

# Opposing effects of the UV lesion repair protein XPA and UV bypass polymerase $\eta$ on ATR checkpoint signaling

Ryan D Bomgarden<sup>1</sup>, Patrick J Lupardus<sup>1</sup>,  
Deena V Soni<sup>1</sup>, Muh-Ching Yee<sup>1</sup>,  
James M Ford<sup>2</sup> and Karlene A Cimprich<sup>1,\*</sup>

<sup>1</sup>Department of Molecular Pharmacology, Stanford University, Stanford, CA, USA and <sup>2</sup>Departments of Medicine and Genetics, Division of Oncology, Stanford University, Stanford, CA, USA

An essential component of the ATR (ataxia telangiectasia-mutated and Rad3-related)-activating structure is single-stranded DNA. It has been suggested that nucleotide excision repair (NER) can lead to activation of ATR by generating such a signal, and in yeast, DNA damage processing through the NER pathway is necessary for checkpoint activation during G1. We show here that ultraviolet (UV) radiation-induced ATR signaling is compromised in XPA-deficient human cells during S phase, as shown by defects in ATRIP (ATR-interacting protein) translocation to sites of UV damage, UV-induced phosphorylation of Chk1 and UV-induced replication protein A phosphorylation and chromatin binding. However, ATR signaling was not compromised in XPC-, CSB-, XPF- and XPG-deficient cells. These results indicate that damage processing is not necessary for ATR-mediated S-phase checkpoint activation and that the lesion recognition function of XPA may be sufficient. In contrast, XP-V cells deficient in the UV bypass polymerase  $\eta$  exhibited enhanced ATR signaling. Taken together, these results suggest that lesion bypass and not lesion repair may raise the level of UV damage that can be tolerated before checkpoint activation, and that XPA plays a critical role in this activation.

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## Introduction

Eukaryotic cells have evolved highly conserved DNA damage response pathways called checkpoints that are responsible for coordinating various stress responses such as cell cycle arrest, lesion repair, adaptation and apoptosis (Zhou and Elledge, 2000; Melo and Toczyski, 2002). In mammalian

cells, these pathways are primarily controlled by two phosphatidylinositol kinase-related protein kinases, ATM (ataxia telangiectasia-mutated) and ATR (ATM and Rad-3 related) (Abraham, 2001; Bakkenist and Kastan, 2004). The ATM kinase has been shown to be primarily activated by double-stranded DNA breaks caused by ionizing radiation (IR). In contrast, ATR is responsible for signaling in response to multiple types of DNA damage, including those induced by ultraviolet (UV) radiation, methyl methanesulfonate, cis-platinum, IR and the DNA replication inhibitors aphidicolin and hydroxyurea.

ATR checkpoint signaling is known to involve the independent translocation of multicomponent protein complexes to sites of damage (Kondo *et al*, 2001; Melo *et al*, 2001; Zou *et al*, 2002; Lee *et al*, 2003), which leads to the phosphorylation of ATR's main effector kinase Chk1 (Zhou and Elledge, 2000; Melo and Toczyski, 2002). Although the exact checkpoint signal is not yet known, it is thought to involve a replication protein A (RPA)-coated, single-stranded DNA (ssDNA) region adjacent to a primer (reviewed by Cortez, 2005; O'Connell and Cimprich, 2005). One process that has been shown to generate an ATR-activating signal is the inhibition of DNA replication (Cliby *et al*, 1998; Kumagai *et al*, 1998; Hekmat-Nejad *et al*, 2000; Liu *et al*, 2000; Brown and Baltimore, 2003). Experiments in the *Xenopus* extract system have shown that both DNA-damaging agents and replication inhibitors stall the replicative polymerases, resulting in continued unwinding of DNA by the MCM helicase (Pacek and Walter, 2004; Byun *et al*, 2005). This uncoupling of the polymerase activity from the helicase activity leads to an accumulation of ssDNA, increased binding of RPA and subsequent phosphorylation of Chk1 by ATR. ATR is thought to be recruited to RPA through its interacting protein, ATRIP, which is also required for ATR phosphorylation of Chk1 (Cortez *et al*, 2001; Zou and Elledge, 2003).

In addition to replication, nucleotide excision repair (NER) has also been implicated in ATR checkpoint activation (O'Driscoll *et al*, 2003; Giannattasio *et al*, 2004a; reviewed in Rouse and Jackson, 2002). In mammalian cells, defects in various NER proteins have been linked to a number of related DNA damage sensitivity disorders including xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (Ford and Hanawalt, 1997; Lehmann, 2003). Seven complementation groups have been identified for XP syndrome (XPA-XPG) and two for CS (CSA and CSB). Proteins involved in NER can be further classified by their roles in either transcription-coupled repair (TCR), a more rapid form of repair for the transcribed strand of expressed genes, or global genomic repair (GGR), which is needed for the rest of the genome. Regardless of the form of repair through which a DNA lesion is recognized (TCR or GGR), lesion processing proceeds through a common mechanism of damaged DNA

\*Corresponding author. Department of Molecular Pharmacology, CCSR, Stanford University School of Medicine, 269 Campus Drive, Rm 3215a Stanford, CA 94305-5174, USA. Tel.: +1 650 498 4720; Fax: +1 650 725 4665; E-mail: cimprich@stanford.edu

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base excision resulting in an ssDNA gap intermediate (de Laat *et al*, 1999; Hanawalt, 2002; Costa *et al*, 2003).

Evidence of a role for NER in contributing to checkpoint activation has come mainly from studies in yeast (Neecke *et al*, 1999; Giannattasio *et al*, 2004a). Mec1, the *Saccharomyces cerevisiae* ATR homolog, has been shown to have reduced activity in Rad14 (XPA) and Rad2 (XPG) mutants, which are defective in NER (Giannattasio *et al*, 2004b). Physical interactions between the checkpoint protein Ddc1 (human Rad9) and the NER protein Rad14 (XPA) have also been observed (Giannattasio *et al*, 2004a). Importantly, these studies were conducted in yeast arrested in G1, providing evidence that NER is needed to generate a checkpoint-inducing signal in the absence of replication. It has been speculated that this signal is an RPA-coated, ssDNA-gapped structure (O'Driscoll *et al*, 2003; Giannattasio *et al*, 2004a). Such a structure could be generated upon excision of the UV lesion and is similar to the checkpoint-activating structure generated at a stalled replication fork.

Whether or not NER directly contributes to activation of the ATR-dependent checkpoint in higher eukaryotes is unclear. A previous study found defects in UV-induced H2AX phosphorylation in non-replicating XPA-deficient cells but not Cockayne syndrome A (CSA)-deficient cells (O'Driscoll *et al*, 2003). This defect was similar to that seen in Seckel syndrome cells, which have reduced ATR expression. In addition, accumulation of p53 is reduced in XPA-deficient cells that are unable to progress through S phase (Nelson and Kastan, 1994). Another study found that cycling cells deficient in XPC, which is needed for the recruitment of XPA during GGR, did not have a defect in ATR phosphorylation of Chk1 in response to UV (Ward *et al*, 2004). Chromatin immunoprecipitation has also revealed that upstream checkpoint proteins are recruited to UV-damaged DNA in XPC- and XPA-deficient cells, and in this study at least some phosphorylation of Chk1 was observed during G1 and S phase in these cells (Jiang and Sancar, 2006). Importantly, none of these prior studies assessed ATR function in NER-deficient cells in comparison to isogenic, complemented control cell lines, nor did they study the role of lesion excision in UV-induced checkpoint activation in mammalian cells.

Similar to NER, it is also not known what role UV *trans*-lesion synthesis plays in checkpoint activation. One *trans*-lesion synthesis polymerase, polymerase  $\eta$  (Pol $\eta$ ), is mutated in variant forms of xeroderma pigmentosum (XP-V) that are proficient in the repair of UV DNA damage (Masutani *et al*, 1999; Lehmann, 2005). Pol $\eta$  is an error-prone polymerase on undamaged DNA templates but can specifically bypass UV-damaged bases during replication in an error-free manner by inserting adenines across from a cyclobutane pyrimidine dimer (CPD) (Kannouche and Strydom, 2003; Lehmann, 2005). Cells deficient in Pol $\eta$  exhibit a prolonged UV-induced S-phase arrest and have greatly enhanced H2AX phosphorylation following UV treatment, indicative of double-strand break formation (Limoli *et al*, 2002). These breaks may arise from damage-induced replication fork collapse, which is also observed upon loss of ATR function (Brown and Baltimore, 2003).

In this study, we sought to determine the contribution of NER and Pol $\eta$ -mediated UV lesion bypass to ATR checkpoint activation. To do so, we assessed translocation of the ATR-associated protein, ATRIP, to sites of UV damage in various

NER-deficient cell lines and in complemented, isogenic controls. We also assessed the activation of ATR following UV treatment in these cells by analyzing the ATR-dependent phosphorylation of Chk1 at S345. We found that cells deficient in XPA have a defect in ATRIP translocation and Chk1 phosphorylation following UV treatment. XPA-depleted *Xenopus* egg extracts also exhibited a defect in Chk1 phosphorylation. Importantly, these defects were observed during S phase. Moreover, we found that XPA-deficient cells have decreased UV-induced RPA chromatin binding and phosphorylation. Chk1 phosphorylation was unaffected in XP-C, CS-B, XP-G and XP-F cell lines. We also found that XP-V cells, which are deficient in Pol $\eta$ , had increased Chk1 phosphorylation relative to their complemented controls following UV treatment. Consistent with these results, we found that damage-induced RPA phosphorylation was also modestly increased in XP-V cells. Overall, our findings suggest that XPA and Pol $\eta$  may have opposing effects on the S-phase activation of ATR following UV-induced DNA damage. They also suggest that although XPA plays a role in Chk1 phosphorylation, excision of the UV-induced lesion is not necessary for S-phase checkpoint activation in mammalian cells.

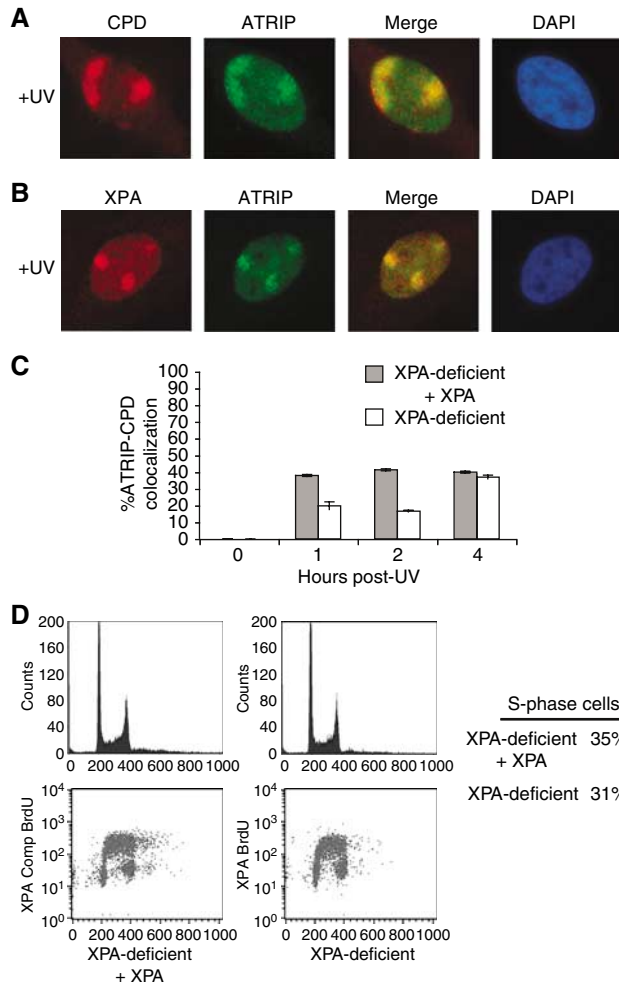
## Results

### ***ATRIP colocalization with UV-induced DNA damage is reduced in XPA-deficient cells***

Both ATR and its associated protein ATRIP have been shown to translocate to punctate nuclear foci following treatment of cells with IR and UV (Tibbetts *et al*, 1999; Cortez *et al*, 2001; Barr *et al*, 2003). This translocation event is thought to be one of the earliest indicators of the ATR-mediated DNA damage response. By locally UV irradiating portions of the nucleus through a 5  $\mu$ m porous filter (Volker *et al*, 2001), it has been shown that ATR staining is enhanced in regions containing UV photoproducts (Ward *et al*, 2004). Using this method, we assessed the translocation of ATRIP to sites of UV damage. As shown in Figure 1A (and data not shown), ATRIP, like ATR, colocalizes with regions of the nucleus also recognized by an antibody to CPDs. We did not observe ATRIP translocation to regions of the nucleus that did not stain positive for CPDs; however, in many cells containing regions that stained positive for CPDs, ATRIP colocalization was not observed. Importantly, when cells were synchronized by a double-thymidine block and released into S phase, the percentage of cells in which ATRIP translocated to sites of UV damage increased significantly, moving from 38% in asynchronous cells to 77% in S-phase synchronized cells (Supplementary Figure 1A). This increase correlates with the increase in the percentage of S-phase cells observed. These observations indicate that ATRIP can translocate to UV lesions during S phase and are consistent with the idea that ATR activation is dramatically enhanced in S-phase cells (Lupardus *et al*, 2002; Ward *et al*, 2004).

As seen in Figure 1B, ATRIP also colocalizes with the NER protein XPA after UV treatment through these filters. Thus, we asked if XPA was required for UV-induced ATRIP translocation. Utilizing the protocol described in Figure 1A, we assessed the colocalization of ATRIP with CPDs at different time points over 4 h in an XPA-deficient cell line and an isogenic line complemented with wild-type XPA. As

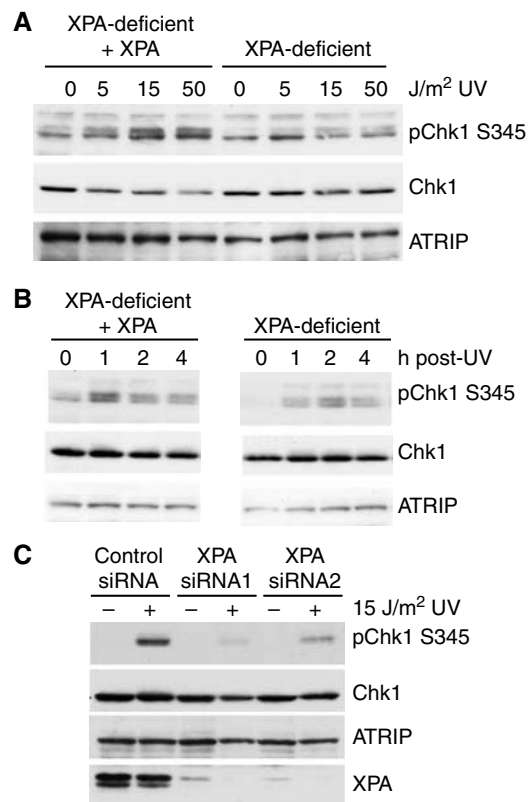
shown in Figure 1C, in the XPA-deficient cell line, there was a marked decrease in the percentage of cells exhibiting colocalization of ATRIP with sites of UV damage 1 and 2 h following UV treatment. At 4 h following UV treatment, however, the localization of ATRIP to sites of UV damage was similar in both XPA-deficient and XPA-complemented cells. The defect in ATRIP translocation at 1 and 2 h does not appear to be due to a difference in the cell cycle distributions of these two cell lines as propidium iodide staining and BrdU incorporation indicate that both have similar cell cycle profiles (Figure 1D).



**Figure 1** XPA facilitates ATRIP recruitment to sites of UV damage. HeLa cells grown on coverslips were exposed to 50 J/m<sup>2</sup> UV through a porous membrane and allowed to recover for 1 h. Cells were fixed and stained with an anti-ATRIP rabbit polyclonal antibody and an anti-CPD mouse monoclonal antibody (A) or an anti-XPA mouse monoclonal antibody (B), followed by staining with a fluorescent goat anti-rabbit antibody Alexa 488 (green) and goat anti-mouse antibody Alexa 595 (red). Nuclei were counterstained with DAPI. Colocalization of labeled proteins is shown as yellow in merge. (C) XPA-complemented cells (gray bar) and XPA-deficient cells (white bar) were UV treated and stained as in panel (A) at the times shown. The recruitment of ATRIP to CPD lesions was determined for a total of 200 nuclei containing CPD foci. Shown graphically is the percentage of nuclei with ATRIP foci that colocalized with CPD foci. Error bars indicate standard error of three samples from a representative experiment ( $n=3$ ) performed in triplicate. (D) Percentage of S-phase cells was determined by flow cytometry using propidium iodide staining (top panels) and by measuring BrdU incorporation (bottom panels).

### UV-induced Chk1 phosphorylation is decreased in XPA-deficient cells

The defect in translocation of ATRIP to sites of UV damage suggests that ATR signaling may be reduced in XPA-deficient cells. Chk1 is a downstream effector of ATR that is phosphorylated on serine 345 in an ATR-dependent manner following several types of DNA damage (Guo *et al*, 2000; Hekmat-Nejad *et al*, 2000; Liu *et al*, 2000), and the phosphorylation of Chk1 is thought to be necessary for Chk1 activation (Zhao and Piwnicka-Worms, 2001). Using an antibody that recognizes Chk1 phosphoserine 345 (S345), we tested whether Chk1 phosphorylation was decreased in XPA-deficient cells. Asynchronous XPA-complemented and XPA-deficient cells were treated with varying doses of UV (Figure 2A) and harvested 1 h post-treatment. In the XPA-complemented cells, UV induced the phosphorylation of Chk1 S345. However, the phosphorylation of Chk1 was greatly reduced in the XPA-deficient cells at all of the doses tested. We then assessed the effect of XPA loss on Chk1 phosphorylation at several times following UV treatment. Consistent with the results shown in Figure 1C for ATRIP localization to CPDs,



**Figure 2** XPA-deficient cells show reduced Chk1 phosphorylation following UV treatment. (A) Isogenic XPA-deficient and XPA-complemented cells were either mock treated or exposed to 0, 5, 15 or 50 J/m<sup>2</sup> UV. Cells were harvested after 1 h and lysates were resolved by SDS-PAGE and analyzed by Western blot using phospho-serine 345 Chk1, Chk1 and ATRIP antibodies. (B) XPA-complemented and XPA-deficient cells were mock treated or exposed to 15 J/m<sup>2</sup> UV and then harvested at the times shown. Lysates were analyzed as described in panel A. (C) HeLa cells were transfected with control siRNA or one of two siRNAs to XPA and treated with 15 J/m<sup>2</sup> UV 48 h later. At 1 h after UV treatment, lysates were prepared and resolved by SDS-PAGE and analyzed by Western blot using phospho-serine 345 Chk1, Chk1, ATRIP and XPA antibodies.

the effect of XPA loss was most evident at early times after UV treatment (Figure 2B).

To further demonstrate a role for XPA in UV-induced ATR activation, we examined the effect of XPA loss on UV-induced Chk1 phosphorylation in HeLa cells. For this, we used small interfering (si) RNA-mediated XPA depletion. Depletion of XPA using either of two different XPA-specific siRNAs resulted in decreased phosphorylation of Chk1 following 15 J/m<sup>2</sup> of UV treatment (Figure 2C). In addition, XPA-deficient mouse embryo fibroblasts (Ichikawa *et al*, 2000) also exhibited poor Chk1 phosphorylation relative to control 3T3 cells (Supplementary Figure 2).

To determine if the role of XPA in the phosphorylation of Chk1 is specific to UV-like DNA-damaging agents, we also analyzed the phosphorylation of Chk1 in XPA-deficient and XPA-complemented cells following several other types of DNA damage. As shown in Figure 3A, Chk1 phosphorylation was significantly decreased in XPA-deficient cells following treatment with the UV mimetic, 4-nitroquinoline oxide (4-NQO). However, there was no defect in Chk1 S345 phosphorylation following aphidicolin treatment (Figure 3B). Likewise, XPA-deficient and XPA-complemented cells had similar levels of IR-induced Chk1 phosphorylation (Figure 3C). Taken together, these results suggest that XPA is necessary for the timely and efficient activation of Chk1 following damage induced by UV and UV-like agents.

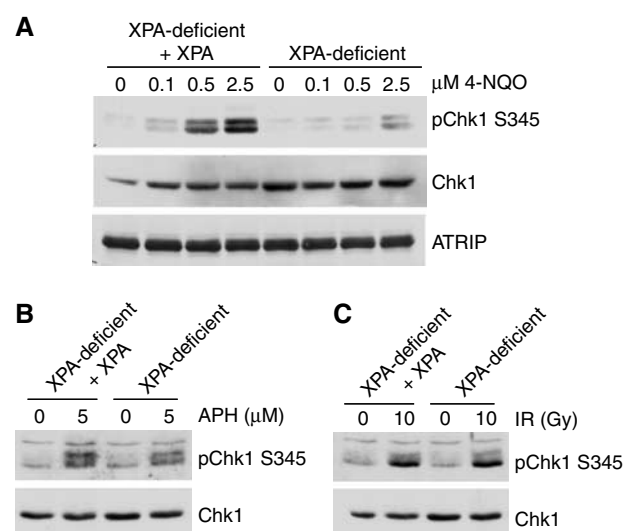
### UV-induced Chk1 phosphorylation is decreased in *Xenopus* egg extracts lacking XPA

The UV-induced phosphorylation of Chk1 has been reported to be restricted to S-phase cells (Ward *et al*, 2004). Consistent with this finding, we have also found that Chk1 phosphorylation is enhanced in S-phase synchronized cells (Supplementary Figure 1B). Because ATRIP translocation to sites of UV damage and Chk1 phosphorylation both appear to occur predominantly in S phase, our results suggest that XPA

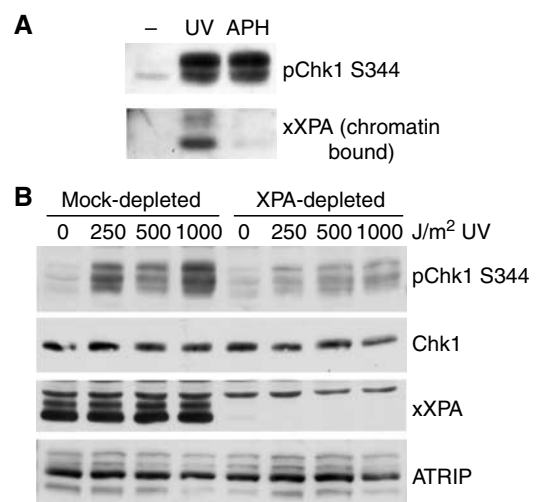
may be required for the S-phase checkpoint. As another way of assessing the role of XPA in this checkpoint, we used the *Xenopus* model system to test whether depletion of XPA from egg extracts also leads to a defect in Chk1 phosphorylation. In *Xenopus* egg extracts, UV-induced phosphorylation of Chk1 requires initiation of replication (Lupardus *et al*, 2002). Using an antibody raised against the full-length, recombinant *Xenopus* XPA (xXPA) protein, we found that xXPA was recruited to chromatin after UV damage but not after aphidicolin treatment (Figure 4A). Notably, when this antibody was used to immunodeplete xXPA from extracts, we observed a significant decrease in the UV-induced phosphorylation of XChk1 on S344, the site analogous to S345 in Chk1 (Figure 4B). These results are consistent with our studies in XPA-deficient mammalian cells and support the idea that the requirement for XPA in ATR checkpoint signaling following UV is primarily in S phase.

### XPC- and CSB-deficient cells show no defect in Chk1 phosphorylation

Because cells deficient in XPA, a protein central to both TCR and GGR pathways (de Laat *et al*, 1999; Hanawalt, 2002; Costa *et al*, 2003), exhibit a defect in the ATR response, we asked whether one of these NER repair pathways was specifically involved. To do this, we utilized XPC- and CSB-deficient cells, which are defective in GGR and TCR, respectively, to perform the ATRIP-CPD colocalization assay described in Figure 1A. As seen in Figure 5A, there is a slight decrease in colocalization of ATRIP with CPD lesions in XPC-deficient cells compared to an isogenic, XPC-comple-

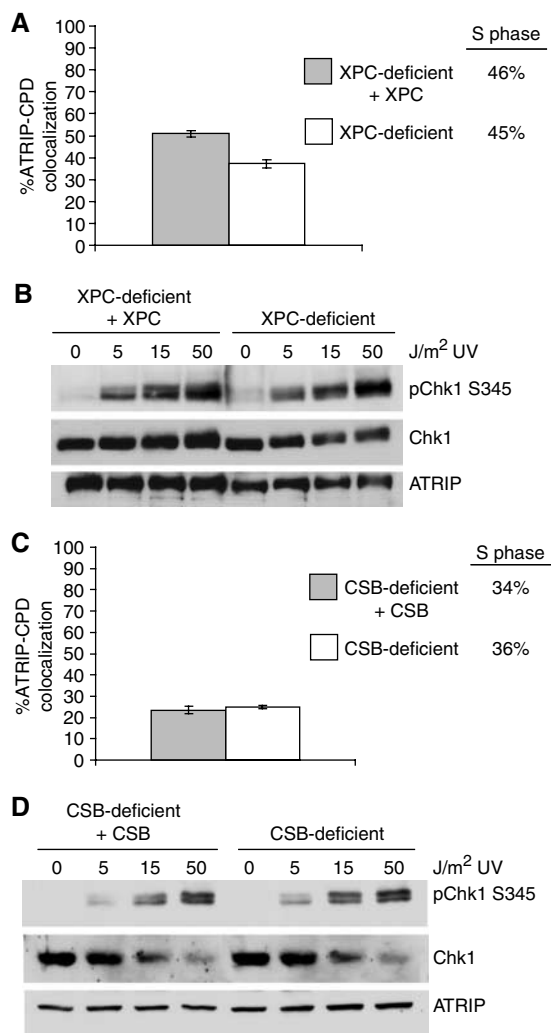


**Figure 3** XPA is required for full Chk1 phosphorylation induced by 4-NQO, but not aphidicolin or IR. Isogenic XPA-complemented and XPA-deficient cells were exposed to increasing concentrations of the UV-mimetic drug 4-NQO for 1 h (A), 5 μM aphidicolin for 6 h (B) or 10 Gy IR followed by a 2 h recovery (C). Lysates were prepared and analyzed as described in Figure 2.



**Figure 4** Depletion of XPA from *Xenopus* egg extracts inhibits UV-induced Chk1 phosphorylation. (A) *Xenopus* egg extracts were incubated with either UV-damaged sperm chromatin (1000 J/m<sup>2</sup>) or treated with 150 μM aphidicolin. After 90 min, total extract samples were taken and an aliquot was removed for analysis of Chk1 S344 phosphorylation. From the remaining sample, chromatin was isolated through a sucrose cushion, sheared and boiled in sample buffer. Samples were resolved by SDS-PAGE and analyzed by Western blot using the indicated antibodies. (B) *Xenopus* extract was either mock-depleted or XPA-depleted using rabbit IgG (mock) or an xXPA antibody coupled to protein A beads. Undamaged or UV-treated sperm chromatin was added to the extracts and incubated for 90 min before addition of sample buffer. Equal amounts of extract were resolved by SDS-PAGE and analyzed by Western blot using the indicated antibodies.

mented cell line. Importantly, however, this decrease was not accompanied by a significant decrease in Chk1 S345 phosphorylation (Figure 5B). These results are in agreement with a previous report that XPC-deficient cells do not have a significant defect in the ATR response (Ward *et al*, 2004). We also found that there was no difference in ATRIP translocation or Chk1 S345 phosphorylation following UV treatment between CSB-deficient and isogenic, complemented cell lines (Figures 5C and D). Furthermore, the percentage of S-phase cells was similar in both XPC- and CSB-deficient and complemented cell lines. These observations indicate that the defect seen in the XPA-deficient cells is not likely attributed to a specific arm of the NER pathway.



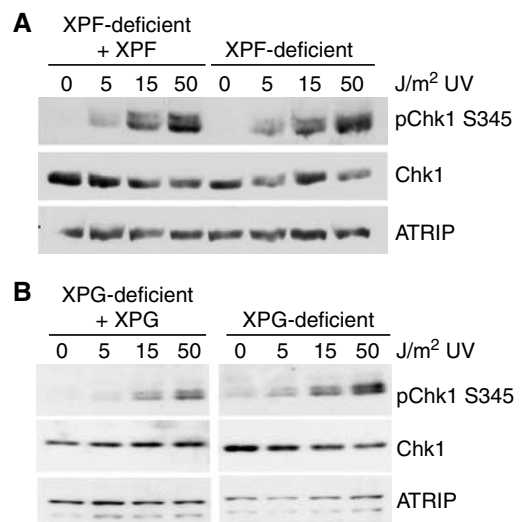
**Figure 5** ATRIP translocation and Chk1 phosphorylation are unaffected by loss of XPC or CSB. Isogenic XPC-deficient (white bar) and XPC-complemented (gray bar) cells (A) or CSB-deficient (white bar) and CSB-complemented (gray bar) cells (C) were UV treated and stained as in Figure 1A. Colocalization of ATRIP and CPD was determined as in Figure 1C. Percentage of S-phase cells was determined by flow cytometry as shown in Figure 1D. XPC-deficient and XPC-complemented cells (B) or CSB-deficient and CSB-complemented cells (D) were treated with increasing amounts of UV and allowed to recover for 1 h. Equal amounts of cell lysate were resolved by SDS-PAGE and analyzed by Western blot using the indicated antibodies.

### XPF- and XPG-deficient cells show no defect in Chk1 phosphorylation

A late step in NER is excision of the DNA surrounding the UV-damaged bases by the endonucleases XPF-ERCC1 and XPG (de Laat *et al*, 1999; Hanawalt, 2002; Costa *et al*, 2003). In *S. cerevisiae*, the XPG homolog Rad2 has been shown to be required for UV-dependent activation of the ATR homolog, Mec1, in G1 cells (Giannattasio *et al*, 2004a). Using cells deficient in either XPF or XPG and their complemented, isogenic lines, we assessed Chk1 S345 phosphorylation 1 h after treatment with UV. As shown in Figure 6A, there was no decrease in Chk1 S345 phosphorylation in XPF-deficient cells. There was also no significant decrease in Chk1 S345 phosphorylation in the XPG-deficient cells relative to control cells (Figure 6B). Together, these results suggest that excision of the UV lesion is not required for Chk1 S345 phosphorylation in asynchronous cells.

### XPA deficiency results in decreased RPA phosphorylation and chromatin binding

RPA is an ssDNA binding protein that is thought to be a component of the ATR checkpoint signal (reviewed in O'Connell *et al*, 2000; Cortez, 2005). RPA accumulates on chromatin following UV treatment in both mammalian cells and *Xenopus* extracts (Lupardus *et al*, 2002; Zou and Elledge, 2003). *In vitro* studies using purified proteins have shown that RPA is sufficient to recruit ATRIP to ssDNA (Zou and Elledge, 2003), although RPA-independent modes of DNA binding and checkpoint signaling also exist (Bomgarden *et al*, 2004; Ball *et al*, 2005; Kim *et al*, 2005). RPA is also phosphorylated during normal cell cycle progression as well as hyperphosphorylated in response to DNA damage, and a subset of these phosphorylation events are ATR-dependent (Barr *et al*, 2003; Binz *et al*, 2004). As Chk1 phosphorylation is decreased in XPA-deficient cells and XPA-depleted *Xenopus* egg extracts, we asked whether RPA chromatin binding and/



**Figure 6** XPF and XPG are not required for UV-induced Chk1 phosphorylation. Isogenic XPF-deficient and XPF-complemented cells (A) or XPG-deficient and XPG-complemented cells (B) were either mock treated or exposed to UV radiation and allowed to recover for 1 h. Equal amounts of cell lysate were resolved by SDS-PAGE and analyzed by Western blot using the indicated antibodies.

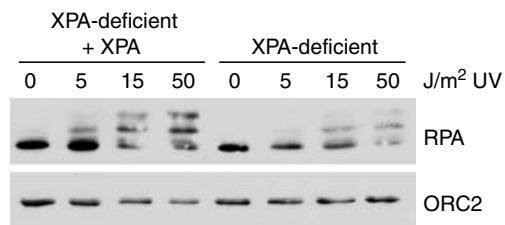
or phosphorylation was altered in XPA-deficient cells. To do so, cells were treated with UV damage and chromatin-bound RPA was isolated by subcellular fractionation. ORC2 binding was also analyzed to normalize for loading of equal amounts of chromatin. As shown in Figure 7, RPA chromatin binding and phosphorylation are significantly reduced in XPA-deficient cells compared to the complemented cells. These observations suggest that XPA may be involved in establishing an ATR-activating structure following UV treatment in S phase.

### Chk1 and RPA phosphorylation are enhanced in UV-treated XP-V cells

Activation of ATR by UV during S phase is thought to be due, at least in part, to the accumulation of ssDNA, which results from uncoupling of helicase and polymerase activities after stalling of the replicative polymerase at the lesion (Byun *et al*, 2005). In the light of this model, one might expect that the loss of NER would lead to increased ATR signaling as the additional unrepaired lesions present in repair-deficient cells could stall an increased number of replicative polymerases. Thus, it is somewhat surprising that loss of XPA leads to a defect in the S-phase checkpoint and that loss of other NER factors has no effect on ATR activation. It is possible, however, that loss of repair does not increase ATR signaling during S phase because other processes are able to compensate for the increased number of lesions.

Lesion bypass by *trans*-lesion synthesis DNA polymerases is one mechanism through which cells can tolerate UV DNA damage (Prakash and Prakash, 2002), and this process might be expected to reduce checkpoint activation by allowing bypass of UV lesions and preventing sustained blockage of replication forks. To test whether lesion bypass affects UV-induced checkpoint activation, the phosphorylation of Chk1 was assessed in XP-V cells, which lack activity of the *trans*-lesion Pol $\eta$ . As shown in Figure 8A, Chk1 S345 phosphorylation was increased in a sustained manner in Pol $\eta$ -deficient cells following treatment with UV relative to the same cell line complemented with the full-length wild-type Pol $\eta$  cDNA. In addition, Pol $\eta$ -deficient cells and Pol $\eta$ -complemented cells exhibited no difference in S345 phosphorylation following aphidicolin treatment (Figure 8B). These data suggest that loss of Pol $\eta$  leads to enhanced ATR signaling following UV damage.

To further test the effects of Pol $\eta$  loss on ATR signaling, RPA chromatin binding and phosphorylation were assessed

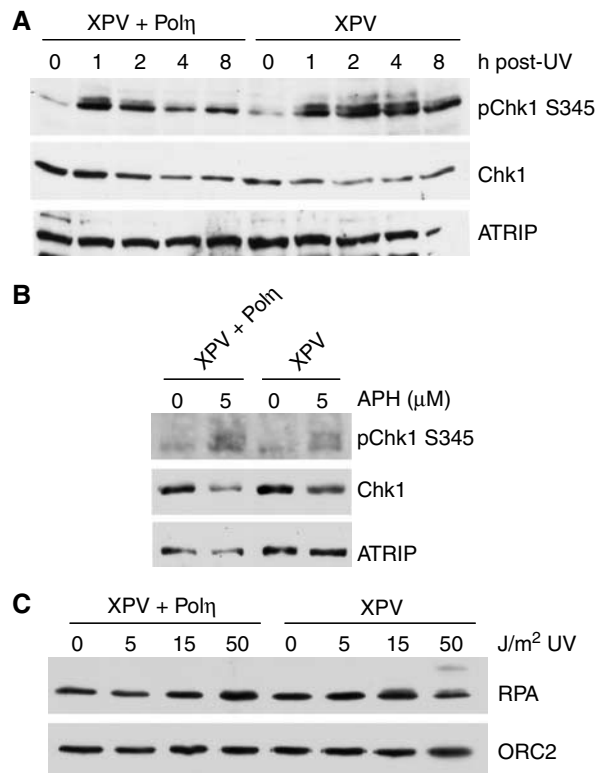


**Figure 7** RPA chromatin binding and phosphorylation is decreased in XPA-deficient cells. XPA-deficient and XPA-complemented cells were treated with increasing amounts of UV radiation and fractionated. Proteins of the insoluble chromatin fraction were resolved by SDS-PAGE and analyzed by Western blot using the indicated antibodies.

following increasing amounts of UV damage. As shown in Figure 8C, RPA chromatin binding was only modestly increased in UV-treated Pol $\eta$ -deficient cells compared to control cells, and the effect was most noticeable at lower UV doses. However, in Pol $\eta$ -deficient cells, RPA phosphorylation was increased compared to complemented controls, particularly after treatment with 50 J/m<sup>2</sup> of UV (Figure 8C). This observation is consistent with the observed increase in Chk1 phosphorylation seen in XP-V cells (Figure 8A). Taken together, these results suggest that a lack of UV *trans*-lesion synthesis causes increased activation of the ATR pathway.

### Discussion

Previous studies in mammalian cells and *Xenopus* egg extracts have shown that activation of the ATR-dependent checkpoint after UV irradiation is dependent upon DNA replication (Lupardus *et al*, 2002; Ward *et al*, 2004). This is thought to be due to stalling of the replicative polymerases by UV DNA damage, resulting in the accumulation of ssDNA and recruitment of ATR-ATRIP to sites of damage (Lupardus *et al*, 2002; Zou and Elledge, 2003; Byun *et al*, 2005). By such a model, one might predict that any process that alters the



**Figure 8** Chk1 and RPA phosphorylation are increased in XPV cells. (A) Pol $\eta$ -deficient (XP-V) and Pol $\eta$ -complemented (XP-V + Pol $\eta$ ) cells were either mock treated or exposed to 15 J/m<sup>2</sup> UV and allowed to recover for the times indicated. Equal amounts of cell lysate were resolved by SDS-PAGE and analyzed by Western blot using the indicated antibodies. (B) Pol $\eta$ -deficient and Pol $\eta$ -complemented cells were treated with 5  $\mu$ M aphidicolin for 6 h and then analyzed as described in panel A. (C) Pol $\eta$ -deficient and Pol $\eta$ -complemented cells were treated with increasing amounts of UV and fractionated. Proteins of the insoluble chromatin fraction were resolved by SDS-PAGE and analyzed by Western blot using the indicated antibodies.

amount of UV-damaged DNA in the nucleus might significantly contribute to checkpoint activation caused by polymerase stalling. Here, we investigated the role of two processes, NER and UV lesion bypass, on ATR checkpoint activation. Interestingly, we found opposing roles for two proteins involved in these processes during S-phase checkpoint activation. After UV damage, cells deficient in the repair protein XPA were found to have a defect in ATR signaling, whereas cells deficient in the *trans*-lesion synthesis polymerase Pol $\eta$  exhibited enhanced ATR signaling. Our results may have also uncovered a role for XPA in checkpoint signaling that is distinct from its function in NER.

To address the role of NER in ATR activation, we analyzed a panel of cell lines that are defective in various steps of this repair pathway. Our results indicate that there is a defect in ATR activation when XPA is lost. This is supported by a decrease in ATRIP and ATR localization to sites of UV damage in XPA-deficient cells, as well as a decrease in UV-induced S345 Chk1 phosphorylation in cells from XPA patients and XPA<sup>-/-</sup> mice, in HeLa cells upon siRNA-mediated knock-down of XPA and in *Xenopus* egg extracts upon depletion of XPA. In addition, we found no substantial defect in ATR activation in XP-C, CS-B, XP-G and XP-F cell lines. Because XPC and CSB function in GGR and TCR, respectively (de Laat *et al*, 1999; Hanawalt, 2002; Costa *et al*, 2003), these results suggest that the defect in ATR activation is not due to a specific branch of NER. However, XPA is required for both types of repair and functions downstream of XPC and CSB (Costa *et al*, 2003). Thus, it is possible that both GGR and TCR must be compromised in mammalian cells in order to observe the reduced ATR activation seen in XP-A cells. Indeed, this is the case in yeast where loss of both GGR and TCR, but neither alone, leads to loss of Rad53 phosphorylation (Giannattasio *et al*, 2004a). In this context, it is worth noting that there was a slight reduction in ATRIP accumulation at sites of UV damage in XPC-deficient cells, suggesting that XPC may have some effect on the ATR response that does not translate to a defect in Chk1 phosphorylation.

The lack of a defect in XPF- and XPG-deficient cells is somewhat surprising. In contrast to XPA, which is involved in damage recognition, XPF and XPG are involved in damage processing, acting as endonucleases to remove the lesion and surrounding nucleotides, and they are needed for both TCR and GGR (de Laat *et al*, 1999; Hanawalt, 2002; Costa *et al*, 2003). Our results differ from a previous study in yeast, which suggests that the NER-dependent activation of ATR's homolog, Mec1, might result from damage processing and generation of a ssDNA gap by XPF and XPG. Importantly, this conclusion is based largely on experiments with G1 synchronized yeast where a defect in Rad53 phosphorylation is observed in mutants of Rad14 and Rad2, the yeast homologs of human XPA and XPG, respectively (Giannattasio *et al*, 2004a). In contrast, the phosphorylation of Chk1 and ATRIP relocalization that we observe following UV treatment occurs predominantly in S-phase cells. This indicates that the activation we are studying is largely S-phase specific and is consistent with our finding that XPA depletion from *Xenopus* egg extracts also reduces Chk1 phosphorylation. It should also be noted that ATR activation could be occurring at significantly lower levels outside of S phase and that our methods are not sensitive enough to detect this activation. Thus, we cannot exclude the possibility that activation of ATR outside of S

phase may still require NER. Indeed, this possibility is consistent with experiments in non-replicating cells showing a requirement for XPA in UV-induced H2AX phosphorylation (O'Driscoll *et al*, 2003).

Altogether, our results provide the first evidence that XPA, but not NER-mediated damage processing, is necessary for ATR checkpoint activation in replicating human cells. The requirement for XPA could indicate that damage recognition by XPA may be sufficient for UV-induced S-phase checkpoint activation, although other unidentified functions of XPA are also possible. Regardless of the mechanism, this role for XPA appears to be specific to bulky lesions, like those produced by UV radiation or 4-NQO because no defect in the aphidicolin-induced phosphorylation of Chk1 was observed upon loss of XPA. We previously proposed a model in which UV lesions and replication inhibitors activate ATR in S phase through a common mechanism that involves slowing down of replicative polymerases and increased unwinding of DNA by the MCM helicases (Pacek and Walter, 2004; Byun *et al*, 2005). This leads to accumulation of RPA-coated ssDNA, which is thought to be involved in recruiting and/or stabilizing the interaction of ATR and ATRIP with DNA (Lupardus *et al*, 2002; Zou and Elledge, 2003). In our experiments, we observed less RPA chromatin binding and phosphorylation in UV-treated XPA-deficient cells. Thus, one possibility is that the specific binding of XPA at sites of UV or UV-like damage may enhance stalling of the replicative DNA polymerases at these lesions, resulting in increased activation of the ATR pathway. Although the naked lesion itself is sufficient to slow replicative polymerases *in vitro* (Fleck and Schar, 2004), the presence of XPA at lesions during DNA replication could facilitate checkpoint signaling by prolonging arrest at these sites, possibly by competing with bypass polymerases. Alternatively, as XPA directly binds to RPA during lesion repair (Matsuda *et al*, 1995; Stigger *et al*, 1998; Costa *et al*, 2003), XPA may help to stabilize the RPA-coated ssDNA intermediate formed when polymerases stall at UV lesions.

It is important to note that complete loss of Chk1 phosphorylation was not observed in cells from XPA patients or upon depletion of XPA. Moreover, at later times after UV treatment, the effect of XPA loss was less pronounced than at earlier times. Similarly, the effect of XPA loss on ATRIP localization to sites of UV damage was most noticeable at earlier times after UV treatment. There are at least two possible explanations for the residual Chk1 phosphorylation observed. As described above, the phosphorylation that does occur could reflect less efficient formation of the ATR-activating structure, ssDNA, through helicase and polymerase uncoupling. However, it is also possible that the Chk1 phosphorylation that is observed is induced in a different manner. Previous studies have linked loss of the ATR-Chk1 pathway to double-strand break formation and replication fork collapse (Osborn *et al*, 2002; Brown and Baltimore, 2003; Zachos *et al*, 2003, 2005). Because XPA is needed for activation of the ATR-Chk1 pathway, the Chk1 phosphorylation observed could result from collapse of replication forks in the absence of XPA, leading to generation of ssDNA from the collapsed fork and activation of ATR by the break and/or ensuing ssDNA. Additional work will be required to discriminate between these and other possibilities.

NER is the major mechanism by which UV DNA damage is repaired in mammalian cells. Because loss of NER activity

leads to an increase in UV damage but does not cause an increase in ATR-mediated checkpoint signaling, it seems likely that another process can compensate for the loss of NER at the doses used here in a manner that affects the ability of UV lesions to stall polymerases in S phase and activate ATR. Supporting this hypothesis, we found that cells deficient in *trans*-lesion synthesis polymerase Pol $\eta$  exhibit increased phosphorylation of Chk1 in response to UV. Because these cells are repair-proficient and the level of damage does not change, these observations suggest that the ability to bypass lesions helps to suppress checkpoint activation. We also observed increased phosphorylation of RPA in XP-V cells as well as a modest increase in RPA chromatin binding. The accumulation of RPA suggests that additional ssDNA is present owing to an inability to efficiently bypass the lesion and is consistent with recent EM studies in yeast showing that cells lacking *trans*-lesion synthesis accumulate small ssDNA gaps along the replicated strands (Lopes *et al*, 2006). Recently, it was reported that cells deficient in Pol $\kappa$ , which is needed to bypass non-UV-mediated bulky adducts, also had increased Chk1 phosphorylation in response to benzo( $\alpha$ )pyrene-dihydrodiol epoxide (Bi *et al*, 2005). Taken together, these findings suggest that *trans*-lesion synthesis polymerases raise the threshold level of damage that can be tolerated before the checkpoint is activated. They are also consistent with a requirement for polymerase stalling in checkpoint activation and suggest that Pol $\eta$  may raise this threshold by reducing replication fork arrest.

Cells that are deficient in XPA and Pol $\eta$  (XPV) are derived from patients suffering from XP. Although defects in these genes result in increased sensitivity to UV, patients lacking XPA have much more severe clinical phenotypes than patients with XP-V (Masutani *et al*, 1999). This difference in severity is generally attributed to the increased accumulation of UV DNA damage in repair-deficient XP-A patients relative to repair-proficient XP-V patients (Lehmann, 2003). Our finding that XPA-deficient cells have decreased ATR checkpoint signaling may suggest an additional reason for the more severe UV-sensitive phenotype of these patients. Likewise, the enhanced ATR signaling we observed in Pol $\eta$ -deficient cells may also result in a less severe UV sensitivity owing to a more active checkpoint and upregulation of p53-mediated response genes such as XPC and DDB2 (Adimoolam and Ford, 2003). Ultimately, further investigation will be required to understand the contribution of ATR checkpoint activation to these UV-sensitive syndromes.

## Materials and methods

### Cell lines and cell culture

HeLa cells purchased from the National Cell Culture Center were maintained in DMEM (Invitrogen) supplemented with 10% FBS, penicillin, streptomycin and glutamine. XPA-complemented (GM15876A) and XPA-deficient (XP12R0) lines purchased from the Coriell Cell Repository were maintained in DMEM. XPA<sup>-/-</sup>MEFs (Ichikawa *et al*, 2000) and NIH 3T3 cells (ATCC) were maintained in DMEM. CSB-complemented and CSB-deficient (CS1AN.S3.G2) cell lines were maintained in MEM plus 400  $\mu$ g/ml G418 (Tuo *et al*, 2002). XPF-complemented and XPF-deficient (GM08437) cell lines were maintained in MEM plus 300  $\mu$ g/ml G418 (Chen *et al*, 2004). XPG-complemented lines were made by stable transfection of XPG-deficient cell lines (94RD270/XP3BR-SV) (Klein *et al*, 1990) with the XPG cDNA and selection in G418 (800  $\mu$ g/ml). Stable colonies were screened for expression of XPG by Western blot and correction of UV sensitivity was assessed using an MTT

assay (data not shown). XPG-complement cells were maintained in McCoy's media with 10% FBS and 200  $\mu$ g/ml G418. Complemented and deficient Pol $\eta$  (GM2359) cell lines were maintained in DMEM plus 200  $\mu$ g/ml G418 (Bassett *et al*, 2004). The following siRNA duplexes were purchased from Dharmacon (Lafayette, CO): GCUACUGGAGGCAUGGCU (XPA siRNA-1) and GGAGACGAUUGU UCAUCA (XPA siRNA-2), the latter of which was previously reported (Lin *et al*, 2006). SiCONTROL (Dharmacon) was used as the non-targeting siRNA. All siRNA transfections were performed using 50 nM of oligonucleotide and Lipofectamine 2000 (Invitrogen).

Cells were washed once with PBS, then exposed to UV-C using a Stratallinker (Stratagene) except when local irradiation was used (see below). The media were replaced and cells were allowed to recover for 1 h in the incubator. 4-NQO (Sigma) treatment was performed by adding drug to cells in serum free media for 1 h. IR treatment was performed using a <sup>137</sup>Cs irradiator followed by a 2 h recovery. Aphidicolin (Sigma) treatment was for 6 h at 5  $\mu$ M in normal media.

### Antibodies

The Chk1 S345 phospho-specific antibody (Cell Signaling), Chk1 antibody (Santa Cruz Biotech), human ORC2 antibody (BD Bioscience), XPA 12F5 antibody (Lab Vision), BrdU antibody (Roche), XPG 8H7 antibody (Santa Cruz) and human RPA AB3 antibody (Oncogene) are commercially available. The anti-CPD mouse monoclonal antibody (TDM2) was a gift of Toshio Mori, Nara Medical University, Nara, Japan (Mori *et al*, 1991). The human ATRIP antibody (Bomgarden *et al*, 2004) and the *Xenopus* ATRIP antibody (Byun *et al*, 2005) were described previously. The xXPA rabbit polyclonal antibody used for immunodepletion was generated at a commercial facility (Josman, Napa, CA) from full-length HIS-xXPA expressed using the Bac-To-Bac system (Invitrogen) in Hi5 insect cells.

### Immunofluorescence staining

For the ATRIP-CPD filter-based assay,  $2 \times 10^5$  cells were plated onto coverslips and incubated 12 h before staining. The published protocol (Fitch *et al*, 2003) was modified as follows. Cells were rinsed once with PBS before treatment with 50 J/m<sup>2</sup> UV-C through a 5  $\mu$ m filter membrane (Whatman) followed by a 1 h recovery. Cells were washed twice with PBS and fixed with 3% paraformaldehyde in PBS plus 0.1% Triton X-100 (PBST) for 20 min. Cells were then washed once with PBS, permeabilized for 15 min with 0.1% PBST and treated with 2 N HCl for 5 min. Acid was neutralized by washing five times with PBS. Cells were then stained with affinity-purified anti-ATRIP (1:600) and anti-CPD (1:150) primary antibodies diluted in 0.1% BSA/PBST for 1 h in a humidified chamber at 37°C. After washing three times with 0.1% BSA/PBST, cells were stained with secondary Alexa rabbit 488 and mouse 595 antibodies (Molecular Probes) diluted 1:400 in 0.1% BSA/PBST. Cells were washed five times with 0.1% BSA/PBST before staining with 1:10 000 DAPI (Sigma) and fixing with Prolong anti-fade (Molecular Probes). All slides were visualized with a Zeiss Axioskop microscope. Images were prepared using Adobe Photoshop.

### Extract preparation and depletions

To prepare whole-cell extracts, cells were harvested by trypsinization, washed in PBS, resuspended in modified TGN buffer (50 mM Tris pH 7.5, 150 mM NaCl, 50 mM  $\beta$ -glycerophosphate, 10% glycerol, 1% Tween 20, 0.2% Nonidet P-40, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT and 10  $\mu$ g/ml leupeptin, pepstatin, aprotinin) for 20 min on ice and cleared by centrifugation (10 min, 16 000 g).

*Xenopus* extracts were prepared as described (Murray, 1991; Lupardus *et al*, 2002). Aphidicolin was used at 150  $\mu$ M, and sperm chromatin was UV damaged in a Stratallinker (Stratagene). Chromatin binding from interphase extracts has been previously described (Lupardus *et al*, 2002). Immunodepletion (three rounds, 30 min each) of XPA from interphase extract was carried out at 4°C using Affiprep Protein-A beads (Bio-Rad). XPA antiserum was bound to beads at a 1:2 ratio of beads to serum. Phospho-S344 Chk1 blots for *Xenopus* Chk1 were performed with a phospho-Ser345 human Chk1 antibody raised by Zymed Inc.

### Chromatin binding

Small-scale nuclear extracts were prepared using  $1 \times 10^6$  cells harvested with trypsin, washed with PBS and resuspended in



200  $\mu$ l buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitor cocktail) plus 5% Triton X-100 for 5 min on ice. Nuclei were pelleted by centrifugation at 1300 g in swinging bucket Eppendorf 5417C centrifuge for 4 min. The nuclei were washed with 200  $\mu$ l buffer A without detergent and respun. The pellet was then resuspended with 200  $\mu$ l buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT), incubated on ice for 10 min and centrifuged at 1700 g for 4 min. Chromatin-bound proteins were washed with buffer B, respun and resuspended in 200  $\mu$ l sample buffer before shearing DNA using a 28-gauge syringe needle.

#### Cell synchronization

Cells were synchronized in S phase by addition of 2 mM thymidine to cell media for 16 h, replacement with fresh media for 8 h and addition of 2 mM thymidine again for 16 h before release with fresh media for S-phase time course. BrdU incorporation was performed

as described previously (Barr *et al*, 2003). Cell cycle analysis was performed using a Beckton Dickinson FACS Caliber.

#### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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