV-induced photoproducts.** (A) UV survival assay of XP30RO-derived cell lines expressing EGFP-polη, EGFP-polη-S601A, and EGFP-polη-S601D plated in the presence of 0.375 mM caffeine. Error bars denote SD of three experiments. (B) Alkaline sucrose sedimentation analysis of DNA from cells that were UV irradiated (8 J/m^2), pulsed for 30 min with ^{3}H-thymidine, and chased for 150 min, all in the presence of 0.375 mM caffeine. XP30RO expressing EGFP-polη-S601A (open triangles) was compared with one cell line proficient in damage bypass (XP30RO complemented with wild-type EGFP-polη, squares) and one defective (XP30RO, diamonds). The inset shows the average molecular weights of the distributions ± SD from three experiments. (C) UV survival assay of XP30RO-derived cell lines expressing the indicated polη mutant constructs. Error bars denote SD of three experiments. (D) As in B, but with cells expressing PIP/S601A and UBZ/S601A double mutants.

used in our experiments, P-Chk1 was similar in XPV cells and in cells expressing wild-type or PIP box mutant polη (Fig. 5 B, lanes 2, 4, and 8). Remarkably however, P-Chk1 was signficantly reduced in cells expressing polη S601A and was barely detectable in cells expressing the UBZ mutant (Fig. 5 B, lanes 6 and 10). These data, showing an effect in the phospho- and UBZ mutant proteins, but neither in the absence of protein nor in the presence of wild-type protein, suggests some kind of dominant-negative effect. Interestingly, ATR- and ATM-mediated phosphorylation of the WRN protein was recently shown to have a dominant-negative effect on recovery of cell cycle progression from a hydroxyurea-mediated replication block (Ammazzalorso et al., 2010). Further studies are needed to unravel these complex phenomena.

Model and concluding remarks

Because the catalytic domain of Polη is contained within the N-terminal 432 aa (Biertümpfel et al., 2010), we consider it very unlikely that phosphorylation affects the catalytic activity of the polymerase. We anticipate that phosphorylation of polη might change the composition of polη-containing complexes within the cell, and we present a speculative model in Fig. 5 C that is consistent with our data. We know that the UBZ motif is necessary for accumulation of polη in the chromatin fraction, which we have shown previously equates with replication factories. By implication, polη needs to bind to a ubiquitinated protein to be retained in the factories. Although ubiquitinated PCNA is an obvious candidate for this protein and contributes to retention of polη in factories (Sabbioneda et al., 2008), several lines of evidence suggest that another ubiquitinated protein must play a role: (1) polη accumulates in replication factories during a normal S phase. This is absolutely dependent on the UBZ motif (Bienko et al., 2005), but ubiquitination of PCNA is negligible; (2) we showed previously that treatment of cells with proteasome inhibitors abolishes PCNA ubiquitination, but has no effect on polη localization (Sabbioneda et al., 2008); (3) using a cell line in which ubiquitination of PCNA is prevented (Niimi et al., 2008), localization of polη is unaffected (unpublished data); (4) the UBZ motif of polη is essential for translesion synthesis in human cell extracts, but ubiquitination of PCNA is dispensable (Schmutz et al., 2010). We therefore postulate a role for a hypothetical ubiquitinated protein X, which is necessary for accumulation of polη into replication factories, where it can be phosphorylated on ser601 by ATR (Fig. 5, step 1). We next propose two possibilities: (A) phosphorylation of polη

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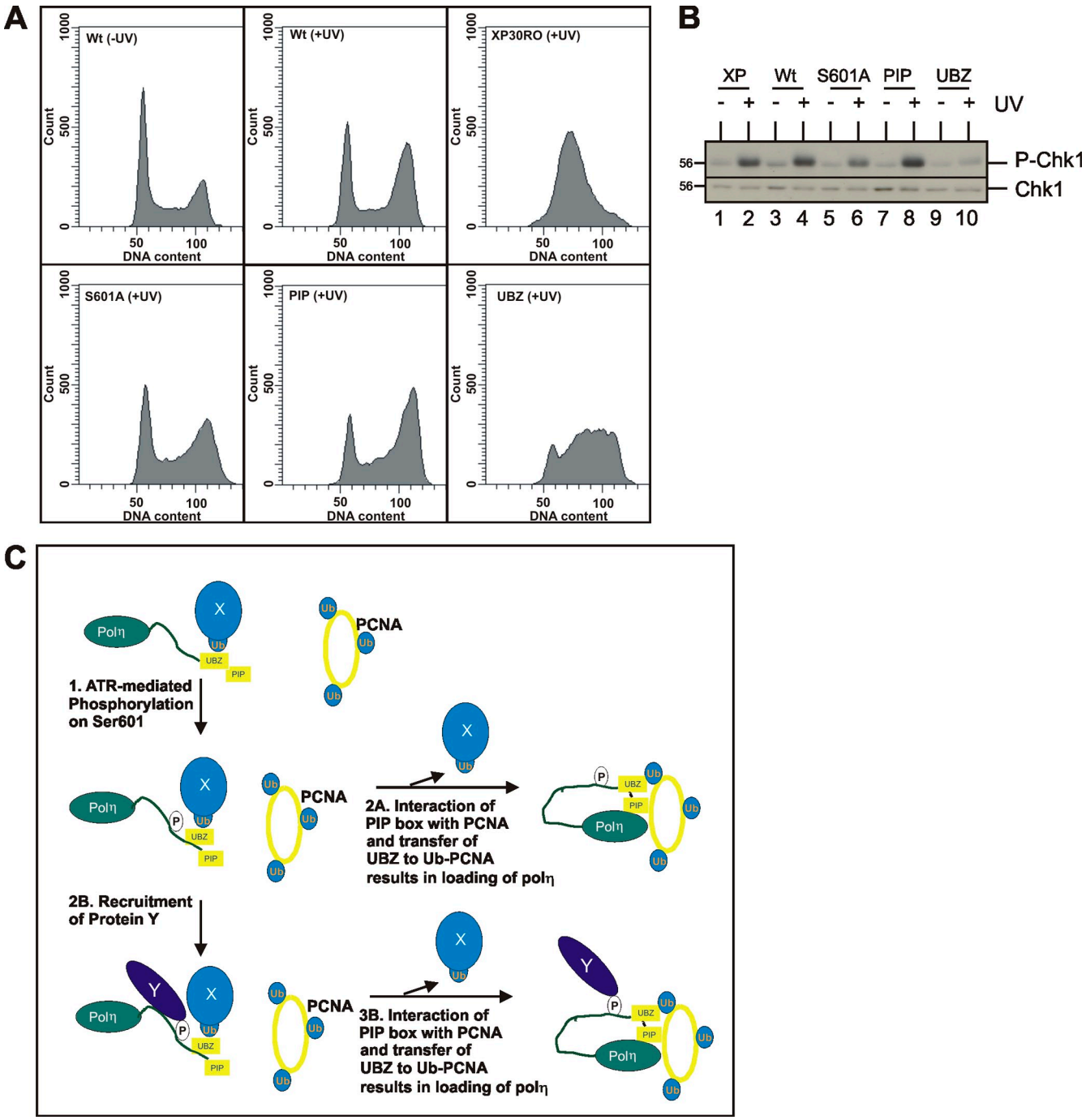

Figure 5. **Polη phosphorylation, cell cycle progression, and checkpoint activation.** (A) Cell cycle profiles of XP30RO cells complemented with EGFP-polη before irradiation (0 h) and of XP30RO cells complemented with the indicated wild-type or mutant forms of polη 24 h after UV irradiation (8 J/m^2). (B) Unirradiated or UV-irradiated (15 J/m^2) XP30RO-derived cell lines expressing EGFP-polη, either wild-type or indicated mutant forms, were incubated for 5 h before analysis. (C) Model to explain epistasis and dependency. (1) Binding of UBZ to hypothetical ubiquitinated protein X stimulates phosphorylation at ser601. (2A) Phosphorylation of polη regulates the interaction with protein X and facilitates the handoff to ubiquitinated PCNA. Alternatively: (2B) the phosphorylation results in binding to hypothetical protein Y. (3B) This stimulates the transfer of the UBZ onto ubiquitinated PCNA. Together with the independent binding of the PIP box to PCNA, this results in polη being loaded onto the DNA to carry out TLS. Note that we have not included the effects on cell cycle and checkpoint activation in the model, as we feel that further investigation is required to fit these observations into the model.

regulates the interaction with protein X and facilitates the handoff to ubiquitinated PCNA (Step 2A). Alternatively (B) phosphorylation results in binding to a second hypothetical protein Y (step 2B), which then facilitates the handoff of the UBZ from protein X to ubiquitinated PCNA (step 3B). In either model, binding to PCNA is independently strengthened by interaction of the PIP box with the interdomain connecting loop of PCNA. Though clearly highly speculative, this model satisfactorily explains the epistasis analysis of Fig. 4 and the dependencies of Fig. 3 D, and may serve as a working hypothesis to test in future experiments. It is consistent with recent findings that the majority of polη is part of a complex

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with other proteins and that the amount (Yuasa et al., 2006) and size (Sabbioneda et al., 2008) of this complex change after UV irradiation. Currently we are investigating whether Ser601 phosphorylation mediates specific protein–protein interactions.

Our finding that ATR kinase activity is involved in PRR provides the first link between DNA damage–induced checkpoint activation and translesion synthesis in mammalian cells and highlights the complexity of TLS polymerase regulation.

Materials and methods

Cell culture, transfection, and sorting

SV40-transformed MRC5 and XP30RO cells were grown in Eagle's MEM (Invitrogen) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 15% fetal calf serum (PAA Laboratories, Inc.). HEK 293 cells were grown in DME supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 15% fetal calf serum.

MRC5 cells were transfected using Fugene6 (Roche) according to the manufacturer's instructions and HEK-293 cells were transfected using the calcium phosphate transfection method.

XP30RO cell lines expressing various EGFP-polη constructs were obtained by Fugene6 transfection, followed by selection in 1 mg/ml G418, and individual clones were isolated. Before use, cells were sorted using a FACSAria cell sorter (BD) to yield a population of cells expressing EGFP-polη at close to physiological levels.

Antibodies

The α-polη phosphospecific antibody was raised against the following peptide: H_2N-MDLAHNS(PO_3H_2)QSMHAS-$CONH_2$ (Eurogentec). An α-polη antibody was raised in rabbit against the full-length protein (Kannouche et al., 2001). Other antibodies used in this work are as follows: ATR (N-19; Santa Cruz Biotechnology, Inc.), PCNA (PC-10; Cancer Research UK), Vimentin (Ab-1; Oncogene), Chk1 (DCS-310; Santa Cruz Biotechnology, Inc.), Phospho-Chk1 (Ser317, 2344; Cell Signaling Technology), and GFP (Roche).

Western blotting

Cells from semi-confluent 10-cm dishes were lysed in Laemmli buffer and the lysate run on SDS-PAGE gels. To detect phosphorylated polη using anti-polη antibody (Figs. 1 and 2), 16-cm gels were used and electrophoresis was performed for 4 h. Transferred proteins were probed with the antibodies indicated in the figure at the following dilutions: anti-polη (1:1,000) and anti P-polη (1:100).

ATR kinase assay

For ATR in vitro kinase assays, Flag-tagged ATR-wt and a kinase-dead version (ATR KD; Tibbetts et al., 1999) were transfected into 293 cells and immunoprecipitated with anti-Flag M2 agarose beads (Sigma-Aldrich). Immunoprecipitates were washed three times in lysis buffer and twice in kinase buffer (25 mM Hepes, pH 7.4, 50 mM NaCl, 10 mM $MnCl_2$, 10 mM $MgCl_2$, and 1 mM DTT) and then incubated with His-tagged polη purified from baculovirus-infected insect cells (Masutani et al., 2000) in the presence of γ-[^{32}P]ATP in ATR kinase buffer at 30°C for 30 min. Phosphorylated proteins were separated by SDS-PAGE. Gels were dried and exposed to film.

RNA interference, immunoprecipitation, and chromatin isolation

RNA interference experiments were performed using HiPerfect transfection reagent (QIAGEN). MRC5 cells were transfected with 40 nM ATR siRNA, 40 nM Chk1 siRNA, and 40 nM Rad18 siRNA (ON-TARGETplus SMART pools; Thermo Fisher Scientific) and UV irradiated at the indicated dose 48 h after transfection. Cell lines expressing His-PCNA K164R were transfected with 100 nM PCNA siRNA [GCCGAGAUCUCAGCCAUAUTT] (Thermo Fisher Scientific) and were UV irradiated 72 h after transfection.

For immunoprecipitation, cells were lysed in CSK buffer (10 mM Pipes, pH 6.8, 50 mM NaCl, 3 mM $MgCl_2$, 10% sucrose, and 1% Triton X-100) containing phosphatase inhibitors and protease inhibitors. Lysates were supplemented with benzonase (EMD) and incubated on ice for 1 h and adjusted to 150 mM NaCl. Cell lysates were centrifuged for 15 min at 4°C, precleared, and immunoprecipitations were performed by incubating cell lysates with the indicated antibodies and protein A–Sepharose overnight at 4°C. Immunoprecipitates were washed three times in lysis buffer containing 150 mM NaCl and subjected to SDS-PAGE. For chromatin isolation, cells were washed with PBS and incubated on ice for 5 min with buffer A (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mm Pipes, pH 6.8, 1 mM EGTA, and 0.2% Triton X-100) supplemented with phosphatase inhibitors and protease inhibitors. Remaining material (chromatin fraction) was resuspended in SDS-loading buffer. The soluble fraction was precipitated and resuspended in the same volume of SDS-loading buffer.

Clonogenic and PRR assay

Clonogenic survival and PRR assays were performed on cells that were FACS sorted immediately before use to ensure equal expression of GFP-polη in all populations, as described previously, except that the concentration of caffeine was 0.375 mM in both types of experiment (Bienko et al., 2010).

Plasmids and site-directed mutagenesis

EGFP-polη construct deficient in monoubiquitination (NLS-4KR) has been described recently (Bienko et al., 2010). EGFP-polη constructs carrying mutations in the PIP box and ubiquitin binding zinc finger (UBZ) have been described elsewhere (Bienko et al., 2005). To generate the serine/threonine to alanine/aspartic acid mutations in polη, EGFP-tagged full-length polη was used as a template, with the following primers: for EGFP-polη S601A, 5′-GATTTGGCCCACAACGCCCAAAGCATGCAGCAGG-3′ as forward and 5′-CGTGCATGCTTTGGGCGTTGTGGGCCAAATC-3′ as reverse primer; For EGFP-polη S601D, 5′-GATTTGGCCCACAACGACCAAAGCATGCACGC-3′ as forward and 5′-CGTGCATGCTTTGGTCGTTGTGGGCCAAATC-3′ as reverse primer.

Flow cytometric analysis

To study cell cycle distribution, cells were either UV irradiated (8 J/m^2) or unirradiated. At the indicated time points, cells were trypsinized, washed in PBS, and fixed in 70% ethanol overnight at −20°C. The cells were resuspended in PBS containing 5 µg/ml propidium iodide. The stained samples were analyzed by flow cytometry (FACS Canto; BD).

Online supplemental material

Fig. S1 shows phosphorylation of polη in response to different DNA-damaging agents. Fig. S2 shows that phosphorylation of polη is not dependent on PCNA or polη ubiquitination and is resistant to low concentrations of caffeine. Fig. S3 shows foci formation of GFP-polη wild-type and GFP-polη S601A. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201008076/DC1.

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References

Acharya, N., J.H. Yoon, H. Gali, I. Unk, L. Haracska, R.E. Johnson, J. Hurwitz, L. Prakash, and S. Prakash. 2008. Roles of PCNA-binding and ubiquitin-binding domains in human DNA polymerase eta in translesion DNA synthesis. *Proc. Natl. Acad. Sci. USA.* 105:17724–17729. doi:10.1073/pnas.0809844105

Alao, J.P., and P. Sunnerhagen. 2009. The ATM and ATR inhibitors CGK733 and caffeine suppress cyclin D1 levels and inhibit cell proliferation. *Radiat. Oncol.* 4:51. doi:10.1186/1748-717X-4-51

Ammazzalorso, F., L.M. Pirzio, M. Bignami, A. Franchitto, and P. Pichierri. 2010. ATR and ATM differently regulate WRN to prevent DSBs at stalled replication forks and promote replication fork recovery. *EMBO J.* 29:3156–3169. doi:10.1038/emboj.2010.205

Bienko, M., C.M. Green, N. Crosetto, F. Rudolf, G. Zapart, B. Coull, P. Kannouche, G. Wider, M. Peter, A.R. Lehmann, et al. 2005. Ubiquitin-binding domains in translesion synthesis polymerases. *Science.* 310:1821–1824. doi:10.1126/science.1120615

Bienko, M., C.M. Green, S. Sabbioneda, N. Crosetto, I. Matic, R.G. Hibbert, T. Begovic, A. Niimi, M. Mann, A.R. Lehmann, and I. Dikic. 2010. Regulation of translesion synthesis DNA polymerase eta by monoubiquitination. *Mol. Cell.* 37:396–407. doi:10.1016/j.molcel.2009.12.039

Biertümpfel, C., Y. Zhao, Y. Kondo, S. Ramón-Maiques, M. Gregory, J.Y. Lee, C. Masutani, A.R. Lehmann, F. Hanaoka, and W. Yang. 2010. Structure and

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mechanism of human DNA polymerase eta. *Nature.* 465:1044–1048. doi:10.1038/nature09196

Chen, Y.W., J.E. Cleaver, Z. Hatahet, R.E. Honkanen, J.Y. Chang, Y. Yen, and K.M. Chou. 2008. Human DNA polymerase eta activity and translocation is regulated by phosphorylation. *Proc. Natl. Acad. Sci. USA.* 105:16578–16583. doi:10.1073/pnas.0808589105

Friedberg, E.C. 2005. Suffering in silence: the tolerance of DNA damage. *Nat. Rev. Mol. Cell Biol.* 6:943–953. doi:10.1038/nrm1781

Hoege, C., B. Pfander, G.L. Moldovan, G. Pyrowolakis, and S. Jentsch. 2002. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature.* 419:135–141. doi:10.1038/nature00991

Johnson, R.E., C.M. Kondratick, S. Prakash, and L. Prakash. 1999. hRAD30 mutations in the variant form of xeroderma pigmentosum. *Science.* 285:263–265. doi:10.1126/science.285.5425.263

Kannouche, P., B.C. Broughton, M. Volker, F. Hanaoka, L.H. Mullenders, and A.R. Lehmann. 2001. Domain structure, localization, and function of DNA polymerase eta, defective in xeroderma pigmentosum variant cells. *Genes Dev.* 15:158–172. doi:10.1101/gad.187501

Kannouche, P.L., J. Wing, and A.R. Lehmann. 2004. Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Mol. Cell.* 14:491–500. doi:10.1016/S1097-2765(04)00259-X

Lehmann, A.R. 2005. Replication of damaged DNA by translesion synthesis in human cells. *FEBS Lett.* 579:873–876. doi:10.1016/j.febslet.2004.11.029

Lehmann, A.R., S. Kirk-Bell, C.F. Arlett, M.C. Paterson, P.H. Lohman, E.A. de Weerd-Kastelein, and D. Bootsma. 1975. Xeroderma pigmentosum cells with normal levels of excision repair have a defect in DNA synthesis after UV-irradiation. *Proc. Natl. Acad. Sci. USA.* 72:219–223. doi:10.1073/pnas.72.1.219

Masutani, C., R. Kusumoto, A. Yamada, N. Dohmae, M. Yokoi, M. Yuasa, M. Araki, S. Iwai, K. Takio, and F. Hanaoka. 1999. The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. *Nature.* 399:700–704. doi:10.1038/21447

Masutani, C., R. Kusumoto, S. Iwai, and F. Hanaoka. 2000. Mechanisms of accurate translesion synthesis by human DNA polymerase eta. *EMBO J.* 19:3100–3109. doi:10.1093/emboj/19.12.3100

McCulloch, S.D., R.J. Kokoska, C. Masutani, S. Iwai, F. Hanaoka, and T.A. Kunkel. 2004. Preferential cis-syn thymine dimer bypass by DNA polymerase eta occurs with biased fidelity. *Nature.* 428:97–100. doi:10.1038/nature02352

Niimi, A., S. Brown, S. Sabbioneda, P.L. Kannouche, A. Scott, A. Yasui, C.M. Green, and A.R. Lehmann. 2008. Regulation of proliferating cell nuclear antigen ubiquitination in mammalian cells. *Proc. Natl. Acad. Sci. USA.* 105:16125–16130. doi:10.1073/pnas.0802727105

Ohmori, H., E.C. Friedberg, R.P. Fuchs, M.F. Goodman, F. Hanaoka, D. Hinkle, T.A. Kunkel, C.W. Lawrence, Z. Livneh, T. Nohmi, et al. 2001. The Y-family of DNA polymerases. *Mol. Cell.* 8:7–8. doi:10.1016/S1097-2765(01)00278-7

Pagès, V., S.R. Santa Maria, L. Prakash, and S. Prakash. 2009. Role of DNA damage-induced replication checkpoint in promoting lesion bypass by translesion synthesis in yeast. *Genes Dev.* 23:1438–1449. doi:10.1101/gad.1793409

Plosky, B.S., A.E. Vidal, A.R. Fernández de Henestrosa, M.P. McLenigan, J.P. McDonald, S. Mead, and R. Woodgate. 2006. Controlling the subcellular localization of DNA polymerases iota and eta via interactions with ubiquitin. *EMBO J.* 25:2847–2855. doi:10.1038/sj.emboj.7601178

Prakash, S., R.E. Johnson, and L. Prakash. 2005. Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annu. Rev. Biochem.* 74:317–353. doi:10.1146/annurev.biochem.74.082803.133250

Sabbioneda, S., I. Bortolomai, M. Giannattasio, P. Plevani, and M. Muzi-Falconi. 2007. Yeast Rev1 is cell cycle regulated, phosphorylated in response to DNA damage and its binding to chromosomes is dependent upon MEC1. *DNA Repair (Amst.).* 6:121–127. doi:10.1016/j.dnarep.2006.09.002

Sabbioneda, S., A.M. Gourdin, C.M. Green, A. Zotter, G. Giglia-Mari, A. Houtsmuller, W. Vermeulen, and A.R. Lehmann. 2008. Effect of proliferating cell nuclear antigen ubiquitination and chromatin structure on the dynamic properties of the Y-family DNA polymerases. *Mol. Biol. Cell.* 19:5193–5202. doi:10.1091/mbc.E08-07-0724

Schmutz, V., R. Janel-Bintz, J. Wagner, D. Biard, N. Shiomi, R.P. Fuchs, and A.M. Cordonnier. 2010. Role of the ubiquitin-binding domain of Polη in Rad18-independent translesion DNA synthesis in human cell extracts. *Nucleic Acids Res.* 38:6456–6465. doi:10.1093/nar/gkq403

Stary, A., P. Kannouche, A.R. Lehmann, and A. Sarasin. 2003. Role of DNA polymerase eta in the UV mutation spectrum in human cells. *J. Biol. Chem.* 278:18767–18775. doi:10.1074/jbc.M211838200

Stelter, P., and H.D. Ulrich. 2003. Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature.* 425:188–191. doi:10.1038/nature01965

Tibbetts, R.S., K.M. Brumbaugh, J.M. Williams, J.N. Sarkaria, W.A. Cliby, S.Y. Shieh, Y. Taya, C. Prives, and R.T. Abraham. 1999. A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev.* 13:152–157. doi:10.1101/gad.13.2.152

Traven, A., and J. Heierhorst. 2005. SQ/TQ cluster domains: concentrated ATM/ATR kinase phosphorylation site regions in DNA-damage-response proteins. *Bioessays.* 27:397–407. doi:10.1002/bies.20204

Watanabe, K., S. Tateishi, M. Kawasuji, T. Tsurimoto, H. Inoue, and M. Yamaizumi. 2004. Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination. *EMBO J.* 23:3886–3896. doi:10.1038/sj.emboj.7600383

Yuasa, M.S., C. Masutani, A. Hirano, M.A. Cohn, M. Yamaizumi, Y. Nakatani, and F. Hanaoka. 2006. A human DNA polymerase eta complex containing Rad18, Rad6 and Rev1; proteomic analysis and targeting of the complex to the chromatin-bound fraction of cells undergoing replication fork arrest. *Genes Cells.* 11:731–744. doi:10.1111/j.1365-2443.2006.00974.x

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