

WRN Is Required for ATM Activation and the S-Phase Checkpoint in Response to Interstrand Cross-Link–Induced DNA Double-Strand Breaks

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Werner syndrome (WS) is a human genetic disorder characterized by extensive clinical features of premature aging. Ataxia-telangiectasia (A-T) is a multisystem human genomic instability syndrome that includes premature aging in some of the patients. WRN and ATM, the proteins defective in WS and A-T, respectively, play significant roles in the maintenance of genomic stability and are involved in several DNA metabolic pathways. A role for WRN in DNA repair has been proposed; however, this study provides evidence that WRN is also involved in ATM pathway activation and in a S-phase checkpoint in cells exposed to DNA interstrand cross-link–induced double-strand breaks. Depletion of WRN in such cells by RNA interference results in an intra-S checkpoint defect, and interferes with activation of ATM as well as downstream phosphorylation of ATM target proteins. Treatment of cells under replication stress with the ATM kinase inhibitor KU 55933 results in a S-phase checkpoint defect similar to that observed in WRN shRNA cells. Moreover, γ H2AX levels are higher in WRN shRNA cells than in control cells 6 and 16 h after exposure to psoralen DNA cross-links. These results suggest that WRN and ATM participate in a replication checkpoint response, in which WRN facilitates ATM activation in cells with psoralen DNA cross-link–induced collapsed replication forks.

INTRODUCTION

Proliferating cells are continuously exposed to agents that cause DNA damage and/or interfere with the progression of DNA replication (i.e., induce DNA replication fork arrest). Because such events can adversely affect genomic stability, cells have evolved S-phase signaling cascades, including DNA damage checkpoint responses and DNA repair mechanisms, to remove or repair DNA damage. In cells with DNA damage, the S-phase checkpoint inhibits the firing of late origins of replication (intra-S checkpoint) and prevents cells from entering mitosis with persistent DNA lesions (S-M checkpoint; Bartek *et al.*, 2004). Together, these mechanisms promote accurate completion of DNA replication before cell division. Cells with defective S-phase cell cycle checkpoints are characterized by high levels of genomic instability.

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Abbreviations used: A-T, ataxia-telangiectasia A-T; CPT, camptothecin; DSBs, double-strand breaks; HU, hydroxyurea; ICLs, interstrand cross-links; MRN, Mre11-Rad50-Nbs1; PUVA, psoralen plus UVA; PFGE, pulse-field gel electrophoresis; WS, Werner syndrome; WRN, Werner protein.

Many commonly used cancer therapeutic agents sensitize cancer cells to replication-blocking DNA damage (Bryant *et al.*, 2005; Farmer *et al.*, 2005). For example, psoralen plus UVA (PUVA) induces interstrand cross-links (ICLs), which covalently bind the complementary strands of the DNA double helix, causing DNA replication fork arrest, and inducing cytotoxic DSBs during S-phase (Rothfuss and Grompe, 2004). Similarly, camptothecin (CPT) induces and traps topoisomerase I-DNA covalent complexes, stabilizing otherwise transient DNA single-strand breaks and ultimately producing cytotoxic DNA double-strand breaks (DSBs) during S-phase (Pommier *et al.*, 2003). Exposure to hydroxyurea (HU), which depletes dNTP pools, has similar effects on cycling cells (Lundin *et al.*, 2002). Although the mechanisms by which these clastogens act are distinct, they all indirectly induce DSBs in the vicinity of stalled DNA replication forks, a process known as replication fork collapse. It is generally thought that the Mre11-Rad50-Nbs1 (MRN) complex senses DSBs induced by ionizing irradiation and radiomimetic chemicals (Uziel *et al.*, 2003; Stracker *et al.*, 2004; Lee and Paull, 2005); however, less is known about the mechanisms involved in the cellular response to DSBs associated with replication fork collapse.

DSB-induced replication arrest is usually transduced through the nuclear protein kinases ATM and ATR, which are members of a family of proteins characterized by a PI3-like domain (Abraham, 2004). ATM, a primary inducer

of the cellular response to DSBs, activates the DSB response network by phosphorylating key players in its numerous branches (Shiloh, 2003, 2006). Loss or inactivation of ATM is associated with the genomic instability syndrome ataxia-telangiectasia (A-T; Chun and Gatti, 2004), which is characterized by neuronal degeneration, immunodeficiency, radiation sensitivity, and cancer predisposition. ATR responds primarily to UV damage and stalled replication forks, but it is also involved in the response to DSBs and shares some protein targets with ATM (Shechter *et al.*, 2004b). ATM and ATR are also involved in initiation and progression of DNA replication in unstressed cells (Abraham, 2001; Shechter *et al.*, 2004a). When ATM or ATR is depleted in *Xenopus* cell free extracts, restart of damaged replication forks can induce DSBs (Trenz *et al.*, 2006). Recent reports suggest that ATM acts upstream of ATR in the S-phase checkpoint response to γ -irradiation; however, this may not be true for cells exposed to UV light or HU, agents that do not directly induce DNA DSBs (Cuadrado *et al.*, 2006; Jazayeri *et al.*, 2006; Stiff *et al.*, 2006). Therefore, it appears that ATM and ATR cooperate in activating the S-phase checkpoint, but their exact roles in this process may differ depending on the nature of the DNA damage and the type of replication-associated stress.

Werner syndrome (WS) is a rare autosomal recessive disorder characterized by premature onset of age-related pathologies and genomic instability. The gene mutated in WS, WRN, encodes a conserved DNA helicase of the RecQ family (Yu *et al.*, 1996). WRN possesses 3' \rightarrow 5' helicase, 3' \rightarrow 5' exonuclease, and DNA-dependent ATPase activities (Gray *et al.*, 1997; Huang *et al.*, 1998). Biochemical and cellular studies in mice provide evidence that WRN copurifies with the 17S multiprotein DNA replication complex via direct binding to proliferating cell nuclear antigen (PCNA; Lebel *et al.*, 1999). In addition, the *Xenopus* orthologue of WRN, FFA-1, is required for the formation of replication foci (Yan *et al.*, 1998). Consistent with these observations, WS fibroblasts show a reduced number of replication initiation sites, a prolonged S-phase, and defective sister chromatid exchange at telomeres in unstressed cells (Takeuchi *et al.*, 1982; Poot *et al.*, 1992; Crabbe *et al.*, 2004; Laud *et al.*, 2005). In addition to a role for WRN in normal DNA replication, RecQ helicases have been implicated in replication checkpoint(s). Deletion of the yeast homolog of WRN, SGS1, leads to defective S-phase checkpoint activation in the stressed cells (Frei and Gasser, 2000). Furthermore, one report showed that activation of ATM is dependent on another RecQ helicase, BLM, after prolonged exposure to HU but not after exposure to the radiomimetic agent bleomycin (Davalos *et al.*, 2004). Interestingly, WS cells are hypersensitive to DNA ICLs, but only mildly sensitive to γ -irradiation-induced DSBs (Poot *et al.*, 2001; Yannone *et al.*, 2001; Bohr *et al.*, 2001). WS cells with persistent DNA ICLs undergo apoptosis in S-phase, which is likely due to accumulation of cytotoxic DSBs during DNA replication (Poot *et al.*, 2002; Rothfuss and Grompe, 2004). Nonetheless, the exact role of WRN in the response to replication fork collapse is not understood.

Although ample evidence suggests that WRN resolves recombinational intermediates to facilitate DSB repair (Prince *et al.*, 2001; Saintigny *et al.*, 2002), relatively little is known about the role of WRN in the initial response to DSBs. Recent reports indicated that recruitment of WRN to sites of chromosome structural changes including DSBs is a rapid response (Lan *et al.*, 2005; Turaga *et al.*, 2007). Although ATR is thought to play a key role in the response to many types of replication stress including high doses of PUVA (Pichierri and Rosselli, 2004; Alderton *et al.*, 2006; Jazayeri *et al.*, 2006), ATM and WRN were independently

identified as regulators of chromosomal replication (Lebel *et al.*, 1999; Shechter *et al.*, 2004a). These results led us to propose and test whether WRN and ATM might cooperate with each other during the response to replication-dependent DSBs. Indeed, the results presented here demonstrate that WRN and ATM cooperatively participate in an intra-S checkpoint in cells with collapsed replication forks.

MATERIALS AND METHODS

Cells and Reagents

The generation and maintenance of WRN short-hairpin RNA (shRNA), ATM shRNA and control cells using the U-2 OS osteosarcoma cell line have been described previously (Cheng *et al.*, 2006; Ziv *et al.*, 2006). The pBSCA-6XHis WRN expression vector was transfected into cells using PolyFect reagent (Qiagen, Chatsworth, CA) as previously described (Cheng *et al.*, 2003). The ATM inhibitor, KU55933, was kindly donated by Dr. Graeme Smith (Kudos Pharmaceuticals). Nocodazole, 8-methoxyypsoralen, camptothecin, HU, and angelicin were purchased from Sigma (St. Louis, MO), and aphidicolin from Calbiochem (La Jolla, CA). All were dissolved in DMSO except for HU, which was dissolved in water.

Flow Cytometric Analysis

Cell monolayers were treated with trypsin, and single cells were fixed overnight in 70% ethanol at -20°C . After centrifugation, RNase A and propidium iodide were added and incubated in the dark using the Cellular DNA Flow Cytometric Analysis Reagent Set according to the manufacturer's suggestion (Roche, Indianapolis, IN). Samples were analyzed using a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Cell cycle analysis on histograms from propidium iodide-stained cells was performed using Multicycle (Phoenix Flow Systems, San Diego, CA), where doublets and debris were modeled by software algorithms.

Checkpoint Assays

For intra-S checkpoint assay that measures DNA synthesis after DNA damage (Jaspers and Zdzienicka 2006), cells were preincubated with [^{14}C]thymidine for 24 h. After extensive washing, cells were treated with 8-methoxyypsoralen (0.1 $\mu\text{g}/\text{ml}$, 8-MOP, Sigma) for 10 min and 365-nm UV light at 1.8 J/cm 2 for 5 min (PUVA; Majumdar *et al.*, 1998). Cells were then pulsed with [^3H]thymidine after the indicated times and collected 2 h later. Alternatively, cells were γ -irradiated (0–20 Gy), immediately pulsed with [^3H]thymidine, and collected 2 h later. For Figure 4C, ethanol-fixed WRN shRNA and control cells were permeabilized with 0.25% Triton X-100 in PBS on ice for 15 min. After centrifugation, cells were stained with phospho-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY) in PBS containing 1% BSA, followed by FITC-conjugated secondary antibody and propidium iodide staining. Mitotic cells were examined by flow cytometry.

Western Blotting Analysis

Whole cell lysates and immunoblot analysis were described previously (Cheng *et al.*, 2003). The following antibodies were used: pS1981 ATM (clone 10H11.E12) and pS139 H2AX (clone JBW301) were from Upstate Biotechnology; ATM (Epitomics, clone Y170); pS966 SMC1 (clone BL311) from Bethyl (Montgomery, TX); pS1423 BRCA1 and H2AX (Novus, Littleton, CO); BRCA1 and WRN (Transduction Laboratories, Lexington, KY); SMC1, pS317 CHK1, and CHK1 (Cell Signaling Technology, Beverly, MA); and lamin B (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluorescence

Cells were grown on Lab-Tek glass chamber slides (Nunc, Rochester, NY). For colocalization studies, cells were treated with bromodeoxyuridine (BrdU, 10 μM) and incubated at 37°C for 30 min in normal culture medium before PUVA treatment. The slides were washed with PBS between every indicated step. After treatment with a hypotonic lysis solution (10 mM Tris-HCl, pH 7.4, 2.5 mM MgCl $_2$, 1 mM PMSF, and 0.5% Nonidet P-40) for 8 min on ice, cells were fixed in 4% paraformaldehyde for 10 min at room temperature (Balajee and Geard, 2001). Fixed cells were permeabilized with PBS + 0.2% Triton X-100 at room temperature for 5 min, blocked in PBS + 2% fetal bovine serum for 1 h at room temperature, and incubated with rabbit anti-ATM mAb (Epitomics, 1:500) previously found to be ATM-specific in immunostaining (Biton *et al.*, 2006) and mouse anti-WRN mAb (clone 2D06) at 37°C for 1 h. Subsequently, cells were incubated with Alexa 633-conjugated anti-mouse IgG and Alexa 568-conjugated anti-rabbit IgG antibodies (Molecular Probes, Eugene, OR, 1:1000) at 37°C for 1 h in the dark. The chamber slides were then fixed again with 4% paraformaldehyde in PBS for 5 min at room temperature, and the DNA was denatured by incubating in 2 N HCl at 37°C for 30 min. After blocking as described above, the slides were immunostained with sheep anti-BrdU antibody (GeneTex, 2 $\mu\text{g}/\text{ml}$) at 37°C for 1 h, followed by incuba-

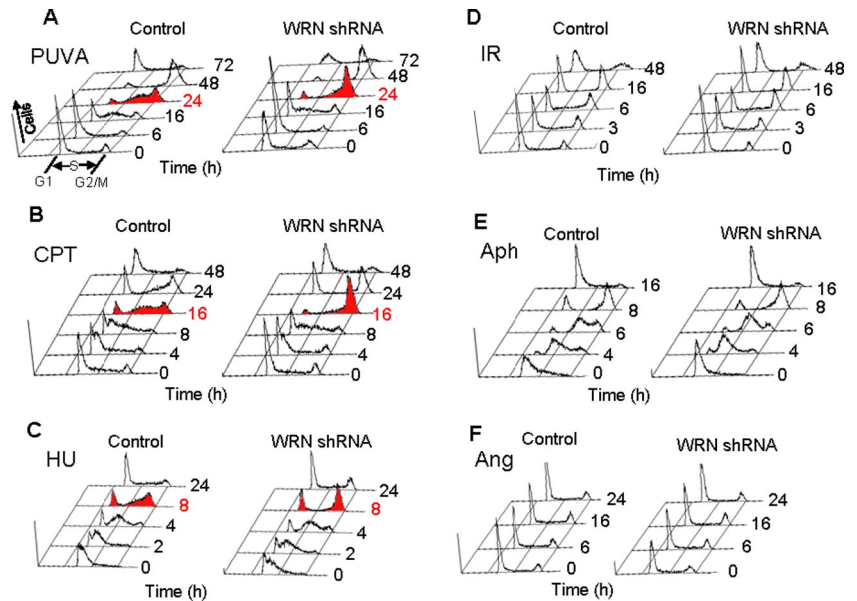


Figure 1. Role of WRN in S-phase checkpoint. Control and WRN shRNA U-2 OS cells were treated with (A) PUVA (0.1 μ g 8-methoxy-psoralen/ml), (B) CPT (1 μ M, 3 h), (C) HU (0.5 mM, 24 h), (D) ionizing radiation (6 Gy), (E) aphidicolin (1 μ g/ml, 24 h), and (F) angelicin (0.1 μ g/ml)+UVA. Cells were harvested at the indicated time points and analyzed by flow cytometry (n = 3–4).

tion with Alexa 488 anti-sheep IgG antibody. DNA was stained with 1 μ M DAPI in PBS for 20 min at room temperature, and the slides were mounted with Prolong Gold antifade reagent (Molecular Probes). Immunofluorescence was analyzed by a confocal microscope (Zeiss 510, Thornwood, NY).

To determine general ATM/ATR phosphorylation, we used the pS/TQ antibody (Cell Signaling), which was raised against a collection of peptides containing the p-SQ or p-TQ motifs. Cells were grown in the glass chamber slides and fixed in 4% paraformaldehyde for 10 min at room temperature and then permeabilized with PBS + 0.2% Triton X-100. Fixed cells were then blocked in 10% goat serum overnight at 4°C, followed by immunostaining with the pS/TQ antibody (1:1500) at 37°C for 2 h. Subsequently, slides were incubated with Alexa 568-conjugated anti-rabbit IgG antibody (1:1000, Molecular Probes). DNA was stained with 1 μ M DAPI in PBS for 20 min at room temperature, and the slide chambers were mounted with Prolong Gold antifade reagent (Molecular Probes). Immunofluorescence signals were visualized by a fluorescence microscope (Zeiss Axiovert 200 M) and quantitated using the software AxioVision 4.5.

Pulse-Field Gel Electrophoresis

U-2 OS cells were treated with the indicated clastogens. After trypsinization, 10^6 cells were melted into 0.7% agarose insert. These inserts were incubated in 0.5 M EDTA (pH 8.0), 1% N-laurylsarcosyl, and proteinase K (1 mg/ml) at 50°C for 48 h and then washed four times in Tris-EDTA buffer before loading onto a 1% agarose (chromosomal grade) gel. Chromosomes were separated by pulsed-field gel electrophoresis for 24 h (CHEF Mapper, Bio-Rad, Richmond, CA; 120° angle, 60–240-s switch time, 4 V/cm). The gel was subsequently stained with ethidium bromide for analysis.

RESULTS

WRN Participates in a S-Phase Checkpoint Activated by Clastogens That Induce Replication-dependent DSBs

The role of WRN in the S-phase checkpoint was examined by treating WRN-deficient and control U-2 OS cells with DNA-damaging agents and examining cell cycle progression. In these experiments, we used WRN-deficient cells that stably expressed a WRN-specific shRNA (WRN shRNA cells) and control cells stably express a nonspecific shRNA (control shRNA cells). Cells were treated with clastogens that cause collapsed replication forks (PUVA, CPT, and HU), DSBs (γ -irradiation), DNA monoadducts (angelicin) or that inhibit replication (aphidicolin) and then were analyzed for cell cycle progression by flow cytometry. The majority of control and WRN shRNA cells treated with PUVA (0.1 μ g 8-methoxy-psoralen/ml, Figures 1A and 2A) arrested in S-phase 16 h after treatment. However, by 24 h after treatment, significantly more control cells were arrested in S-phase and

relatively more WRN shRNA cells appeared at G2/M phase. Similar results were obtained in cells treated with CPT or HU. Both cell types arrested in S-phase 8 h after removal of CPT from the culture media (1 μ M for 3 h), but WRN shRNA cells progressed through S-phase at a faster rate than control cells (Figures 1B and 2B; 16-h time point). As such, the population of WRN shRNA cells contained more G2/M and fewer S-phase cells 16 h after CPT removal. For cells treated with HU, the majority of control and WRN shRNA cells arrested in S-phase 4 h after removal of HU from the culture media (0.5 mM for 24 h), but fewer WRN shRNA cells remained in S-phase 8 h after HU removal (Figures 1C and 2C). Because PUVA, CPT, and HU all induce DSBs during DNA replication, these data suggest that WRN may play a role in the S-phase checkpoint induced by collapsed replication forks. Lack of such replication checkpoint in WRN shRNA cells may compromise cell proliferation, as we have shown previously that WRN shRNA cells are more sensitive than control cells to PUVA-induced cell death (Cheng *et al.*, 2006). The cell doubling times for unstressed U-2 OS cells are ~20 h, consistent with the time frame of the observed S-phase checkpoint defect in WRN shRNA cells after replication stress.

The ability of replication-independent γ -irradiation induced DSBs to induce an S-phase checkpoint was also tested in control and WRN-deficient cells. Cells were treated with 6 Gy γ -irradiation and analyzed by flow cytometry. The number of control and WRN shRNA cells in S-phase was higher at 3 and 6 h after γ -irradiation, and most of these cells arrested in G1 or at G2/M by 16 h after irradiation (Figure 1D and Supplemental Figure S1). However, during the first 48 h after γ -irradiation, there was no apparent difference in cell cycle profiles in control and WRN shRNA cells. Aphidicolin is a known replication inhibitor that does introduce significant DSBs (Saleh-Gohari *et al.*, 2005). After treatment with aphidicolin (1 μ g/ml, 24 h), control and WRN shRNA cells accumulated in S-phase by 4 h and at G2/M by 8 h after removal of aphidicolin; both cell types progressed through the cell cycle with similar kinetics (Figure 1E and Supplemental Figure S2). In addition, angelicin (0.1 μ g/ml), an agent that forms DNA monoadducts but not DNA ICLs in the presence of UVA, did not stimulate a WRN-dependent

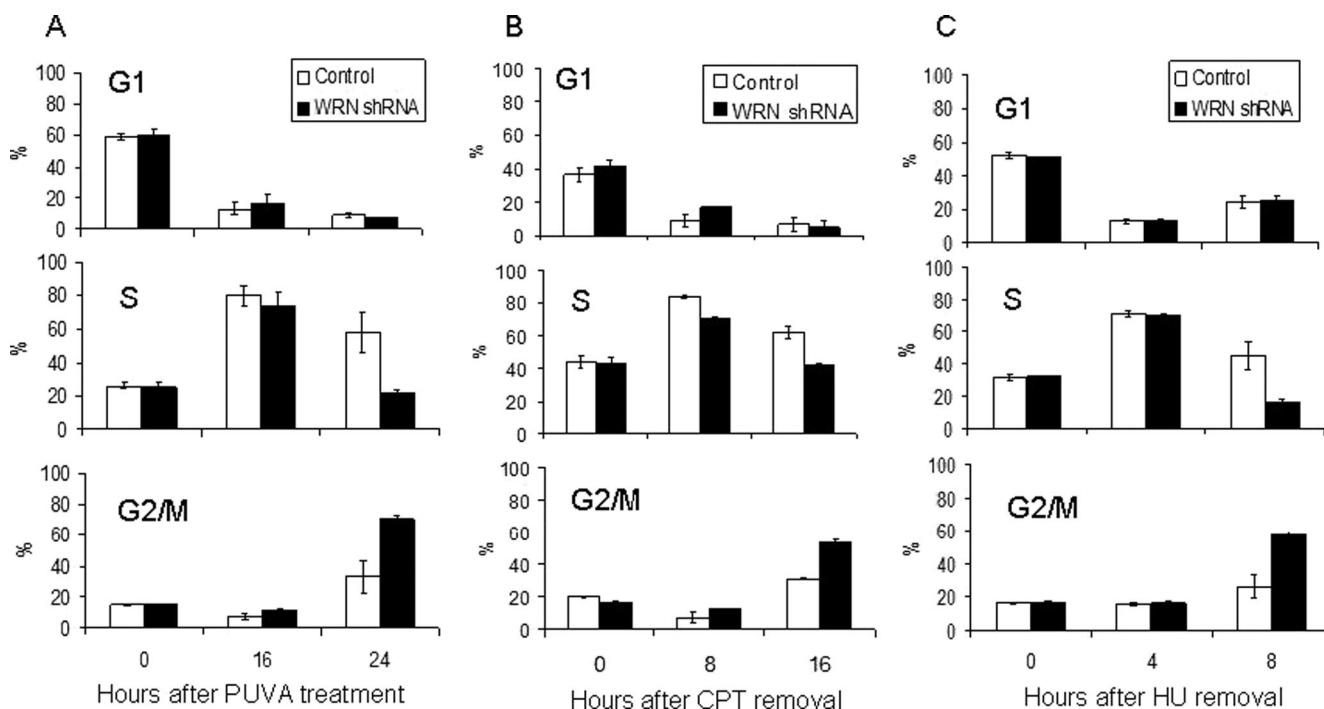


Figure 2. Quantification of cell cycle profiles as described in Figure 1. (A) PUVA; (B) CPT; (C) HU.

S-phase checkpoint (Figures 1F and Supplemental Figure S3). Taken together, the WRN-dependent S-phase checkpoint regulation likely requires formation of sufficient amount of replication-dependent DSBs.

Pulse-field gel electrophoresis (PFGE) was performed to confirm the presence or absence of DSBs in treated cells (Figure 3; see also Saleh-Gohari *et al.*, 2005). The results confirmed that DSBs were generated under the following conditions: PUVA (0.1 μ g 8-methoxy-psoralen/ml, 24 h), CPT (1 μ M for 3 h, followed by 16-h recovery), and γ -irradiation (6 Gy, 30 min recovery; Figure 3). In contrast, DSB induction by exposure to aphidicolin (1 μ g/ml for 24 h, followed by 6-h recovery) is only marginal, the level being much lower than those of γ -irradiation, PUVA, CPT, or HU.

We have previously shown that U-2 OS cells exhibit increased endogenous chromosome breaks as evidenced by

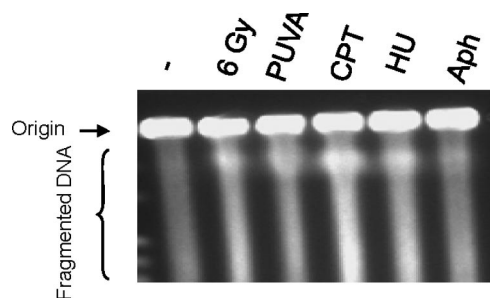


Figure 3. Pulse-field gel electrophoresis of control shRNA U-2 OS cells. DNA from U-2 OS cells was analyzed by PFGE after exposure to DNA damaging agents as follows: 30 min after 6 Gy γ -irradiation, 24 h after PUVA treatment (0.1 μ g 8-methoxypsoralen/ml), 16-h recovery after CPT treatment (1 μ M for 3 h), 8-h recovery after HU treatment (0.5 mM for 24 h), and 6-h recovery after Aph treatment (1 μ g/ml for 24 h).

PFGE (Bartkova *et al.*, 2006). This is consistent with the background DNA fragments observed in unstressed U-2 OS cells (Figure 3). The specificity of WRN knockdown was confirmed by complementing WRN shRNA cells with ectopic His-tagged WRN. It is our experience that Western analysis allows us to detect exogenously expressed WRN in WRN shRNA cells, suggesting that the system of WRN overexpression outpaces that of WRN knockdown. Twenty-four hours after treatment with PUVA, 26 \pm 9% of WRN-knockdown cells arrested in S-phase, whereas 60 \pm 9% of WRN knockdown cells expressing ectopic WRN arrested in S-phase; thus, complementation with wild-type WRN restored near wild-type response to PUVA (69 \pm 11% in S-phase, Supplemental Figure S4). These results strongly support the observation that WRN plays a significant role in the S-phase checkpoint in the PUVA-treated U-2 OS cells.

PUVA-induced Activation of the ATM Pathway Requires WRN

There are at least two possible roles that WRN might play in the S-phase checkpoint: WRN might regulate early S-phase checkpoint kinases, such as ATM, or it might act downstream of checkpoint signaling cascades. The effect of WRN on ATM activation was examined by determining ATM autophosphorylation on Ser-1981. Human ATM activation involves autophosphorylation on Ser-1981, Ser-367, and Ser-1893 (Bakkenist and Kastan, 2003; Kozlov *et al.*, 2006). Interestingly, ATM autophosphorylation on Ser-1981 was deficient in WRN shRNA cells (Figure 4A, top panels, left and right) 3–16 h after PUVA treatment using Western blotting analysis. However, ATM autophosphorylation on Ser-1981 was proficient in control shRNA cells from 3 to 16 h (Figure 4A) and gradually returned to the control level during the period from 24 to 72 h after PUVA treatment (not shown). Similarly, ATM autophosphorylation on Ser-1981 was severely repressed in WRN shRNA cells, but was proficient in control shRNA cells 0–24 h after recovery from HU treat-

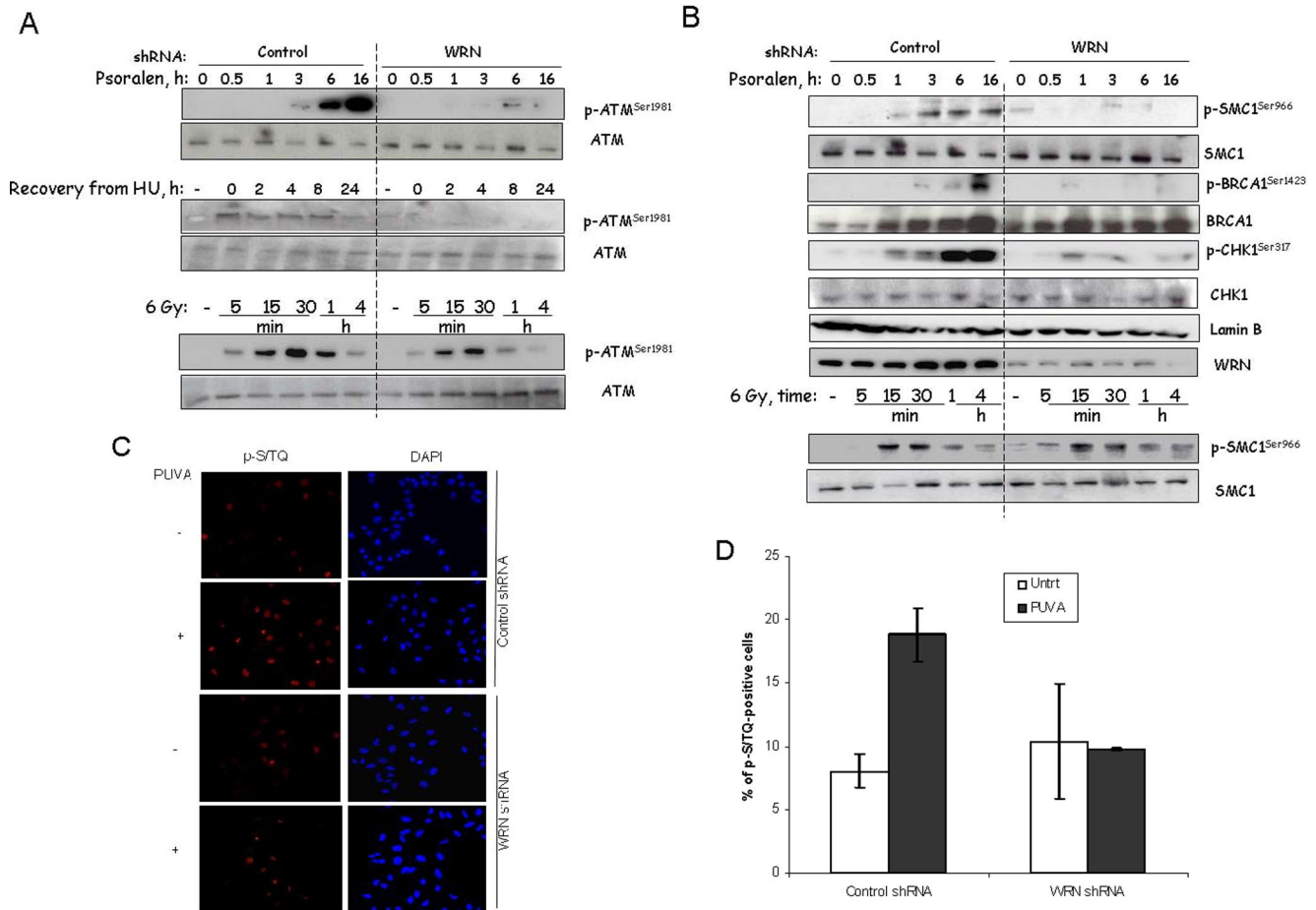


Figure 4. WRN-dependent ATM activation. (A) ATM autophosphorylation on Ser-1981 was quantified in control and WRN shRNA cells treated with PUVA (0.1 μ g 8-methoxy-psoralen/ml), HU (0.5 mM), or γ -irradiation. Cell lysates were prepared at the indicated time points as described for Western analyses (see *Materials and Methods*). (B) As in A, except Western blot was probed with antibodies to ATM substrates. (C) Phosphorylation of ATM substrates in control and WRN shRNA U-2 OS cells 16 h after treatment with PUVA (0.1 μ g 8-methoxy-psoralen/ml). Representative images were shown. (D) Fluorescence intensity after staining with the anti-phospho-(S/TQ) antibody was averaged over fields of cells using the softwares Axio Vision 3.1 and AIM Rel 3.2 (Zeiss).

ment (Figure 4A, middle panels). ATM autophosphorylation on Ser-1981 was not detectable in WRN shRNA cells 0–24 h after recovery from HU treatment, whereas ATM autophosphorylation subsided in control shRNA cells at 24 h, a time point when cells have been released from S-phase arrest (Figure 1C). Thus, our results suggest that replication stress-induced activation of the ATM pathway is diminished, but not delayed, in WRN shRNA cells. Altogether, the results suggest that wild-type WRN is required for optimal ATM autophosphorylation in cells after PUVA treatment, and this might be a general event in response to collapsed replication forks based on the HU treatment data. In contrast, ATM autophosphorylation does not appear to be WRN-dependent in cells treated with 6 Gy of γ -irradiation (Figure 4A, bottom panels).

The results shown in Figure 4A predicted that downstream events in the ATM signaling pathway might also be defective in WRN shRNA cells treated with PUVA. To test this possibility, we determined phosphorylation of SMC1 on Ser-966, BRCA1 on Ser-1423, and CHK1 on Ser-317, all of which have been reported as ATM phosphorylation targets during activation of the S-phase checkpoint (Gatei *et al.*, 2000; Xu *et al.*, 2001; Kitagawa *et al.*, 2004). Indeed, WRN shRNA cells were deficient in PUVA-induced phosphoryla-

tion of SMC1, BRCA1, and CHK1 at these phosphorylation sites (Figure 4B). In contrast, ATM substrates such as SMC1 were phosphorylated at normal levels in WRN shRNA cells exposed to γ -irradiation (Figure 4B, bottom panels). In addition, the amount of total SMC1, BRCA1, CHK1, and lamin B was similar in control and WRN shRNA cells under all conditions tested. Remarkably, it appears that WRN affects ATM activation and regulates S-phase checkpoint with similar kinetics in PUVA- or HU-treated cells (Figures 1 and 4). Thus, WRN is required for the ATM pathway activation in the response to PUVA, but not to γ -irradiation.

These results were confirmed using immunostaining for phosphorylated substrates of ATM; the experiment utilized an antibody that recognizes the ATM phosphorylation motif phosphoserine/phosphothreonine followed by glutamine (pS/TQ; Uziel *et al.*, 2003). We found that there were significantly more pS/TQ-positive control shRNA cells than WRN shRNA cells 16 h after PUVA treatment (19 vs. 10%, $p < 0.05$; Figure 4, C and D). There are comparable results as evidenced by phosphorylation of ATM target motifs (Figures 4, C and D) and specific pS/TQ-containing proteins (Figure 4A), further supporting the conclusion that WRN regulates PUVA-induced ATM pathway activation.

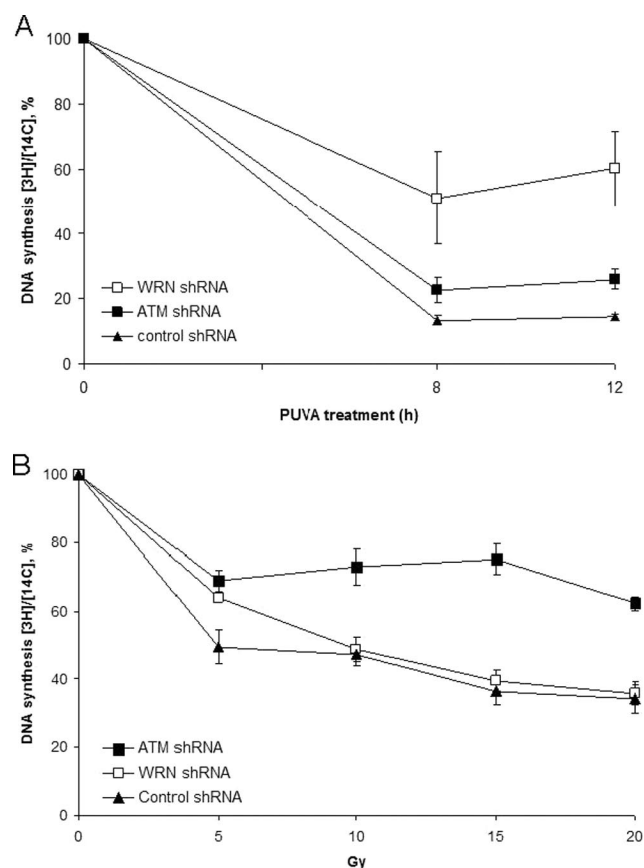


Figure 5. Role of WRN and ATM in intra-S-phase checkpoint in cells treated with PUVa and γ -irradiation. (A) Rate of DNA synthesis in control, WRN and ATM shRNA U-2 OS cells after exposure to PUVa (0.1 μ g 8-methoxypsoralen/ml). Cells were harvested at the indicated time points ($n = 3$). DNA synthesis was measured as described in *Materials and Methods*. (B) Rate of DNA synthesis in control, WRN and ATM shRNA U-2 OS cells 2 h after exposure to the indicated doses of ionizing radiation ($n = 3$).

WRN and ATM Function in a PUVa-induced Intra-S-Phase Checkpoint

The above studies implicate that the ATM-mediated downstream events such as intra-S checkpoint were also impaired. To address this hypothesis, we tested whether WRN and ATM might inhibit DNA synthesis in replicating cells treated with PUVa (0.1 μ g 8-MOP/ml). The results showed an initial decrease in the rate of DNA synthesis similarly in all the three cells, but the rates were higher in WRN and ATM shRNA cells than in control shRNA cells 8 and 12 h after PUVa treatment (Figure 5A). In contrast, the kinetics of DNA synthesis was similar in control and WRN shRNA cells exposed to IR (Figure 5B). Previous studies showed that A-T and Nijmegen breakage syndrome (NBS) cells are characterized by a defect in inhibition of DNA synthesis, known as intra-S checkpoint, after exposure to γ -irradiation (Jaspers and Zdzienicka, 2006). Similarly, ATM shRNA cells failed to inhibit DNA replication after γ -irradiation (Figure 5B). Collectively, our results here expand the role of ATM in the intra-S checkpoint to the more general response to collapsed replication forks. However, the extent of PUVa-resistant DNA synthesis is greater in WRN than in ATM shRNA cells, suggesting a central and upstream role of WRN, in relative to ATM, in PUVa-induced intra-S checkpoint.

The presence of an incomplete replication fork can inhibit cells from entering mitosis (Bartek *et al.*, 2004). We next examined DNA content and the extent of histone H3 Ser-10 phosphorylation (H3-Ser10P) after exposure to PUVa. The relative amount of H3-Ser10P was used as an indicator of cells that have completed DNA replication and entered mitosis (Ajiro *et al.*, 1996). The results showed that the fraction of H3-Ser10P-positive cells was higher in WRN shRNA cells than in control cells 12 h after treatment with PUVa (Supplemental Figure S5). This result is consistent with the presence of a defective S-phase checkpoint in WRN shRNA cells.

Enhanced Level of PUVa-induced γ H2AX in WRN shRNA Cells

It is known that PUVa can induce replication-dependent DSBs (Rothfuss and Grompe, 2004), a well-established trigger of ATM activation. Because we found a deficiency in PUVa-induced ATM activation in WRN shRNA cells (Figure 4), we asked whether PUVa-induced DSB formation is inhibited in WRN shRNA cells. Immunofluorescence (Figure 6A) and Western analysis (Figure 6B) results show that γ H2AX levels increase in a time-dependent manner, and the extent of induction is greater in WRN shRNA than in control shRNA cells 16 h after PUVa treatment. In contrast, γ -irradiation (6 Gy) induces a fast mode of γ H2AX formation, the level of which is similar in WRN and control shRNA cells (Figure 6). The amount of total H2AX protein is similar in WRN and control shRNA cells under all conditions tested (Figure 6B). Taken together, the deficiency in PUVa-induced ATM activation in WRN shRNA cells is unlikely to be attributed to the extent of PUVa-induced DSB formation. Possibly, kinases other than ATM are responsible for H2AX phosphorylation in PUVa-treated WRN shRNA cells. Thus, the results support the view that WRN functionally regulates ATM pathway activation.

WRN and ATM Colocalize at Sites of DNA Replication in the Absence of PUVa

Next, we tested if WRN and ATM colocalize to replication foci by performing coimmunofluorescence for ATM and WRN in U-2 OS cells (Balajee and Geard, 2001). Cells were pulse-labeled with BrdU to identify replication foci. Soluble nucleoplasm was extracted, and cells were fixed before immunostaining. In unstressed cells (no PUVa treatment), ATM colocalizes with WRN at most BrdU-positive replication foci (Supplemental Figure S6A). Interestingly, WRN and ATM colocalize less frequently in non-S-phase cells, which are BrdU-negative (arrow, Supplemental Figure S6B). This result indicates that ATM and WRN constitutively associate with replication foci in S-phase.

WRN and ATM Function in a Common Process of Cellular Response to PUVa

The above data suggest that ATM and WRN may be epistatic in the cellular response to collapsed replication forks. This hypothesis was tested by treating control and WRN shRNA cells sequentially with the ATM kinase inhibitor KU55933 and PUVa. The results showed that PUVa-treated control cells underwent S-phase arrest 24 h after treatment and that this S-phase checkpoint was inhibited to a similar extent by WRN shRNA, KU55933, or both (Figure 7A). However, the fraction of G1 cells increased in cells treated with KU 55933 (open arrows, Figure 7A). The latter result may reflect the fact that ATM may also play a role in G2/M checkpoints (Shiloh, 2003). Thus, cells were cotreated with PUVa and the microtubule blocker nocodazole for 24 h, to trap cells in G2/M phase. Cells arrested in S-phase by PUVa

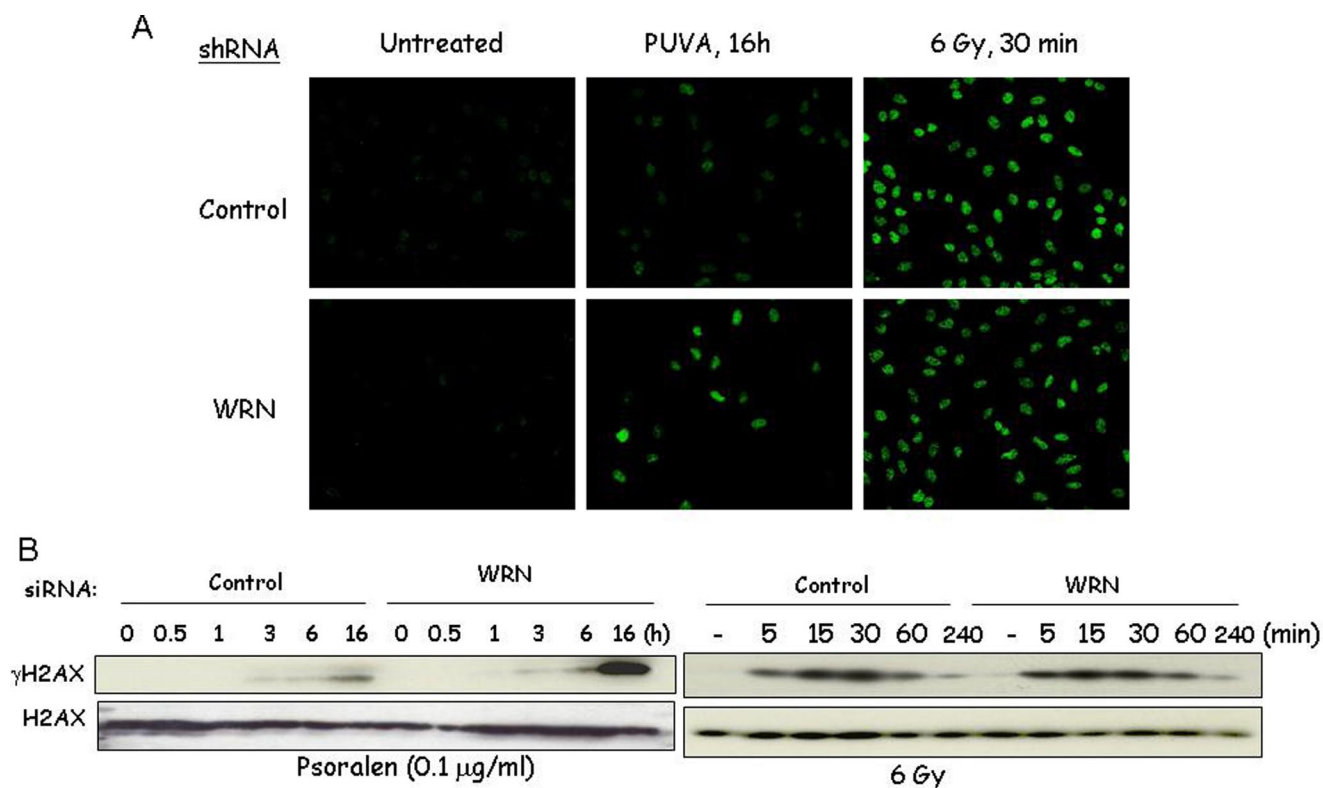


Figure 6. Role of WRN in γ H2AX formation in cells treated with PUVA and γ -irradiation. Control and WRN shRNA U-2 OS cells were treated with PUVA (0.1 μ g 8-methoxypsoralen/ml), and ionizing radiation. (A) Cells were fixed and stained with mouse antibodies against γ H2AX, followed by detection using confocal microscope. (B) Cell lysates were prepared at the indicated time points and were analyzed by Western analyses using antibodies against γ H2AX and H2AX (see *Materials and Methods*).

were not affected by nocodazole until they progress into the G2/M phase, whereby they were trapped. As expected, trapped cells that progressed beyond S-phase arrested at G2/M phase. Although most control cells arrested in S-phase, the distribution of cells through the cell cycle was similar in WRN shRNA cells treated with or without KU 55933 (Figure 7B). These results are consistent with the hypothesis that WRN and ATM kinase function in the same pathway during the S-phase checkpoint response to PUVA interstrand cross-link-induced DNA DSBs.

DISCUSSION

This study describes a novel upstream role of WRN in the response to PUVA-induced DNA DSBs. Based on flow cytometric, DNA synthesis, immunofluorescence, Western and PFGE results, we conclude that WRN is required for an intra-S-phase checkpoint in the response to replication-dependent DSBs, but not for the response to acutely formed DSBs induced by γ -irradiation. Furthermore, WRN appears to modulate ATM activation during the S-phase checkpoint,

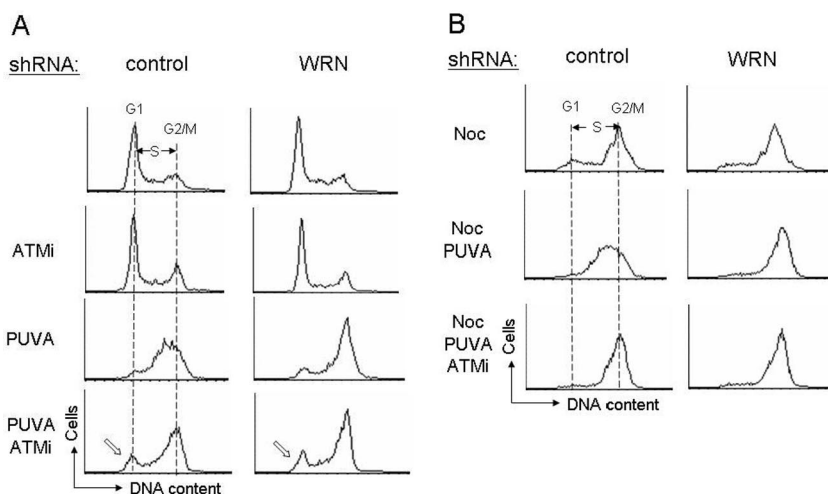


Figure 7. Convergence between WRN and ATM in the cellular response to PUVA. (A) Control and WRN shRNA U-2 OS cells were treated with PUVA (0.1 μ g 8-methoxypsoralen/ml), ATM inhibitor (ATMi, KU55933, 10 μ M), or both and harvested at 24 h. (B) Control and WRN shRNA U-2 OS cells were cotreated with nocodazole, PUVA, and ATMi as described in A.

facilitating autophosphorylation of ATM on Ser-1981 and phosphorylation of downstream ATM protein substrates. Lack of WRN increases PUVA-induced γ H2AX formation, suggesting that lack of the WRN-dependent ATM pathway activation leads to increased genomic instability. These findings demonstrate that WRN cooperates with ATM to implement an intra-S-phase checkpoint in cells with collapsed replication forks. Because ATM is known to act upstream of DNA damage response, WRN and ATM may be poised for a rapid cellular response to collapsed replication forks.

Previous studies of the intrinsic catalytic properties of WRN and functional protein-protein interactions involving WRN suggested that WRN plays an important role in homologous recombination and base excision repair subpathways (Opresko *et al.*, 2004a). For example, WRN helicase activity is stimulated by replication protein A (RPA; Brosh *et al.*, 1999), a ssDNA-binding protein that play a role in DSB repair. WRN helicase activity is stimulated by Nbs1, a component of the MRN complex that is required for DSB-induced S-phase checkpoint and homologous recombination (Tauchi *et al.*, 2002; Cheng *et al.*, 2004). These previously published reports document a downstream role for WRN in DSB repair; this study reports that WRN also acts upstream in cellular signaling pathways, facilitating an intra-S-phase checkpoint in cells with collapsed replication forks. It is of future interests to determine whether WRN's catalytic activities are required for ATM pathway activation after replication fork collapse.

Recent studies demonstrate that the ATM and ATR pathways interact during the response to DNA damage (Cuadrado *et al.*, 2006; Jazayeri *et al.*, 2006; Stiff *et al.*, 2006). In most cells in S-phase, ATM acts upstream of ATR during the response to DSBs (Cuadrado *et al.*, 2006; Jazayeri *et al.*, 2006); in contrast, ATR acts upstream of ATM in response to stalled replication forks or UV treatment (Stiff *et al.*, 2006). Our past work suggests that WRN is not significantly involved in the UV response pathway (Karmakar and Bohr, 2005). Consistent with this, we observed that WRN appears to play only an ancillary role in UV-induced S-phase arrest (Supplemental Figure S7). A previous report suggests that ATR, but not ATM, is required for inhibition of DNA synthesis after cellular exposure to PUVA (Pichierri and Rosselli, 2004). However, they used a time course up to only 9 h, and most likely the majority of cells are not yet arrested in S-phase under these conditions, and it is too early for the accumulation of collapsed forks. DNA cross-links per se do not induce DNA breaks; they are converted into DNA DSBs during DNA replication in S-phase (Rothfuss and Grompe 2004).

It is known that ATM and ATR share some phosphorylation targets. Although CHK1 Ser-317 is generally thought to be a ATR phosphorylation target, CHK1 phosphorylation by ATM in response to γ -irradiation has also been reported (Gatei *et al.*, 2003). In cells treated with PUVA, CHK1 Ser-317 is phosphorylated before ATM and H2AX are phosphorylated (Figures 4, A and B, and 6B), suggesting that at an early stage ATR may phosphorylate CHK1 in response to PUVA-induced DNA ICLs without the involvement of replication-dependent DSBs. Because CHK1 phosphorylation at 1 h occurs in both types of cells, this event appears to be WRN-independent. Furthermore, we earlier showed that collapsed replication forks after inhibition of PARP-1 clearly activates ATM, but does not activate ATR (Bryant and Helleday, 2006). Therefore, DNA breaks may be a major determinant for ATM and/or ATR involvement in replication stress. Although this report focuses on WRN-dependent ATM pathway activation involving replication-dependent DSBs, additional studies are required to define the exact sequence

of events and molecular mechanism of pathways that may involve additional protein partners such as ATR.

This study shows that WRN is required for the ATM pathway activation in cells with PUVA-induced DSBs during replication. It is worth noting that WRN binds DNA substrates that resemble replication intermediates (von Kobbe *et al.*, 2003), whereas ATM does not. In fact, ATM has no intrinsic DNA binding capability (Lee and Paull, 2005). In this context, it is possible that WRN increases the accessibility of ATM to collapsed replication forks and that a functional interaction exists between these two proteins. Here, we also show that WRN and ATM colocalize to DNA replication foci in unstressed cells, which is in accordance with previous studies showing that WRN colocalizes with PCNA at replication foci (Opresko *et al.*, 2004b). DNA DSB mediators, such as MDC1 and 53BP1, contain the BRCT phosphoprotein interaction domain that facilitate transient binding to γ H2AX (Schultz *et al.*, 2000; Goldberg *et al.*, 2003; Lou *et al.*, 2006). 53BP1 also contains the tudor domain that binds Lys-79 methylated histone H3 when the site is exposed by DNA DSBs (Huyen *et al.*, 2004). Moreover, chromatin relaxation is triggered in the vicinity of a DSB after DNA damage induction (Kruhlak *et al.*, 2006), likely resulting in increased accessibility of DSB mediators and ATM to damaged nucleosomes. In comparison, before the fork encounters a nick, chromatin structures 5' to collapsed replication forks are already relaxed as a result of the progression of the replication machinery. Because WRN does not contain BRCT or tudor domains and does not bind directly to γ H2AX (Cheng *et al.*, 2005), WRN may not function as a DNA damage mediator. Rather, WRN has a HRDC domain that is required for binding to DSBs (Lan *et al.*, 2005). Taken together, this suggests that under conditions of replication-dependent DSBs, WRN is required for ATM pathway activation.

Previous studies show that ATM is required for the cellular response to replication stress (Bolderson *et al.*, 2004). This study shows that WRN can play an important role in activating ATM and suggests that WRN may increase the accessibility of ATM to damaged/collapsed replication forks. The exact mechanism by which this might occur is not yet known; however, it may involve direct interaction or shared interaction partners between WRN and ATM. One candidate is the MRN complex that also directly interacts with and activates ATM (Lee and Paull, 2005). Importantly, the MRN complex binds directly to both WRN and ATM (Cheng *et al.*, 2004; Lee and Paull, 2005). Thus, it is possible that WRN and the MRN complex function together to signal the presence of collapsed replication forks, resulting in ATM activation. Future studies are needed to determine whether WRN directly stimulates ATM activation independent of MRN or whether MRN plays a direct role in WRN-dependent activation of ATM.

Studies in yeast suggest that WRN participates in the early response to replication stress. Rqh1 and Sgs1 are the sole members of the RecQ helicase family in fission and budding yeasts, respectively. Rqh1 is not essential for a HU-induced S-phase checkpoint, but prevents aberrant recombination events during checkpoint recovery (Stewart *et al.*, 1997). In contrast, Sgs1 has been implicated in checkpoint activation and in preventing the accumulation of aberrant recombinational intermediates in response to replication stress (Frei and Gasser, 2000; Liberi *et al.*, 2005). In humans, earlier studies suggested that WRN and RPA prevent aberrant recombination intermediates in HU-arrested S-phase cells (Constantinou *et al.*, 2000; Saintigny *et al.*, 2002). Therefore, WRN likely plays dual roles in the response to replication-dependent DSBs: first to stimulate activation of ATM and

second to resolve recombination intermediates. It is not yet understood what factors might regulate these two functions of WRN. However, it is possible that posttranslational modifications of WRN regulate WRN functions and activity. For example, phosphorylation of WRN in stressed cells is ATM- and ATR-dependent (Pichierri *et al.*, 2003) and phosphorylation of WRN alters its DNA binding properties (J. Lee and V. Bohr, unpublished data).

The findings presented here may be related to the observation that WRN-deficient cells are much more sensitive to CPT-, HU-, and ICL-induced cytotoxicity than to γ -irradiation (Poot *et al.*, 2001; Yannone *et al.*, 2001; Cheng *et al.*, 2004). This is consistent with the observation that the WRN-dependent ATM-dependent response pathway is not activated by γ -irradiation. This early function of WRN after DNA damage can potentially impact on many ATM-dependent genome surveillance pathways. Indeed, WRN shRNA cells exhibit decreased replication stress-induced phosphorylation of SMC1 on Ser-966, BRCA1 on Ser-1423, and CHK1 on Ser-317, as well as defective S-phase checkpoint pathways; these proteins are important downstream effectors of the ATM-dependent S-phase checkpoint (Xu *et al.*, 2001; Kim *et al.*, 2002; Gatei *et al.*, 2003). WRN could also act at a later step during resolution of intermediates in recombinational DNA repair. Loss of these two WRN functions would increase genomic instability as well as the cytotoxicity associated with unrepaired DSBs in replicating cells. This is supported by the observation that there is increased γ H2AX formation in WRN shRNA cells. Other kinases are apparently responsible for H2AX phosphorylation in PUVA-treated WRN shRNA cells when ATM activation is defective. It should be noted that although WRN plays a role in ATM pathway activation at the later time point after PUVA or HU treatment, this should not be interpreted as a late response. Considering the nature of PUVA and HU in inducing DNA breaks during DNA replication, this supports a specific role of WRN in S-phase where replication-dependent DNA breaks occur. It is also tempting to speculate that WRN could ensure that homologous recombination is only initiated when S-phase arrest has been achieved.

In summary, this study provides evidence that WRN and ATM function cooperatively at the interface between cell cycle checkpoints and DNA replication. We propose a scenario (Figure 8) in which the WRN-ATM complex may play a role at damaged replication forks. If replication-dependent

DSBs are induced, WRN facilitates ATM activation, which in turn activates downstream effectors and intra-S-phase arrest. In contrast, γ -irradiation-induced ATM activation does not require WRN. Although there is a difference in the premature aging phenotypes between WS and A-T, our results suggest that a deficiency in the WRN-ATM pathway could increase DNA damage-related symptoms of aging. Mutations in either WRN or ATM cause several common cellular features, including senescence, chromosome breaks, and telomere instability. Thus, the novel WRN-dependent ATM activation and its substrate phosphorylation described here may have significant implications for our understanding of aging as well as the complex relationship between cellular checkpoint mechanisms and genomic stability.

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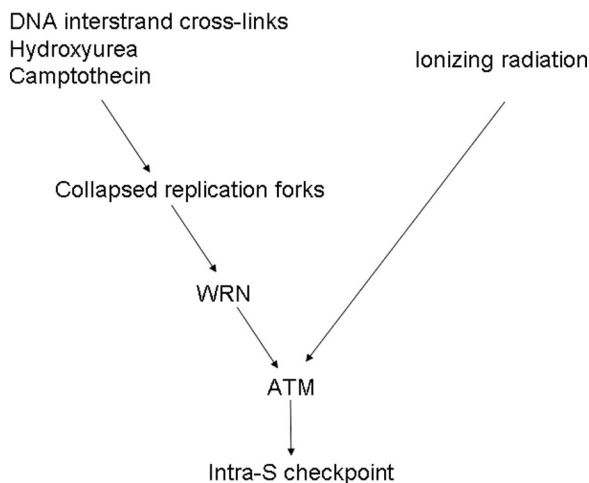


Figure 8. Model of the Role of WRN and ATM in response to collapsed replication forks or DSBs.

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