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DNA Double Strand Break Formation upon UV-Induced Replication Stress Activates ATM and DNA-PKcs Kinases

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Summary

The phosphatidylinositol 3-kinase-like protein kinases (PIKK), including ATM, ATR, and DNA-PKcs, are the main kinases activated following various assaults on DNA. Although ATM and DNA-PKcs kinases are activated upon DNA double strand breaks (DSBs), evidence suggests that these kinases are rapidly phosphorylated by ATR kinase upon UV irradiation; thus these kinases may also participate in the response to replication stress. Using UV-induced replication stress, we further characterize whether ATM and DNA-PKcs kinase activities are also involved in the cellular response. Contrary to the rapid activation of the ATR-dependent pathway, ATM-dependent Chk2 and KAP-1 phosphorylations, as well as DNA-PKcs Ser2056 autophosphorylation, reach their peak level at four to eight hours after UV irradiation. The delayed kinetics of ATM and DNA-PKcs dependent phosphorylations also correlated with a surge in H2AX phosphorylation, suggesting that DSBs formation resulting from collapse of replication forks is responsible for the activation of ATM and DNA-PKcs kinases. In addition, we observed that some phosphorylation events initiated by ATR kinase in the response to UV were mediated by ATM at a later phase of the response. Furthermore, the S-phase checkpoint after UV irradiation was defective in ATM deficient cells. These results suggest that the late increase of ATM activity is needed to complement the decreasing ATR activity for maintaining a vigilant checkpoint regulation upon replication stress.

Keywords

UV; replication stress; DNA double-strand breaks; ATM; DNA-PKcs

Introduction

Irradiation with ultraviolet light (UV) induces cyclobutane pyrimidine dimer (CPD) and 6-4 pyrimidine photoproduct (6-4PP) DNA photolesions. If not removed through nucleotide excision repair (NER), these bulky photolesions inhibit normal DNA replication processes as DNA replication forks cannot pass through them. The stalled replication forks can be resolved through a translesion DNA synthesis (TLS) mechanism that allows bypass of the DNA lesions

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[1]. If unresolved, stalled replication forks will collapse and lead to the formation of DNA double strand break (DSB) [2]. When a replication fork stalls, uncoupling of the minichromosome maintenance (MCM) helicase complex from the DNA replication core machinery allows the continued unwinding of the DNA double helix; this leads to a long stretch of single-stranded DNA (ssDNA) at the stalled replication fork [3]. Subsequent binding of RPA to ssDNA and recruitment of ATR/ATRIP complex by RPA-ssDNA filament activates the ATR kinase [4]. It is generally accepted that ATR is the main kinase activated upon replication stress. ATR plays important roles in replication stress signaling events and cell cycle checkpoint regulation and also influences replication origin firing and the recovery of stalled replication forks [5; 6]. While much is known about the role of ATR, it is less clear whether the DSB-responsive ATM and DNA-PKcs kinases are also involved in the replication stress response.

ATR, ATM, and DNA-PKcs are members of the phosphoinositol 3-kinase related kinase (PIKK) family and are activated in the cellular response to various assaults on DNA [7]. Conventional wisdom suggests that ATM and ATR are mainly required for signal transduction upon DSB and replication stress, respectively, whereas DNA-PKcs participates primarily in DSB repair. This viewpoint has been challenged recently by a growing body of evidence indicating that the roles of these PIKK kinases overlap. For example, ATR is activated not only by replication stress but also by ionizing radiation (IR) during late-S and G2 phases in an ATM and MRN complex dependent manner [8]. Conversely, DNA-PKcs and ATM are required for cellular resistance to UV irradiation and IR [9; 10], indicating that these kinases are required for cellular response to UV-induced replication stress. This notion is further supported by recent evidence that DNA-PKcs and ATM are rapidly phosphorylated in response to UV and hydroxyurea treatments; these replication stress-induced phosphorylations are mediated by ATR kinase [11; 12].

In light of these evidences, we carried out further analyses to examine the significance of ATM and DNA-PKcs in cellular response to replication stress. Using UV-induced replication stress as a model, we have demonstrated distinctive differences in kinetics of ATR-mediated phosphorylations and ATM/DNA-PKcs downstream signaling events upon UV irradiation. Although UV induced the onset of ATR-mediated phosphorylations within one hour, ATM and DNA-PKcs downstream phosphorylations, including KAP-1 and Chk2 phosphorylations and DNA-PKcs Ser2056 autophosphorylation, peaked at 4 to 8 hours. This later induction of ATM and DNA-PKcs downstream phosphorylations also coincided with the peak of H2AX phosphorylation. Thus, our results suggest that DSB formation, resulting from collapsed replication forks, rather than the stalled replication forks or ATR-dependent phosphorylations activates ATM and DNA-PKcs.

Results

Activation of ATM signal pathway in response to UV irradiation

Cells lacking a functional ATM kinase are sensitive to both IR and UV irradiation [10]; therefore, we examined the involvement of the ATM signaling pathway in the response to UV. Exponentially growing HeLa cells were subjected to 10 J/m^2 of UV irradiation and were harvested at various time points after UV irradiation. Western blot analysis revealed that ATM Ser1981 phosphorylation increased rapidly and could be detected within 1 hour after UV irradiation. Phosphorylation at Ser1981 was maximal at 4 hours and this level was sustained for 16 hours after UV treatment. In contrast, the phosphorylation level at the site after IR irradiation reaches a peak level within five minutes [13]. The prolonged phosphorylation of ATM at Ser1981 after UV is similar to that of UV-induced SMC1 phosphorylation at Ser966 (Fig. 1) and DNA-PKcs phosphorylations at the Thr2609 cluster [11]. Furthermore, UV induced a slower or delayed increase of ATM downstream KAP-1 Ser824 and Chk2 Thr68 phosphorylations (Fig. 1). KAP-1 and Chk2 phosphorylations reached peak level at 8 hours

after UV, as did ATM phosphorylation at Ser1981. By contrast, ATR dependent Chk1 phosphorylation at Ser345 was promptly induced by UV, reached maximum intensity at 2 hours, and declined by 4 hours after UV treatment (Fig. 1). The induction of ATM and ATM-dependent phosphorylations were also analyzed in HeLa cells after lower dose UV irradiation. Similar results were obtained except that there was a slight reduction in phosphorylation intensities (Supplemental Fig. 1). Thus the kinetics of ATM-mediated phosphorylations were delayed following UV treatment relative to that observed after IR. Importantly, the peak activation was independent of ATR activity.

Nucleotide excision repair is not required for UV induction of ATM signal pathway

The nucleotide excision repair (NER) pathway is an evolutionarily conserved DNA repair mechanism that eliminates CPD and 6-4 PP photolesions generated upon UV exposure [14]. It was reported that the NER mechanism mediates ATR-dependent phosphorylation of ATM at Ser1981 upon UV [12]. To test whether activation of the ATM signaling pathway involves an NER-mediated process, we analyzed UV-induced phosphorylations of ATM and its downstream targets in the wild-type VA13 human fibroblast cell line and in the XP4PA cell line, which lacks XPC (Xeroderma pigmentosum group C) protein and is defective in NER [15]. As shown in Figure 2A, UV induction of ATM Ser1981 and KAP-1 Ser824 phosphorylations in XP4PA cells was compatible to that in VA13 cells without significant change in kinetics, indicating that XPC is not essential for ATM or downstream target phosphorylation after UV treatment. Similarly, there was no difference in levels of UV-induced SMC1 Ser966 phosphorylation between these two cell lines. Further analysis with immunofluorescent staining demonstrated that UV induction of KAP-1 Ser824 phosphorylation (pSer824) occurred only in replicating cells labeled with bromo-deoxyuridine (BrdU) (Fig. 2B). We have analyzed greater than 200 cells from each cell lines. Of the KAP-1 pSer824 positive VA13 cells, 94% (78 out of 83) were positive with BrdU staining; whereas 93% (82 out of 88) of the XP4PA cells were positive. These results thus suggest that UV photolesion recognition by XPC is not essential for UV induction of the ATM signaling pathway.

UV induces an ATM signaling pathway predominantly in S-phase

We reported previously that replication stress causes the increase in DNA-PKcs phosphorylation at the Thr2609 cluster in response to UV [11]. To determine whether replication stress is required to activate the ATM signaling pathway, HeLa cells synchronized with mitotic arrest/release were UV irradiated at different time points after release. Western blotting revealed that ATM Ser1981, Chk2, KAP-1, and SMC1 phosphorylations were significantly induced by UV in S-phase cells and these increases were correlated with the high levels of expression of CyclinA, an S-phase marker (Fig. 3A). Similar results were obtained from normal human skin fibroblasts (HSFs) synchronized at G1- or S-phases followed by UV irradiation (Supplemental Fig. 2). In both experiments, we observed a slight increase in ATM and SMC1 phosphorylations in G1-phase. However, KAP-1 and Chk2 phosphorylations were not detected in G1-synchronized HeLa cells or HSFs.

The connection between UV-induced replication stress and the ATM signaling pathway was further examined by immunofluorescent staining. Exponentially growing HeLa cells were pulse-labeled with BrdU then UV irradiated. Four hours after UV, cells were harvested and immunostained for BrdU and KAP-1 Ser824 phosphorylation. As shown in Figure 3B, KAP-1 phosphorylation occurred predominantly in BrdU-positive cells after UV irradiation but not in BrdU-negative or unirradiated cells. Among 200 plus cells analyzed, greater than 90% of cells positive for KAP-1 phosphorylation were also positive for BrdU staining (85 out of 90). Additionally, as shown in Figure 3C, we observed that UV induction of Chk2 Thr68 phosphorylation overlapped with UV-induced γH2AX (75 out of 88 Chk2-positive cells) and

DNA-PKcs Thr2609 phosphorylation (70 out of 81 Chk2-positive cells), both of which are elicited in response to UV-induced replication stress [11; 16]. Taken together, our results suggest that the maximal activation of the ATM pathway in the late response to UV irradiation requires DNA replication rather than the processing by NER.

Overlapping roles of ATR and ATM in response to UV irradiation

Although ATR is the main contributor to UV-induced S/TQ phosphorylation events, our results suggest that ATM can also be activated by UV and may contribute to S/TQ phosphorylation events at a later stage. To test this scenario, we analyzed SMC1 phosphorylation status in U2OS cells expressing wild type ATR (ATRwt) or a dominant-negative kinase-dead form of ATR (ATRkd). Our results showed that ATR was required for initial UV induction of SMC1 phosphorylation as the phosphorylation was attenuated in ATRkd cells at 1 hour relative to levels in ATRwt cells (Fig. 4A). However, we observed a significant increase of SMC1 phosphorylation in ATRkd cells at 8 hours after UV. This delayed induction is likely contributed by the activated ATM kinase as treatment with the ATM kinase-specific inhibitor, KU55933, effectively reduced the level of phosphorylation (Fig. 4A). Similar results were obtained in experiments using a small inhibitory RNA (siRNA) against ATR kinase (Fig. 4B). Conversely, inhibition of ATM activity in MCF7 cells with stable short hairpin RNA against ATM (shATM) attenuated UV-induced SMC1 phosphorylation at 8 hours but not initially at 1 hour after UV (Fig 4C). In the presence of ATR inhibitor, caffeine, SMC1 phosphorylation at 8 hours was further reduced to the background level in shATM cells (Fig. 4C), suggesting that the functions of ATR and ATM are redundant at this late time point. Finally, UV-induced KAP-1 Ser824 phosphorylation at 8 hours was independent of ATR kinase and was completely diminished in the absence of ATM activity further demonstrating the importance of late ATM activation (Supplemental Fig. 3).

We previously reported that UV induces rapid DNA-PKcs phosphorylation at the Thr2609 cluster but not at Ser2056 [11], an authentic DNA-PKcs autophosphorylation site *in vivo* [17]. However, the kinetics analysis shown here revealed that DNA-PKcs Ser2056 autophosphorylation was significantly induced at 8 hours after UV but not at earlier time points (Fig. 5). The late increase of DNA-PKcs Ser2056 autophosphorylation was also observed in cells treated with hydroxyurea but not thymidine (Fig. 5). Replication stress induced by hydroxyurea, but not by thymidine, is known to yield detectible DSBs [18], suggesting that DSB formation upon replication stress may contribute to the activation of ATM and DNA-PKcs. This view is further supported by our data indicating that camptothecin, which induces replication-associated DSB formation [19], rapidly induced DNA-PKcs Ser2056 autophosphorylation (Fig. 5) as well as ATM dependent KAP-1 Ser824 phosphorylation (Supplemental Fig. 4). Similarly, it was reported that DSB formation observed upon UV-induced replication stress is the primary cause of UV-induced cytotoxicity [2].

The connection between UV-induced DSB formation and activation of ATM and DNA-PKcs was further supported by the kinetics of H2AX phosphorylation or γH2AX formation. Although an increase in γH2AX was observed at early time points after UV irradiation, it reached a peak at 4 to 8 hours (Fig. 6A and Supplemental Fig. 1). Furthermore, UV induction of γH2AX at 8 hours overlapped with positive TUNEL staining of DNA strand breaks (92 out 97 strongly positive γH2AX cells from >300 cells analyzed), whereas no TUNEL staining was detected at 1 hour after UV (Fig. 6B). The initial UV induction of γH2AX is dependent on the kinase activity of ATR [16] and was attenuated by treatment with an siRNA targeting ATR (Fig. 6C). In addition, it was significantly reduced in U2OS cells expressing a kinase-dead form of ATR at 1 hour after UV (Fig. 6D). Neither treatment with siRNA against ATR kinase nor the presence of the ATR kinase-dead mutant affected UV induction of γH2AX at 8 hours. Similar results were obtained from immunofluorescent analyses showing that ATR is essential only

for the early onset of γH2AX formation (Fig 6E). Taken together, these results suggest that DSB formation at late time points after UV treatment activates ATM (and/or DNA-PKcs) kinase activity which then contributes to the increase of γH2AX. In the absence of ATM, γH2AX is attenuated at the late stage but not at the early stage after UV irradiation (data not shown), which is similar to kinetics of SMC1 Ser966 phosphorylation (Fig. 4C).

ATM signal pathway is required for cell cycle checkpoint regulation upon UV irradiation

ATR and ATM are both capable of eliciting the intra-S checkpoint in response to replication stresses or DSBs [7; 20]. However, it is not clear whether ATM also contributes to the cell cycle checkpoint regulation upon UV or other types of replication stresses. To test this possible scenario, ATM-proficient 1BR3 cells and ATM-deficient AT5 cells were pulse-labeled with BrdU and then were UV irradiated. The progression of BrdU-labeled replicating cells was then monitored by flow cytometry analysis at different time points after UV irradiation (Fig. 7A). In the absence of UV treatment, AT5 cells progressed to S-phase slightly slower than did 1BR3 cells (Fig. 7B). Upon UV irradiation, S-phase progression was attenuated in both 1BR3 and AT5 cells with a tighter S-phase checkpoint response observed in 1BR3 cells. At 12 hours after UV treatment, about 57% of BrdU-positive 1BR3 cells remained in S-phase as compared to 39% of BrdU-positive AT5 cells. In addition to the reduction in S-phase cell population, we observed a significant increase in BrdU-positive G1 population (cells that have gone through mitosis) in UV-irradiated AT5 cells as compared to UV-irradiated 1BR3 cells at 8 and 12 hours (Fig. 7C).

To further confirm the involvement of ATM, we examined the effects of the ATM kinase inhibitor KU55933 on the S-phase checkpoint after UV treatment. KU55933 alone did not affect cell cycle progression in unirradiated 1BR3 cells but it attenuated the S-phase checkpoint response in UV-irradiated 1BR3 cells (Fig. 7D). This indicates that ATM activity is required for the maintenance of a vigilant intra-S checkpoint induced by replication stresses. Taken together, these results suggest that the late activation of the ATM signaling pathway is required to keep cell cycle progression in check even upon UV irradiation. It is likely that the ATM pathway plays a supporting role that complements ATR signaling pathway in intra-S checkpoint and prevents re-initiation of DNA replication under replication stress conditions.

Discussion

Members of the PIKK family of kinases, including ATM, ATR, and DNA-PKcs, are activated in response to various types of DNA damage. It is generally accepted that ATM and ATR are required for signal transduction upon DSBs and replication stress, respectively, whereas DNA-PKcs primarily participates in DSB repair. The simplicity of this model has been challenged by a growing body of evidence suggesting that these PIKK kinases have overlapping roles in the resolution of different types of DNA damage [8; 11; 12]. In the current study, we provide further evidence for the involvement of ATM and DNA-PKcs kinases in response to UV-induced replication stress. We particularly focused on KAP-1 Ser824 and DNA-PKcs Ser2056 phosphorylation as these phosphorylation events are absolutely dependent on ATM and DNA-PKcs, respectively [17; 21; 22]. ATM and DNA-PKcs dependent phosphorylation events were induced by UV with delayed kinetics. ATM-mediated Chk2 and KAP-1 phosphorylations as well as DNA-PKcs Ser2056 autophosphorylation increased slightly at 1 hour and reached peaks at 4 to 8 hours after UV irradiation. These delayed kinetics were in contrast to the rapid increases in levels of ATR-dependent phosphorylations. Clearly, there are distinct phases of signaling events upon UV-induced replication stress: ATR is activated immediately, whereas ATM and DNA-PKcs kinases are activated at later time points. The delayed kinetics or late activation of ATM and DNA-PKcs kinases also coincided with a dramatic increase of UV-induced γH2AX (Fig. 6), which occurred predominantly in response to UV-induced replication stress [16; 23].

Furthermore, UV-induced γH2AX at 8 hours overlapped with positive TUNEL staining (Fig. 6B). These evidence are consistent with the previous study that unrepaired UV photolesions are converted into DNA breaks during DNA replication [2]. Taken together, these results imply that the late-phase activation of ATM and DNA-PKcs kinases is due to DSB formation at sites of collapsed replication forks.

ATM is known to play an important role in S-phase checkpoint upon IR treatment or DSB induction [7; 20]; however, it was not clear whether ATM is involved in S-phase checkpoint upon replication stresses. The late activation of ATM-Chk2 signaling pathway upon UV irradiation suggests that ATM compensates for the decreased activity of the ATR pathway at late time points in order to maintain vigilant cell-cycle checkpoint regulation in the presence of stalled replication forks. This notion is supported by our finding that S-phase progression in ATM-defective cells is faster than that in ATM-proficient cells following UV irradiation (Fig. 7), indicating a defective S-phase checkpoint or radioresistant DNA synthesis (RDS) phenotype in ATM-deficient cells. By titration of UV doses, we found that 0.5-1 J/m^2 of UV irradiation is sufficient to induce ATM-mediated Chk2 phosphorylation in HeLa cells (Supplemental Fig. 5). Even after this minimal UV dose, we observed a rapid degradation of Cdc25A in S-phase-synchronized HeLa cells (data not shown). Cdc25A degradation after IR treatment is one of the key events in ATM-mediated S-phase checkpoint [20; 24] and ATM-Chk2 pathway activation after UV treatment is likely required for UV-induced Cdc25A degradation. In addition to its role in S-phase checkpoint response, ATM has also been implicated in regulation of G2-M checkpoint after IR [25]. The increase of BrdU-positive G1 population suggests that UV-irradiated AT5 cells are able to continue cell cycle beyond G2-M phases (Fig 7C), thus confirming that ATM activity is also required for G2-M checkpoint upon replication stresses.

In addition to cell cycle checkpoint regulation, the increase of ATM activity at late time points after UV irradiation may contribute to and sustain some of the S/TQ phosphorylations initiated by ATR. It is well established that ATM and ATR share many common downstream targets [7]. While ATR is essential for the initial increase of SMC1 and H2AX phosphorylations after UV treatment, our results show that there are no significant differences in phosphorylation levels between wild-type and ATR-deficient cells at 8 hours after UV irradiation (Fig. 4 and 6). It is likely that overlapping activities of ATR and ATM kinases are required for proper maintenance of these phosphorylation events in response to UV irradiation. Thus, defects in kinases or lack of S/TQ phosphorylations will lead to an increase of cellular sensitivity toward UV irradiation [10; 26].

The late activation of DNA-PKcs may contribute to DSB repair activity through the nonhomologous DNA end joining (NHEJ) pathway. Cells lacking functional DNA-PK components are known to have elevated sensitivity toward UV irradiation [9] and we reported previously that DNA-PKcs phosphorylations at the Thr2609 cluster are required for cellular resistance against UV irradiation [11]. Furthermore, a recent study indicated that the DNA polymerase inhibitor aphidicolin induces a surge of DSBs in replicating cells and that DNA-PKcs is required for the repair of these DSBs [27]. This data suggests that DNA-PKcs kinase is important for the repair of replication-associated DSBs. Additionally, DNA-PKcs activity may be involved in cell cycle checkpoint regulation after UV irradiation as it was reported that DNA-PKcs-deficient cells exhibit a radioresistant DNA synthesis (RDS) phenotype [28], an indication that these cells are defective in intra-S checkpoint. However, the precise role of DNA-PKcs in replication stress-induced checkpoint regulation remains to be clarified.

Although our results clearly demonstrate that UV-induced ATM and DNA-PKcs phosphorylations occur predominantly in replicating cells with stalled replication forks, we do not rule out the possibility that ATM and DNA-PKcs phosphorylations may be involved in the

nucleotide excision repair (NER) mechanism as we did observe a slight increase in ATM Ser1981 phosphorylation in G1-phase synchronized cell population after UV irradiation (Fig. 3A). The induction of ATM Ser1981 phosphorylation in G1-phase may participate in the NER mechanism as ATM deficient cells are sensitive to UV irradiation and are defective in repair of UV photolesions [10]. Similarly, it was reported that the NER pathway can initiate checkpoint activation in non-proliferative cells [29] and is required for UV-induced γH2AX in G1-phase, although maximum induction of γH2AX occurs during S-phase [23]. This is consistent with our results that UV irradiation leads to a significant induction of KAP-1 and Chk2 phosphorylations in S-phase synchronized cells (Fig. 3A and Supplemental Fig. 2). In XPC-proficient and XPC-deficient cells there was no clear difference in levels of UV-induced ATM and KAP-1 phosphorylations and that KAP-1 phosphorylation occurred in BrdU-positive replicating cells in the presence and absence of XPC (Fig. 2). Taken together, these data suggest that replication stress is the main cause of activation of the ATM signaling pathway in response to UV irradiation.

In summary, our current study revealed the involvement of ATM and DNA-PKcs in the replication stress response. ATM and DNA-PKcs were both activated by UV irradiation at late time points when DSB formation occurs. In conjunction with early onset of ATR activity, increase of ATM and DNA-PKcs activities likely contribute to cell cycle checkpoint regulation and DSB repair for the maintenance of genomic stability.

Materials and Methods

Cell culture

All cell lines, including human cervical adenocarcinoma HeLa cells, normal human skin fibroblasts (HSF), human osteosarcoma U2OS cells expressing inducible wild-type ATR (GW33), and U2OS cells expressing inducible dominant-negative kinase-dead mutant ATR (GK41), were grown in α-minimum essential medium supplemented with 10% fetal calf serum and penicillin/streptomycin and were maintained in a humidified atmosphere with 5% CO_2. XPC-deficient cells XP4PA [15] and normal VA13 human fibroblasts were generous gifts from Dr. Lisa McDaniel (UT Southwestern). Retroviral vector mediated expression of small hairpin RNA targeting ATM [30] and U2OS cell lines expressing wild-type and kinase-dead mutant ATR were previously described [31]. HeLa cells were synchronized with 40 ng/mL nocodazole for 17 hours to arrest the cell cycle at M-phase and were then reseeded. Cells were mock treated or UV irradiated at the indicated time points after reseeding and were harvested 2 hours after UV treatment. siRNA oligonucleotides complementary to ATR and transfection procedures were previously described [11].

UV irradiation

For UV irradiation, exponentially growing cells on culture dishes were washed once with phosphate-buffered saline and then subjected to UV-C (254 nm) at the rate of 0.5-1 J/m^2/sec to achieve the cumulative desired doses (10 J/m^2 in most experiments). Fresh culture medium was added to the culture dishes immediately after irradiation.

Flow cytometry analysis

For flow cytometry analysis, cells were harvested and fixed in 70% ethanol. Prior to propidium iodide (PI) staining, cells were washed twice with PBS and were re-suspended in PI solution (0.1 mg/mL RNase A, 0.1% Triton X-100, 20 mg/mL PI in PBS) at a concentration of 1×10^6 cells per mL. The cell suspension was tumbled at 4°C for 30 minutes in darkness. DNA content was measured by FS500 flow cytometer and cell cycle compartments were analyzed using the CXP cytometry analysis program (Beckman Coulter). Cell cycle checkpoint analysis was performed as described before [32]. Briefly, cells were pre-incubated with 50 μM bromo-

deoxyuridine (BrdU) for 10 minutes and washed with PBS prior to UV irradiation (3 J/m^2). Cells were continuously incubated and harvested at indicated time points after UV irradiation. Cells were processed as described above, treated with 0.1 mg/mL RNase A and 2 N HCl sequentially for 30 minutes at 37°C and incubated with anti BrdU antibody conjugated with FITC (Invitrogen) for 2 hours. Cells were then subjected to flow cytometry.

Western blotting and immunofluorescent staining

Nuclear extract preparation and western blotting was performed as described previously [33]. Whole cell extracts were prepared by suspending cell pellets in RIPA buffer on ice for 20 minutes followed by centrifugation to remove insoluble material. For immunofluorescent staining, cells were fixed in 4% paraformaldehyde for 10 minutes or cold methanol for 20 minutes, permeabilized in 0.5% Triton X-100 for 10 minutes, and then blocked in 5% normal goat serum or BSA for 1 hour at room temperature. The cells were incubated with primary antibodies for 1 hour and washed three times in PBS, and then incubated with rhodamine red- and Alexa-488-conjugated secondary antibodies for 30 minutes (Molecular Probes). Cells were then washed three times in PBS and mounted in Vectashield mounting medium with 4,6 diamidino-2-phenylindole (Vector Laboratory). TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche) as previously described [34]. After washing with PBS, cells were counter stained with anti-γH2AX antibody. Fluorescence images were captured using Zeiss Axiovert 200M microscope equipped with AxioCam MRm digital CCD camera.

Antibodies

Phospho-specific anti-DNA-PKcs antibodies (anti-pSer2056 and anti-pThr2609) were described previously [17; 22]. Anti-ATM Ser1981 phospho-specific antibody (Rockland), anti-DNA-PKcs mouse monoclonal antibody (mAb) (NeoMarkers), anti-γH2AX mAb (Upstate), anti-cyclobutane-pyrimidine dimmer (CPD) mAb (MBL International), anti-ATR (Bethyl Laboratories), anti-CHK1 and anti-CHK2 antibodies (Cell Signaling), anti-KAP-1 and anti-SMC1 antibodies (Bethyl Laboratory) were purchased from indicated vendors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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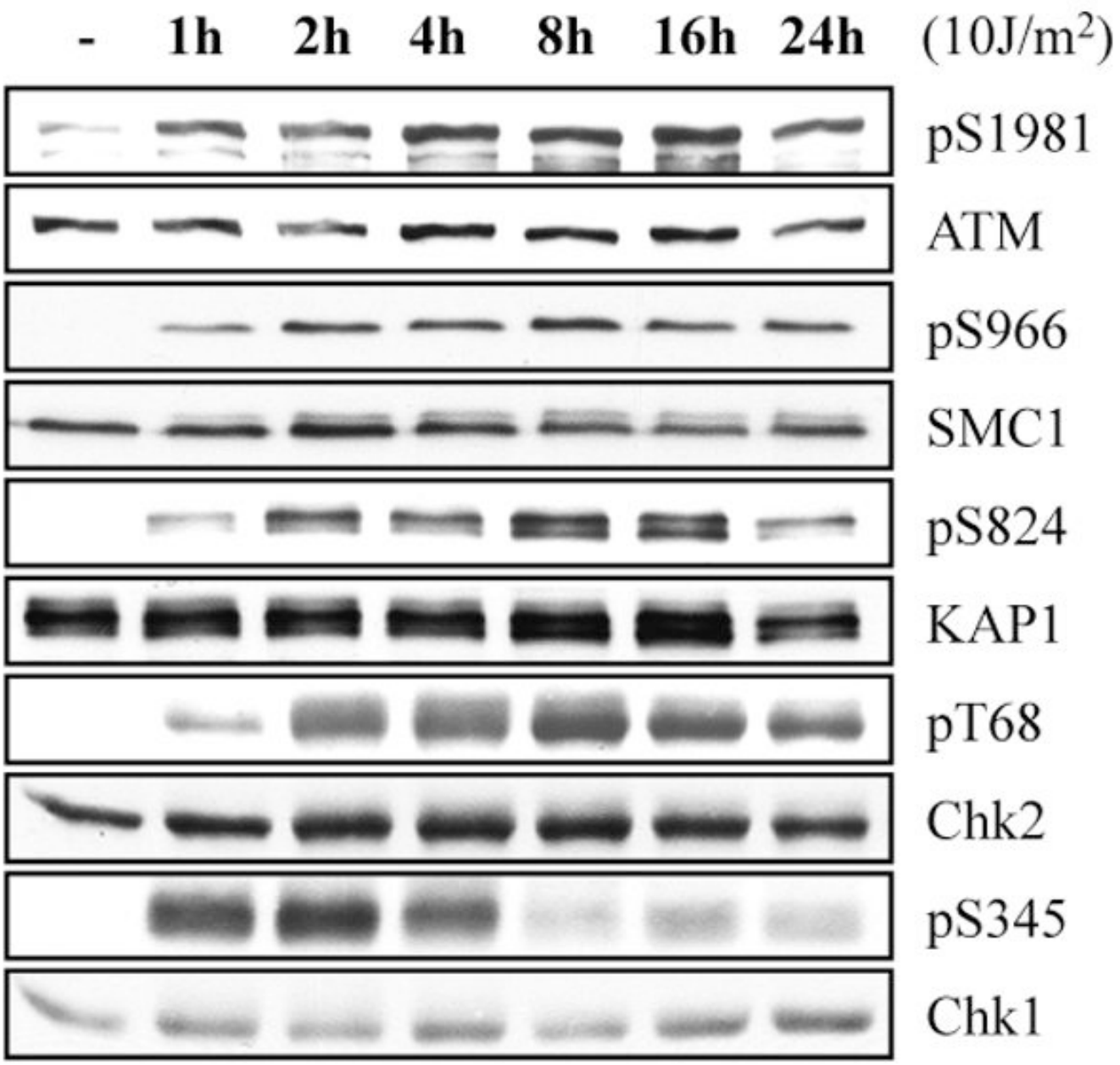


Figure 1.
Activation of ATM signal pathway in response to UV irradiation. Exponentially growing HeLa cells were mock treated or subjected to 10 J/m^2 of UV irradiation. The cells were harvested at the indicated time points and were analyzed for UV-induced phosphorylation events.

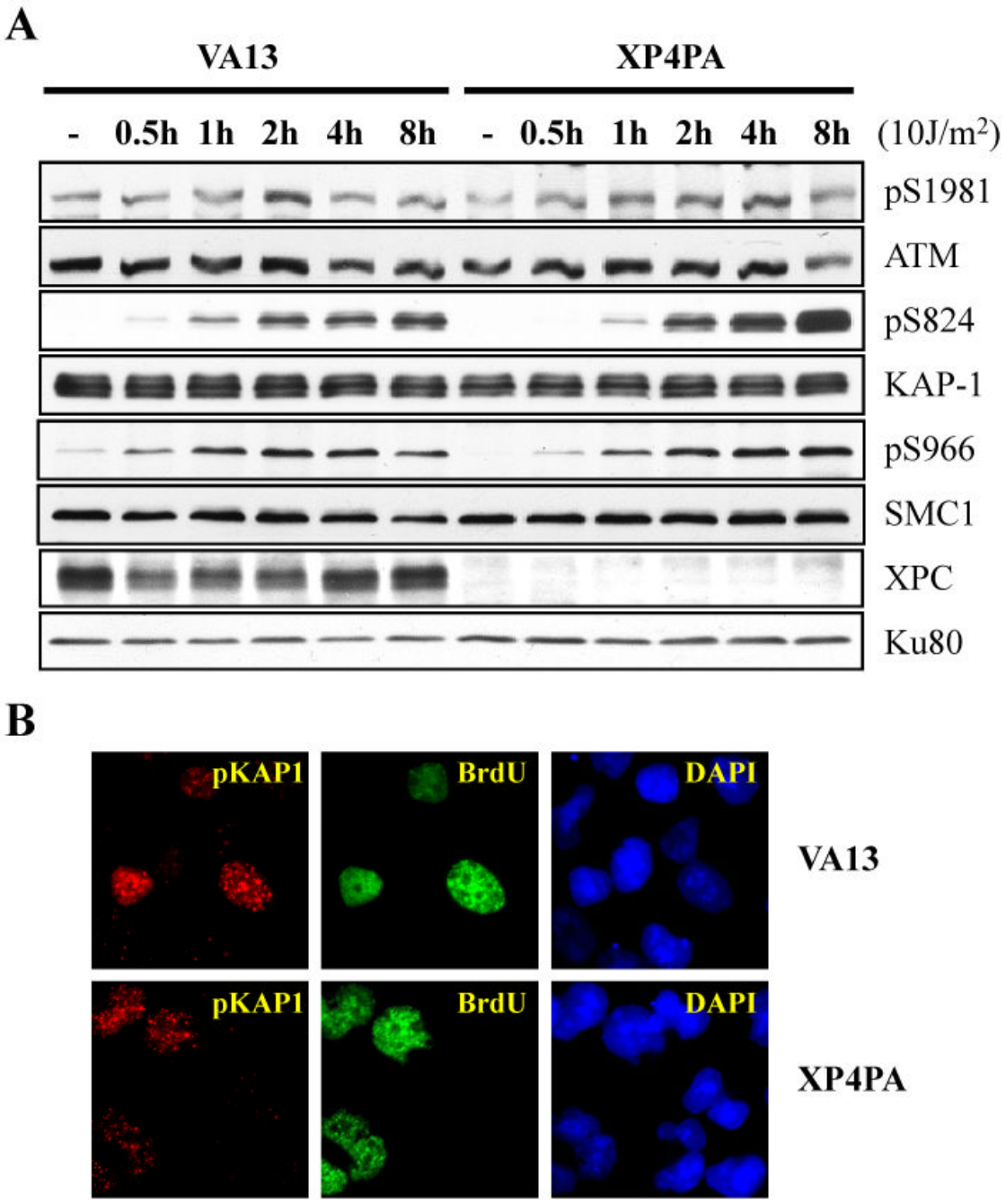


Figure 2.

Nucleotide excision repair is not required for UV induction of ATM signal pathway. (A) Wild-type (VA13) and XPC-deficient (XP4PA) fibroblasts were mock treated or subjected to 10 J/m^2 of UV irradiation. The irradiated cells were harvested at indicated time points and were analyzed for UV-induced phosphorylation of ATM, KAP-1, and SMC1. (B) VA13 and XP4PA cells were pulse-labeled with BrdU for 10 minutes and were UV irradiated (10 J/m^2). Two hours after UV irradiation, the irradiated cells were fixed and were immunostained for BrdU (green) and KAP-1 Ser824 phosphorylation (red). More than 200 cells were analyzed for each cell line.

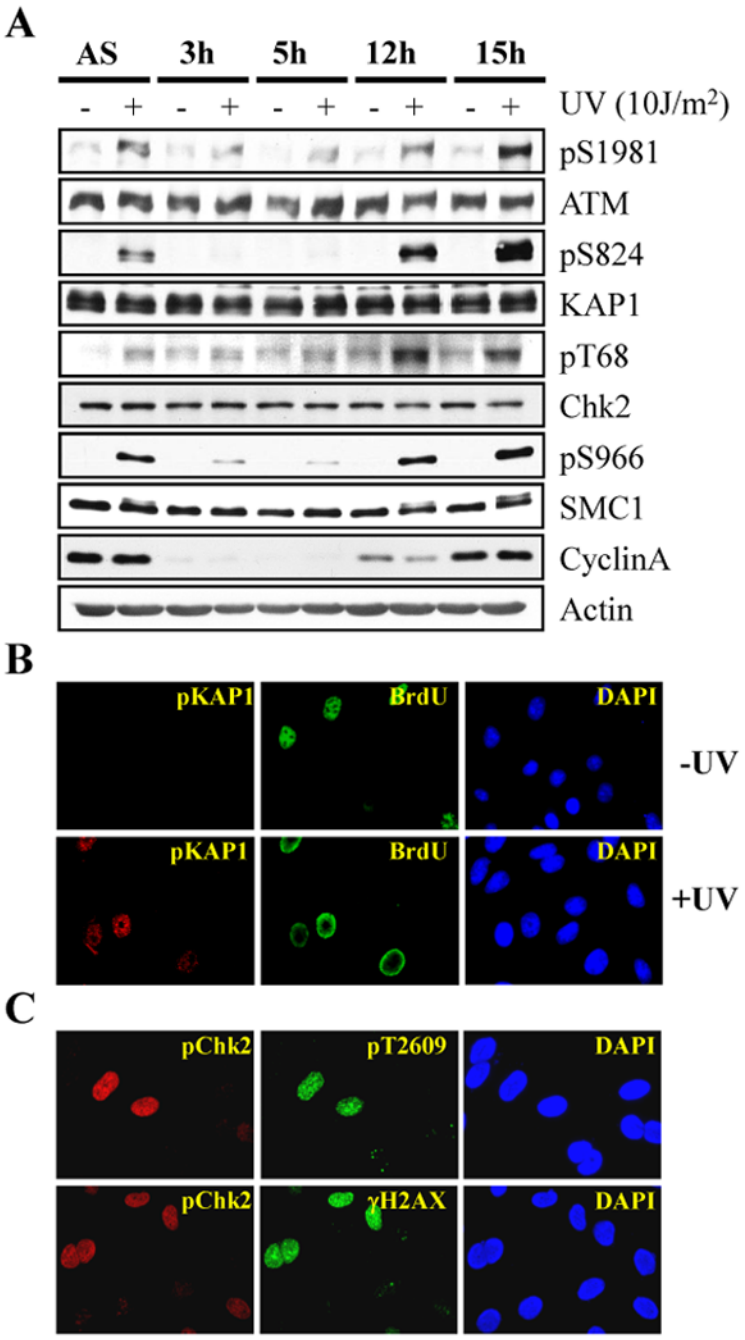


Figure 3.

UV induces ATM signal pathway predominantly in S-phase. (A) HeLa cells were released from nocodazole block and were UV irradiated (10 J/m^2) at the indicated time points after release. Nuclear extracts were prepared at three hours after UV treatment for western blot analysis. (B) Exponentially growing HeLa cells were pulse-labeled with BrdU and were UV irradiated (10 J/m^2). Four hours after UV treatment, cells were fixed and immunostained for KAP-1 Ser824 phosphorylation (red) and BrdU (green). (C) UV-irradiated HeLa cells were co-immunostained for Chk2 Thr68 phosphorylation (red) and either DNA-PKcs Thr2609 or H2AX S139 phosphorylation (green).

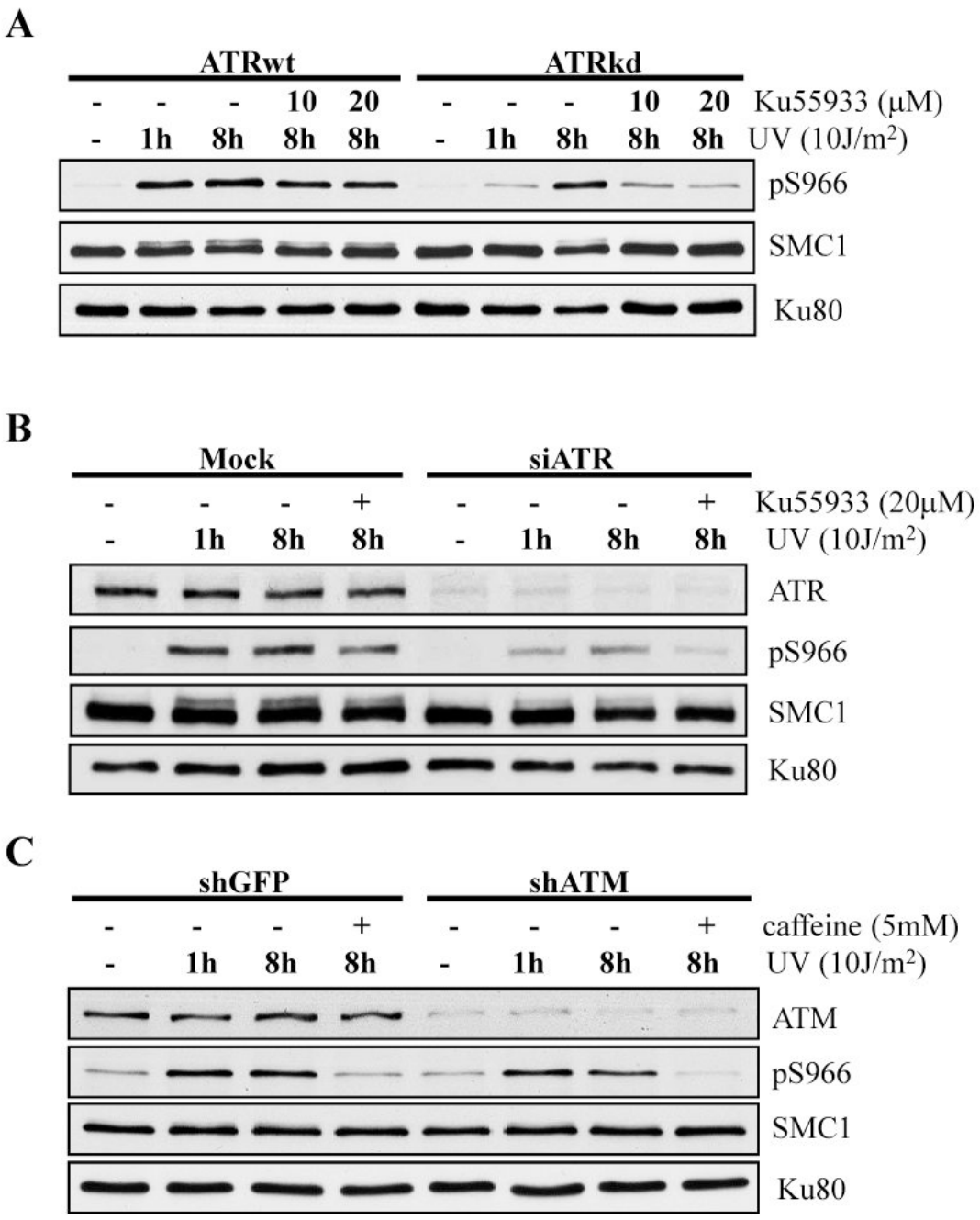


Figure 4.

Overlapping role of ATR and ATM in response to UV irradiation. (A) Human osteosarcoma U2OS cells expressing wild-type ATR kinase (ATRwt) or dominant-negative kinase dead mutant ATR (ATRkd) were UV irradiated (10 J/m^2) and harvested at indicated time points. ATM kinase inhibitor KU55933 was added for the 2 hours prior to harvest if indicated. Whole cell extracts were analyzed for SMC1 phosphorylation. (B) HeLa cells were mock-treated or transfected with siRNA targeting the ATR kinase. Three days after transfection, the cells were UV irradiated in the absence or presence of KU55933 as indicated. (C) MCF7 cells stably expressing shRNA targeting either green fluorescent protein (GFP) or ATM were UV irradiated (10 J/m^2). The cells were treated with ATR kinase inhibitor caffeine for the last 2 hours prior

to harvest as indicated. Whole cell extracts were analyzed for SMC1 phosphorylation. Ku80 blotting is shown as a loading control.

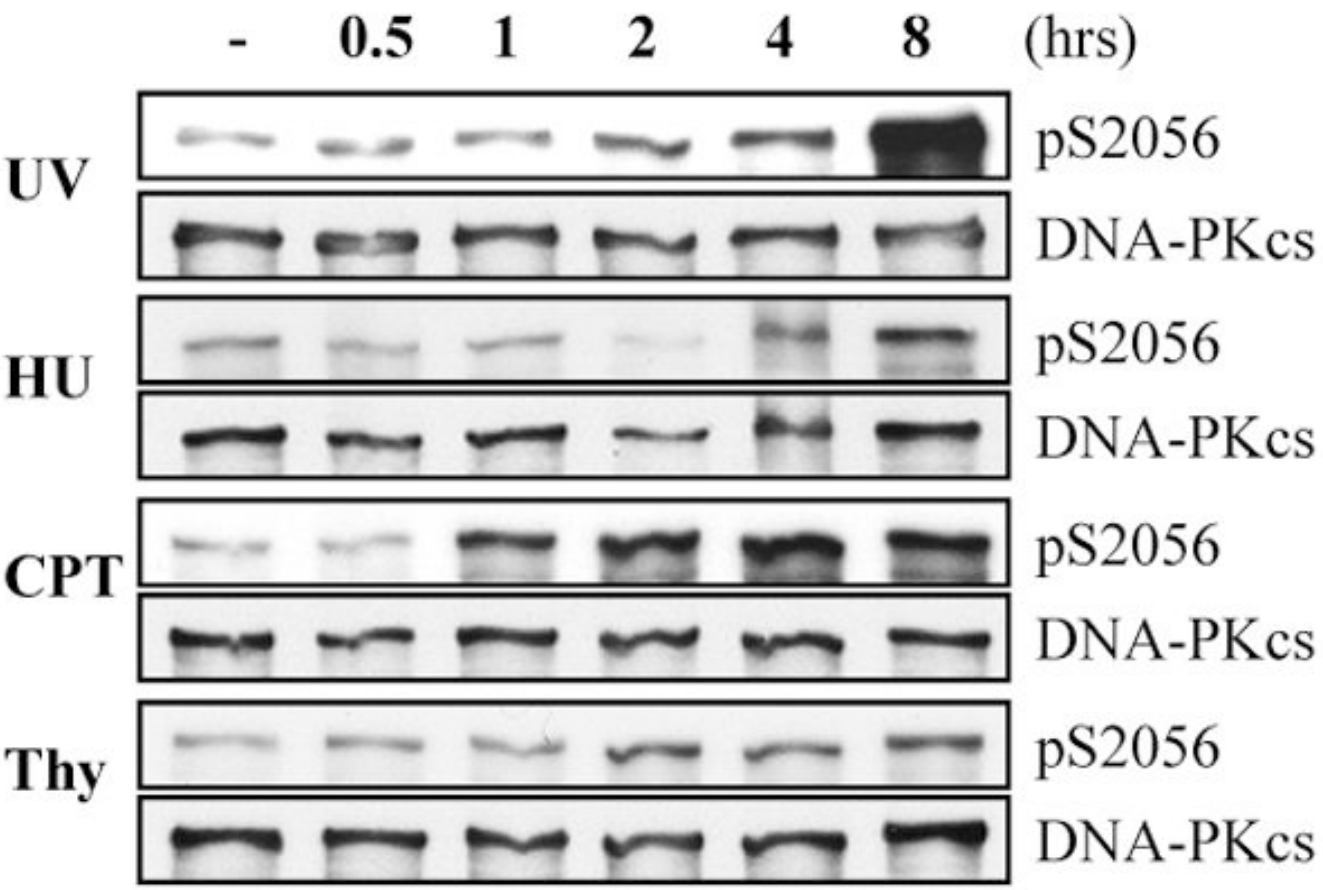
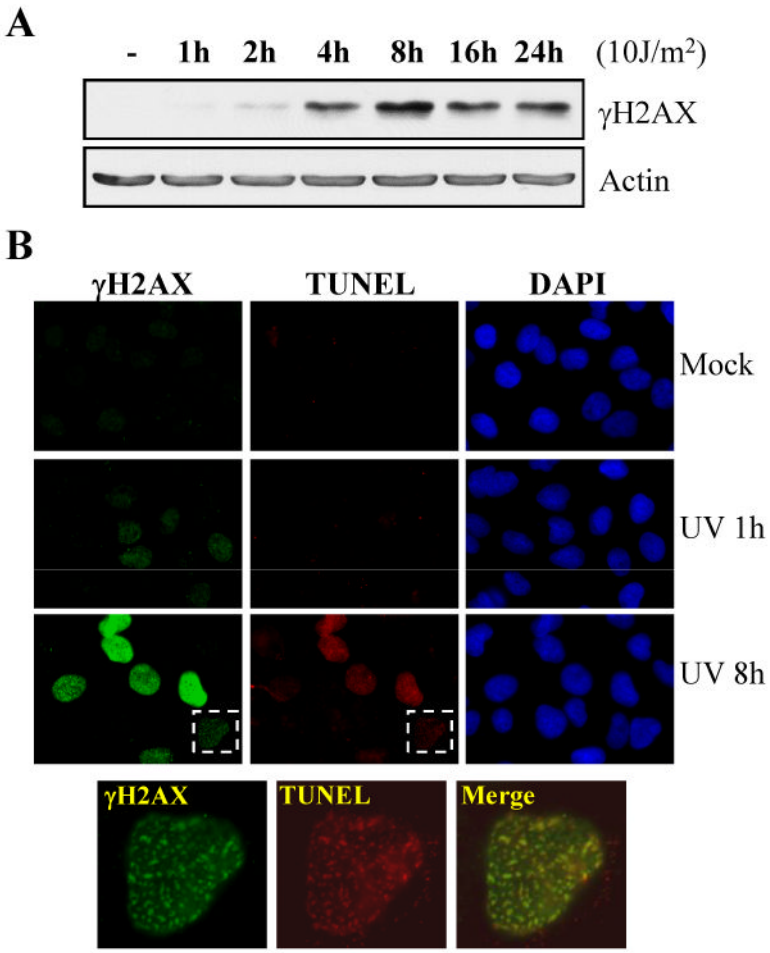


Figure 5.
Induction of DNA-PKcs Ser2056 autophosphorylation in response to various replication stresses. Exponentially growing VA13 cells were mock treated or subjected to various replication stress inducing agents: UV (10 J/m^2), 3 mM hydroxyurea (HU), 1 μM camptothecin (CPT), and 30 mM thymidine (Thy). After treatment, the cells were harvested at the indicated time points for analysis of DNA-PKcs Ser2056 autophosphorylation.



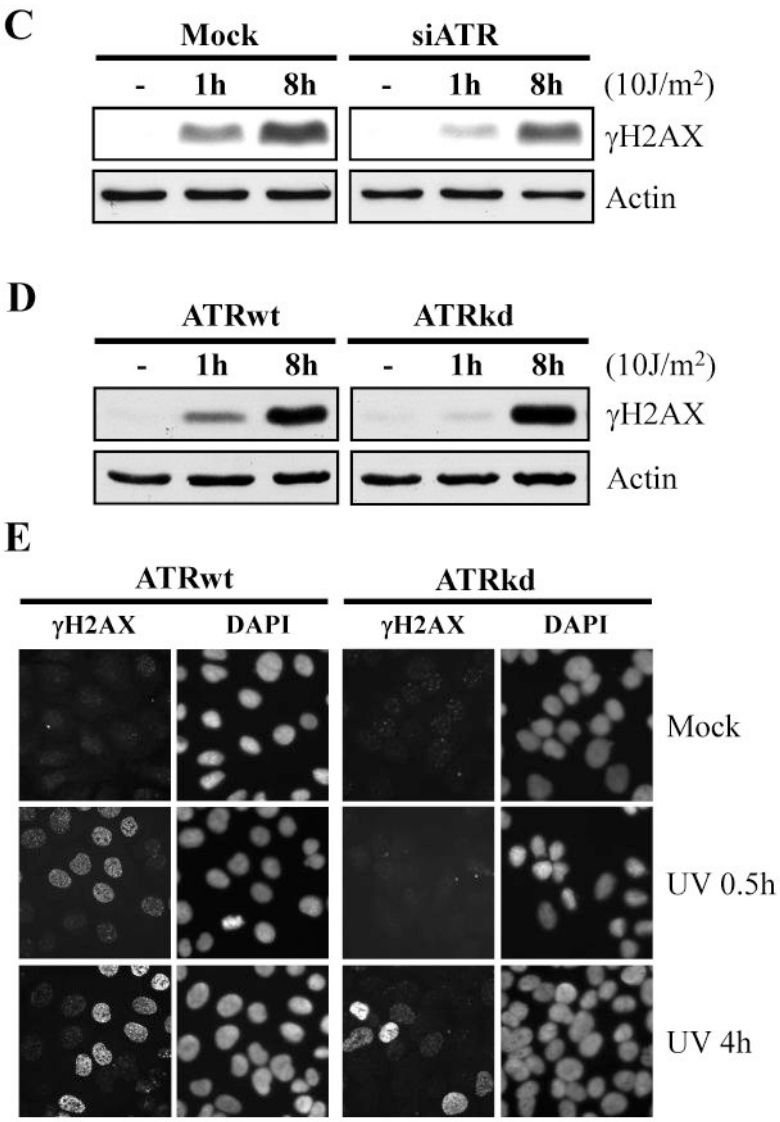


Figure 6.

Late phase activation of ATM signal pathway is associated with DNA double-stranded breaks. (A) Exponentially growing HeLa cells were UV irradiated (10 J/m^2) and were analyzed for the kinetics of UV-induced H2AX phosphorylation. (B) Mock and UV-irradiated HeLa cells were subjected to TUNEL staining followed by immunofluorescent staining with anti-γH2AX antibody. The bottom panel shows the magnified images of positive TUNEL and γH2AX staining from the squares in the 8-hour images. (C) HeLa cells were mock-treated or transfected with siRNA against ATR kinase. Three days after transfection, the cells were UV irradiated and were analyzed for H2AX phosphorylation. (D, E) U2OS cells expressing ATRwt or ATRkd were analyzed for UV-induced H2AX phosphorylation at the indicated time points by western blot (D) or by immunofluorescent staining. (E).

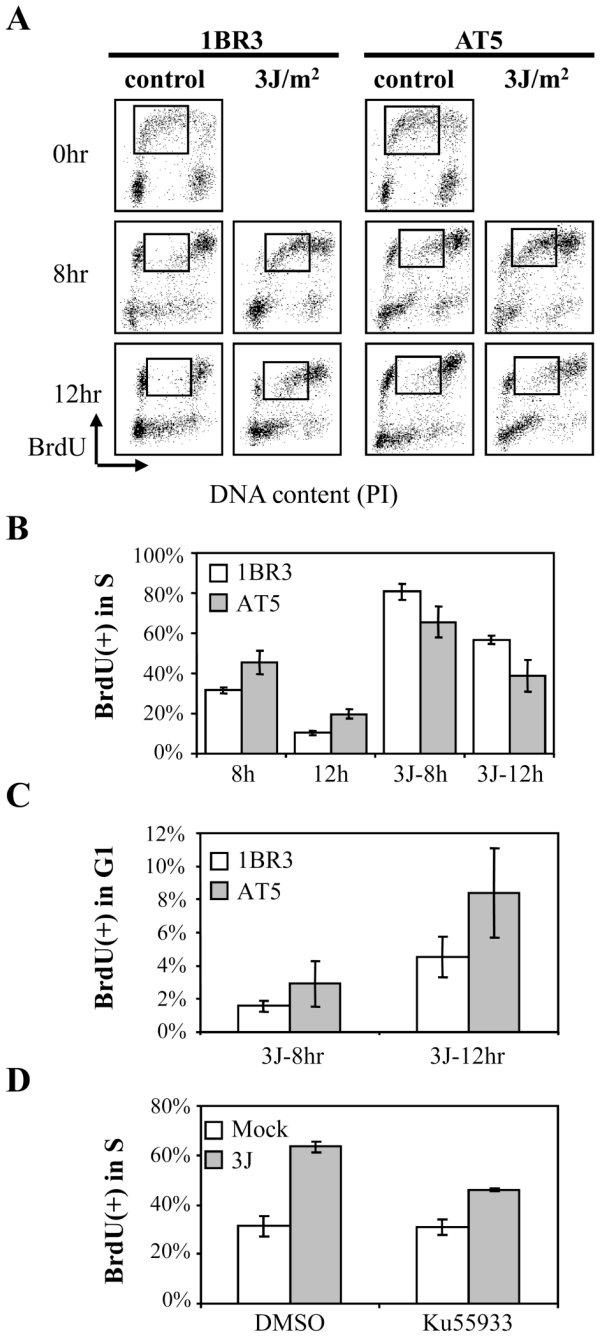


Figure 7.

The ATM signal pathway is required for cell cycle checkpoint regulation upon UV irradiation. (A) Wild-type 1BR3 fibroblasts and ATM-deficient AT5 fibroblasts were pulse-labeled with 1 μM BrdU for 20 minutes and were UV irradiated (3J/m^2). Cell cycle progression of BrdU-labeled cells was monitored by FACS analysis. (B) Percentage of remaining BrdU-positive S-phase cells as compared to that at 0 hr (immediately after BrdU labeling). (C) Percentage of BrdU-positive G1 cells as compared to the initial BrdU-labeled S phase cells at 0 hr. (D) 1BR3 fibroblasts were subjected to the same assay in the presence or absence of ATM kinase inhibitor Ku55933. The remaining BrdU-positive S-phase cells at 8 hrs were as compared to that those at 0 hr. Results in B-D were generated from at least two independent experiments.

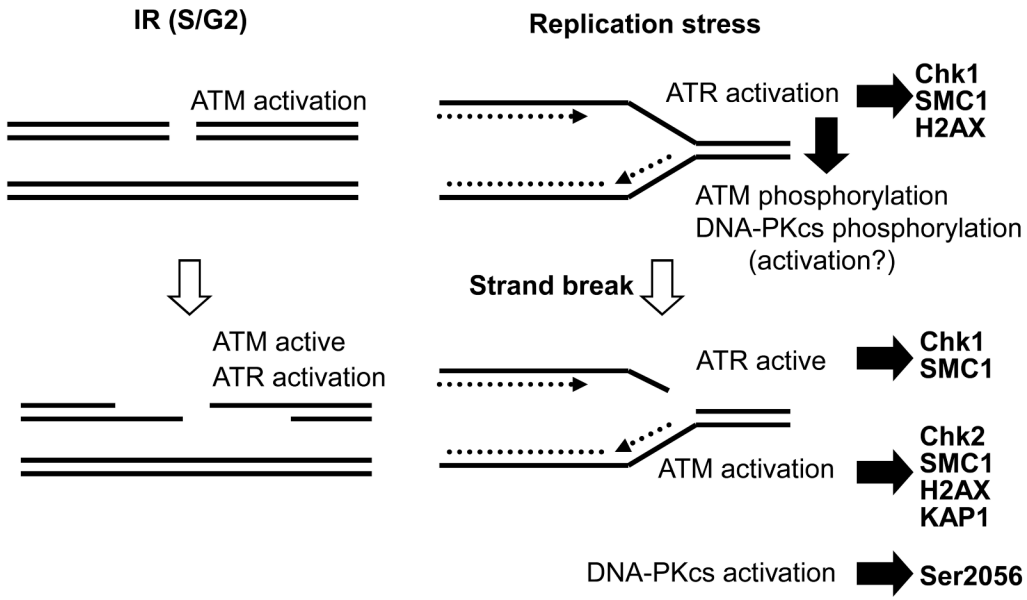


Figure 8.
A model of ATM and DNA-PKcs activation in response to UV-induced replication stress.