

ATM-dependent phosphorylation of the checkpoint clamp regulates repair pathways and maintains genomic stability

Min Hwa Shin,¹ Ming Yuan,¹ Hao Zhang,² Joseph B. Margolick² and Mihoko Kai^{1,*}

¹Department of Radiation Oncology and Molecular Radiation Sciences; Johns Hopkins University School of Medicine; Baltimore, MD USA;

²Department of Molecular Microbiology and Immunology; Bloomberg School of Public Health; Johns Hopkins University; Baltimore, MD USA

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Upon genotoxic stress and during normal S phase, ATM phosphorylates the checkpoint clamp protein Rad9 in a manner that depends on Ser272. Ser272 is the only known ATM-dependent phosphorylation site in human Rad9. However, Ser272 phosphorylation is not required for survival or checkpoint activation after DNA damage. The physiological function of Ser272 remains elusive. Here, we show that ATM-dependent Rad9^{Ser272} phosphorylation requires the MRN complex and controls repair pathways. Furthermore, the mutant cells accumulate large numbers of chromosome breaks and induce gross chromosomal rearrangements. Our findings establish a new and unexpected role for ATM: it phosphorylates the checkpoint clamp in order to control repair pathways, thereby maintaining genomic integrity during unperturbed cell cycle and upon DNA damage.

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Introduction

Cells protect themselves from genotoxic stresses by activating cell cycle checkpoints and DNA repair pathways. Coordination of these pathways needs to be tightly regulated in order to prevent genomic instability.¹⁻³ Mutations in checkpoint and repair genes can cause various diseases, including human cancers.⁴⁻⁶ ATM (ataxia-telangiectasia-mutated) and ATR (ataxia telangiectasia and Rad3-related) kinases belong to the phosphatidylinositol-3-kinase-related kinase (PI-3 kinase) family and are critical components of checkpoint activation. ATM and ATR kinase cascades lead to activation of downstream targets such as Brca1, Chk1, Chk2, p53 and Nbs1 in order to regulate cell cycle progression, apoptosis and DNA repair. ATM pathways are initiated primarily by double-strand breaks (DSBs),⁷ while ATR responds to a broad spectrum of DNA damage and replication disruption, especially during S phase.⁸

Choreography of the DNA damage response pathways has been shown in response to DSBs in budding yeast. The MRE11 and the ATM-related Tel1 kinase are the first proteins detected at DSBs. Next, the replication protein A (RPA) single-strand DNA (ssDNA) binding protein relocalizes to the break and recruits checkpoint proteins, such as RAD24 (Rad17 homolog), DDC1 (Rad9 homolog) and DDC2 (ATRIP homolog). Later, and only in S and G₂ phase, the homologous recombination proteins assemble at the site. Although the checkpoint clamp loader and clamp are recruited to the site before the homologous recombination proteins, they are not required for the recruitment of the

homologous recombination proteins.⁹ It is also shown in mammalian cells that ATM and MRE11 nuclease generate RPA-coated ssDNA, and ATR is recruited to DSB sites subsequently. MRE11 is required for ATM activation.¹⁰⁻¹³ ATM-dependent ATR activation in response to DSBs is restricted to the S and G₂ cell cycle phases.¹⁴ Other components of the ATR pathway, such as the checkpoint clamp complex (Rad9-Rad1-Hus1), are implicated in regulating repair pathways in both mammals and yeasts.¹⁵ Function of the checkpoint clamp complex in response to DSBs is still largely unknown.

Numerous substrates of ATM and ATR kinases have been identified, including Rad9.¹⁶⁻²² Several phosphorylation sites on human Rad9 have been mapped.^{23,24} Among these sites, only Ser272 becomes phosphorylated in response to DNA damage. Moreover, it does so in an ATM-dependent manner. This is unexpected, given that the checkpoint clamp complex (Rad9-Rad1-Hus1) is required for ATR-dependent, rather than ATM-dependent signaling.^{25,26} What is the function of this ATM-dependent Rad9 phosphorylation?

Results

ATM phosphorylates Rad9^{Ser272} upon damage, but phosphorylation is not required for survival or checkpoint activation upon DNA damage. To elucidate the function of the Rad9^{Ser272} phosphorylation, we created human Rad9-S272C and Rad9-S272A mutant cell lines. The mutant cell lines stably express exogenous Rad9-S272C or Rad9-S272A mutant proteins, and endogenous

*Correspondence to: Mihoko Kai; Email: mkai2@jhmi.edu

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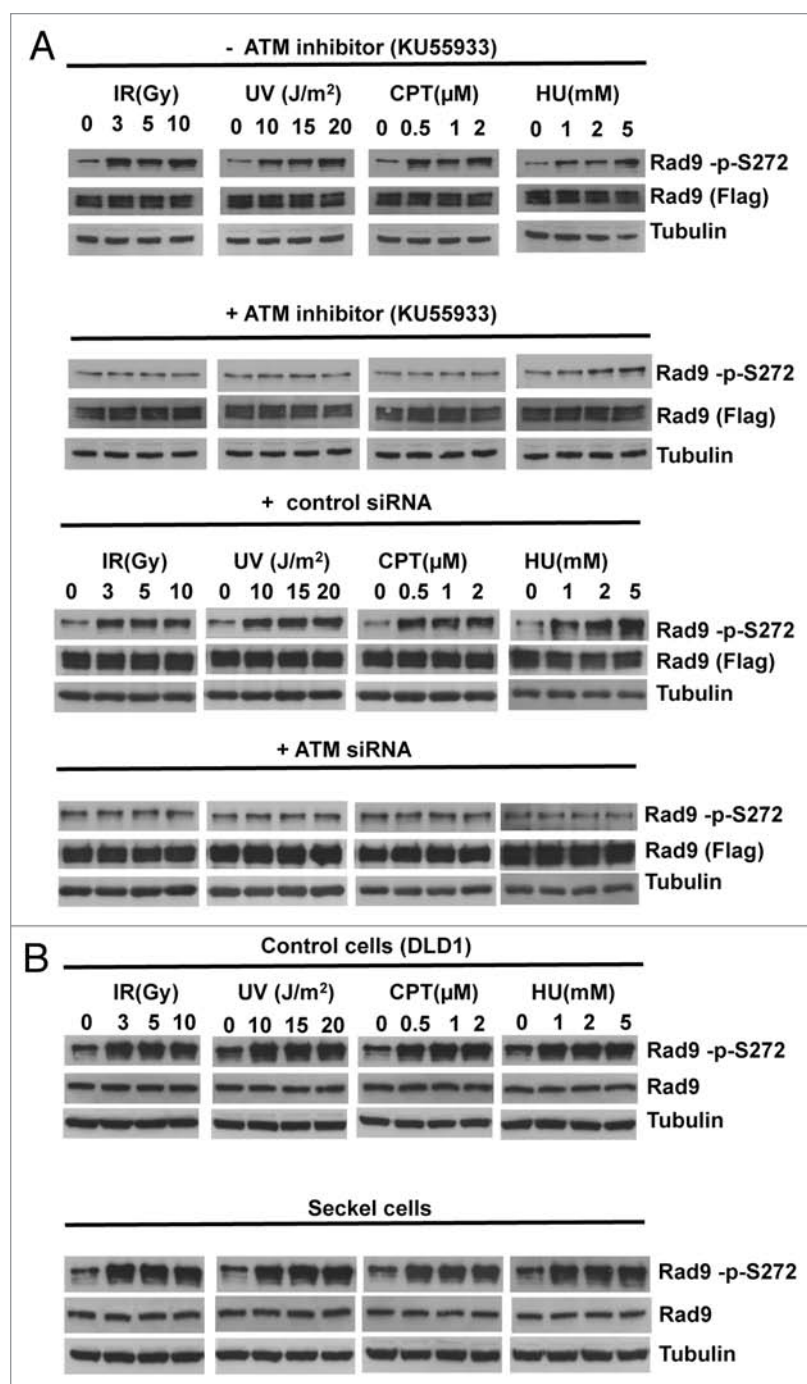


Figure 1. Rad9^{Ser272} is phosphorylated in an ATM-dependent manner. (A) Rad9^{Ser272} is phosphorylated in response to DNA damage. Rad9 becomes phosphorylated at Ser272 in response to IR, UV, CPT (6 h) and HU (1 h) and phosphorylation is inhibited by an ATM-specific inhibitor KU55933 (10 µM) (left part) and ATM-specific siRNA (right part). (B) Rad9^{Ser272} phosphorylation is not affected in ATR-Seckel cells after damage.

Rad9 expression is stably knocked down by its shRNA on the 3' UTR region (hereafter Rad9-S272C and Rad9-S272A cell lines). We also created cell lines (hereafter Rad9 cell lines) in which exogenous wild-type Rad9 proteins are stably expressed, and endogenous Rad9 expression is stably knocked down (Fig. S1A). All experiments were performed under this condition.

Rad9-knockdown cells showed defects in Chk1 activation after IR (ionizing radiation), UV (UV radiation), CPT (camptothecin, a topoisomerase I inhibitor) and HU (hydroxyurea). Chk2 activation in Rad9-knockdown cells was defective after UV, CPT and HU but not IR (Fig. S4). These results are consistent with previous observations that Rad9 is not required for ATM-dependent Chk2 activation after IR.²⁵ Chk1 and Chk2 became phosphorylated in response to damage in the Rad9-S272C and Rad9-S272A mutant cells, indicating that checkpoint responses of the mutant cells to DNA damage, including IR, UV, CPT and HU were normal (Fig. S4, data not shown). No significant sensitivity to these DNA damaging agents was detected in both proliferation and clonogenic survival assays, although Ser272 became phosphorylated (Figs. S4 and S5). In fact, the Rad9-S272C cells showed slightly better survival after IR, UV and CPT treatments compared with the wild-type control ($p < 0.001$, Student t-test) (Fig. S5). Furthermore, damage-induced phosphorylation was abolished by an ATM-specific inhibitor KU55933 as well as two independent ATM siRNAs, indicating that damage-induced Ser272 phosphorylation depends on ATM (Fig. 1A; Fig. S1B).²⁷ Importantly, damage-induced Ser272 phosphorylation was abolished in MRE11-, Rad50- and Nbs1-knockdown cells, indicating that damage-induced phosphorylation requires ATM activation (Fig. 2).

UV and HU treatments are known to activate mainly the ATR-dependent pathway.^{28,29} However, UV- and HU-induced phosphorylation was abolished or significantly reduced by the ATM-specific inhibitor and ATM siRNAs (Fig. 1A). Damage-induced Rad9^{Ser272} phosphorylation was not affected by ATR-specific siRNAs and in ATR-Seckel cells (Fig. 1B; Fig. S2A). ATR-Seckel cells express very low levels of ATR protein and are shown to be defective in phosphorylating its substrates.^{30,31} Furthermore, knockdown of another PI3-kinase, DNA-PKc, by its siRNAs did not affect phosphorylation (Fig. S2B). Nuclear localization of Rad9 protein and damage-induced Rad9 foci formation were not affected by the ATM-specific siRNAs (Fig. S3). These results demonstrate that Rad9^{Ser272} becomes phosphorylated in response to DNA damage in an ATM-dependent manner.

The Rad9-S272C mutant cells are defective in homologous recombination. To investigate the function of Rad9^{Ser272} phosphorylation in controlling DNA repair pathways, we examined *I-SceI*-induced recombination frequencies in the Rad9-S272C and S272A mutant cells using a green fluorescent protein (GFP)-based recombination assay. This system utilizes GFP to measure recombination and the *I-SceI* endonuclease to introduce DSBs. The gene conversion events are

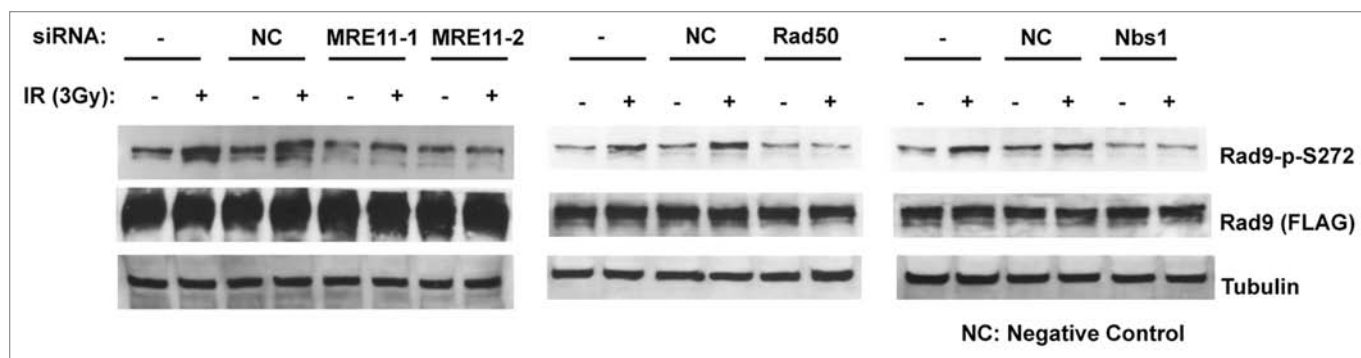


Figure 2. Rad9^{Ser272} is phosphorylated in a MRN complex-dependent manner. Damage-induced Ser272 phosphorylation is inhibited by siRNAs of MRE11, Rad50 and Nbs1.

readily detected by flow cytometry.³² The *I-SceI*-induced recombination frequency was lower in the mutant cells compared with the wild-type controls ($p < 0.01$, Student t-test) (Fig. 3). This result indicates that the importance of Ser272 phosphorylation lies in controlling homologous recombination.

Homology-directed repair requires the Rad51-dependent homologous recombination pathway.³³ We therefore checked Rad51 foci formation and found elevated levels of Rad51 foci in the mutant without DNA-damaging agents. Rad51 foci disappeared 120 min after IR treatment in wild-type cells. However, the foci persisted even after 240 min in the Rad9 mutant cells, implying that Rad51-dependent recombination repair is not efficient in the mutant cells (Fig. 4A). We also observed γ H2AX foci during S phase in untreated Rad9-S272C mutant cells but not in control cells, indicating that DSBs are generated in the mutant cells during unperturbed cell cycle (Fig. S6). Persistent γ H2AX, phosphorylated Chk1 and Chk2 were detected after IR, showing delayed recovery from checkpoint activation in the mutant cells (Fig. 4B). All these observations confirm that there are more DNA breaks caused by defective repair control.

ATM-dependent Rad9^{Ser272} phosphorylation occurs during unperturbed cell cycle. The defect in homologous recombination repair implies that Ser272 phosphorylation might function to regulate repair pathways during the cell cycle. Indeed, we observed Ser272 phosphorylation throughout unperturbed S to G₂ phase in synchronized culture. Unexpectedly, this S-G₂ phase-dependent phosphorylation was abolished by KU55933 treatment and ATM-specific siRNAs, indicating that Rad9^{Ser272} became phosphorylated in an ATM-dependent manner during unperturbed cell cycle (Fig. 5A). It is possible that the double-thymidine block to synchronize the cell cycle could cause DNA damage. However, Chk1, Chk2 and histone H2AX were not phosphorylated in this condition (Fig. 5A). Therefore, it is unlikely that the double-thymidine block induced DSBs that activate ATM-dependent checkpoint pathways. We further confirmed ATM and MRN complex-dependent Rad9^{Ser272} phosphorylation in S to G₂ phase with sorted cells (Fig. 5B; Figs. S7A and S8). Rad9^{Ser272} phosphorylation was not affected in ATR-Seckel cells (Fig. S7B). These results demonstrate that ATM, not ATR, is the main kinase that phosphorylates Ser272, not only in response to DNA damage, but during unperturbed S-G₂ phase as well.

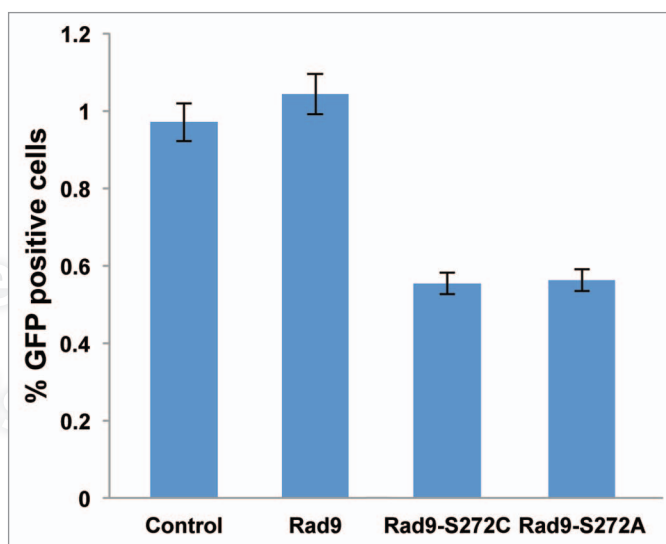


Figure 3. ATM-dependent Rad9 phosphorylation at Ser272 is important to control recombination pathways. The Rad9-S272C/A mutant cells show decreased homologous recombinations induced by I-SCEI, $p < 0.01$ (Student t-test).

ATM is activated during unperturbed S and G₂ phases. It is generally thought that ATM is activated in response to DSBs.^{7,34-39} However, we observed ATM-dependent phosphorylation of Rad9 without exogenous DNA damage (Fig. 5A; Fig. S7). Thus, we examined the autophosphorylation status of ATM. Autophosphorylation of ATM at Ser1981 was detected during S-G₂ phase in unperturbed cells. Furthermore, we observed SMC1^{Ser957} phosphorylation that depends on ATM.^{40,41} Interestingly, however, Chk1, Chk2 and H2AX phosphorylation was not detected in unperturbed cells (Figs. 5A; Figs. S6 and S7). It is possible that this ATM activation can be seen only in transformed cells. Thus, we investigated the phosphorylation status of Rad9 and the autophosphorylation status of ATM in a primary cell line MRC5. Both phosphorylation during S-G₂ phase was observed (Fig. S9). These results demonstrate that ATM is active during unperturbed S-G₂ phase of the cell cycle and phosphorylates certain substrates without a detectable level of DSBs.

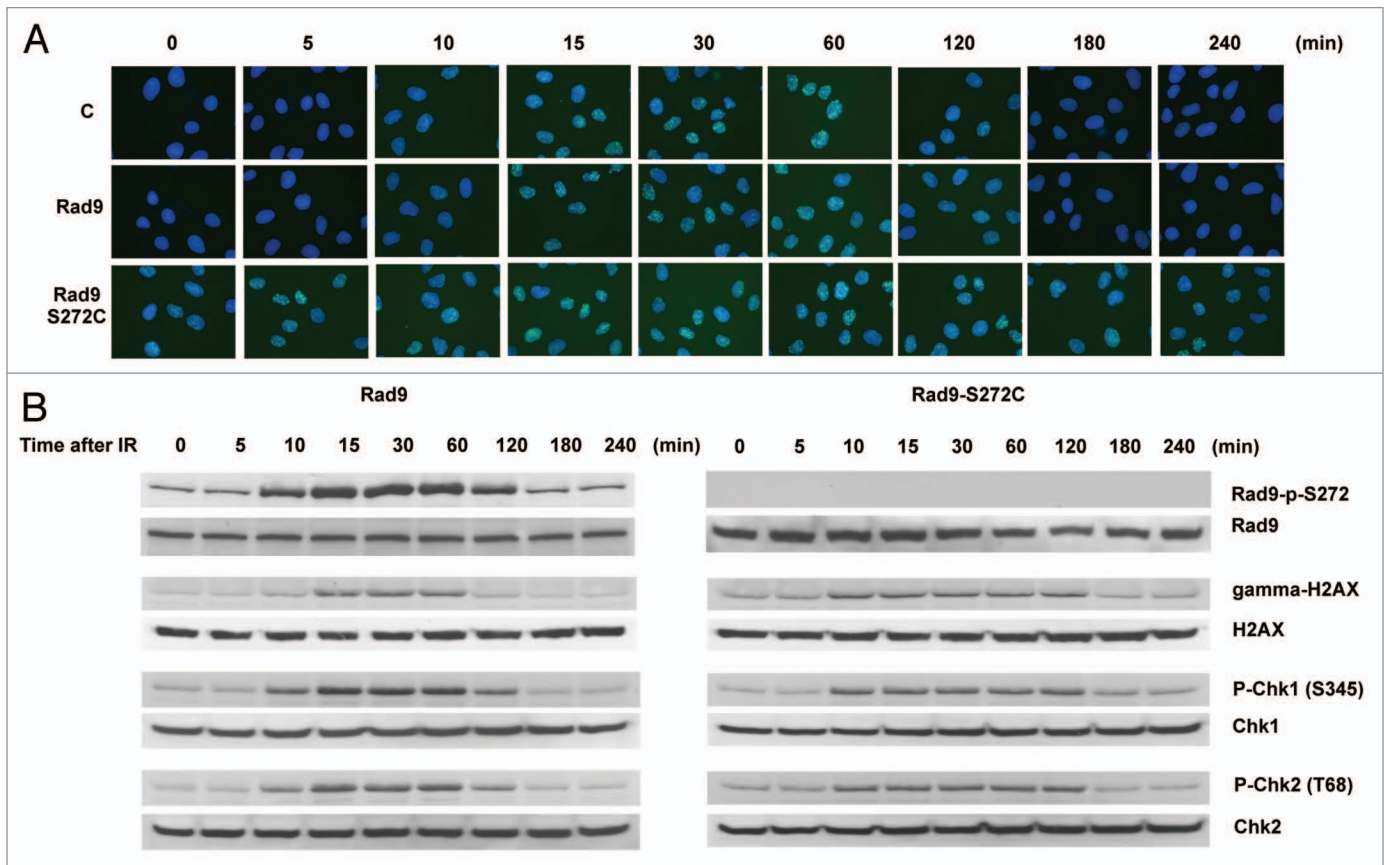


Figure 4. The Rad9-S272C mutant cells accumulate DNA damage. (A) Rad51 foci formation in Rad9 and Rad9-S272C cells before and after IR (3 Gy). (B) Timing of Rad9^{Ser272}, H2AX, Chk1 and Chk2 phosphorylation in response to IR (3 Gy).

We further investigated whether Rad9^{Ser272C} mutation affects phosphorylation of other ATM substrates. We observed intact phosphorylation of ATM substrates SMC1, NBS1, BRCA1, 53BP1 and Rad17 (Fig. S10).

The Rad9-S272C mutant cells induce chromosome instability. To assess the impact of these unscheduled repairs on chromosomal stability, we examined metaphase chromosome spreads. Fully 59% of the Rad9-S272C mutant cells exhibited more than two chromosomal breakages per cell 12 h after IR compared with 21% of wild-type Rad9 cells. A similar result was observed after CPT treatment (Rad9-S272C cells 51%, Rad9 cells 9%). The contrast was even more pronounced for spontaneous chromosomal breakages (Rad9-S272C cells 22%, Rad9 cells 1%) (Fig. 6A). These results confirm that Rad9^{Ser272} phosphorylation is required for proper repair control and maintenance of replication fork stability during unperturbed cell cycle. ATM phosphorylates Rad9 in order to prevent chromosomal breakage during replication.

The accumulation of chromosomal breakages alone might not necessarily be disastrous, if the breaks are correctly repaired. Incorrect repairs, however, will cause deleterious long-term effects, such as gross chromosomal rearrangements (GCRs). To look for these, we sought to perform karyotype analysis using human normal primary (MRC5) cells. However, the Rad9-S272C MRC5 cells were not viable, confirming the importance

of phosphorylation during normal cell cycle in non-transformed cells. We therefore employed a transformed (HCT116) cell line for karyotype analysis. We did not observe significant genomic instability in p53^{+/+} cells (data not shown). However, the Rad9-S272C mutant cells (endogenous Rad9 knocked down) with p53^{-/-} background showed significantly higher frequency of GCRs compared with the wild-type controls (Fig. 6B). We conclude that the improved survival of the Rad9 mutant after DNA damage comes at an unacceptable long-term cost.

Discussion

Our results demonstrate that ATM-dependent phosphorylation of Rad9 acts to maintain chromosome stability by coordinating repair pathways. This is unexpected, particularly during S-G₂ phase, which is independent of Rad9-dependent checkpoint activation pathways. The absence of ATM-dependent phosphorylation induces DSBs, which are improperly repaired in the mutant. ATM detects improper DNA structures and/or replication fork collapses and signals to repair pathways through the checkpoint clamp during S to G₂ phase. We created Rad9-S272C mutant cell lines that express the mutant proteins stably, and the endogenous Rad9 protein is knocked down. Originally, we chose Rad9-S272C mutation instead of Rad9-S272A mutation, because it has been reported that Rad9 phosphorylation site mutation on

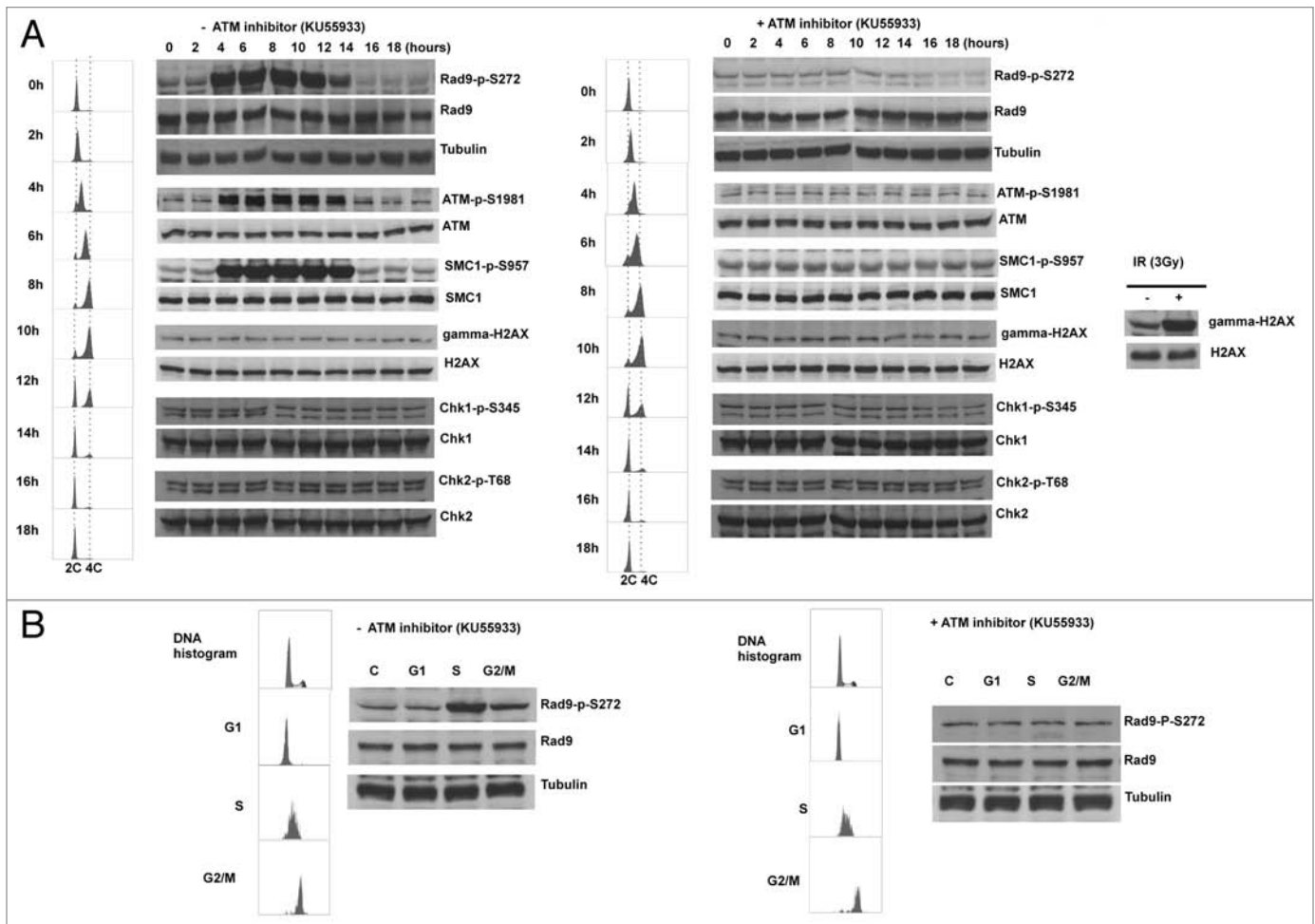


Figure 5. Rad9^{Ser272} is phosphorylated during normal cell cycle in an ATM-dependent manner. (A) Cell cycle progression and Rad9^{S272} phosphorylation after double thymidine block with (right) and without KU55933 treatment (10 μ M) (left). (B) Rad9^{Ser272} phosphorylation in sorted cells with (right) and without KU55933 (10 μ M) treatment (left).

the PCNA-like domain causes a more severe DNA damage sensitivity, possibly due to structural defect in yeast.⁴² We repeated experiments with Rad9-S272A mutant cells and obtained similar results as with Rad9-S272C mutant cells. Importantly, the defects of Rad9-S272C and Rad9-S272A mutant cells in repair regulation do not reduce cell survival in transformed cells. On the contrary, the mutant cells show slightly improved survival after DNA damage (Fig. S5). The mutant cells show defects in *I-SCEI*-induced homologous recombination. This deficiency is modest compared with other checkpoint/homologous recombination protein mutants, such as BRCA1. The DSBs caused by *I-SCEI* are probably repaired by error-prone pathways, such as nonhomologous end joining and single-strand annealing. Repairs are not properly done in the mutant cells, leading to serious genomic instability. Given that the Rad9-S272C mutant cells induce deregulation of recombination and GCRs (Figs. 3 and 6), failure of phosphorylation at this site could lead to further mutations in tumor suppressor genes. Furthermore, increased survival of the mutant cells after damage would confer a selection advantage on these cells in the event of, e.g., chemotherapy treatment. Therefore, ATM-dependent phosphorylation of the checkpoint

clamp Rad9^{Ser272} might function to prevent progression of tumor development and malignant transformation of tumor cells.

ATM activation during unperturbed S-G₂ phase of cell cycle. It is generally accepted that ATM becomes activated in response to DSBs. However, we observed autophosphorylation of ATM and phosphorylation of ATM substrates in unperturbed cells (Fig. 5A and B; Fig. S8). This result is indeed consistent with the results shown in 293T cells that showed ATM^{Ser1981} autophosphorylation without exogenous damage. Autophosphorylation was not detected in primary fibroblasts, 1070S.⁴³ However, we detected phosphorylation of ATM^{Ser1981} and ATM substrates in primary MRC5 cells that were enriched in S-G₂ phase (Fig. S10). Interestingly, Chk2 and H2AX phosphorylation were not observed during unperturbed cell cycle (Fig. 5A and B; Figs. S7 and S8). Therefore, ATM phosphorylates a subset of substrates during S to G₂ phase of the cell cycle without “detectable” DSBs, but does not activate the checkpoint pathway. However, it is possible that ATM is activated by an undetectable level of DSBs caused by spontaneous replication fork collapse during S phase. Indeed, transient activation of ATM has been observed in *Xenopus* egg extracts with undamaged DNA.

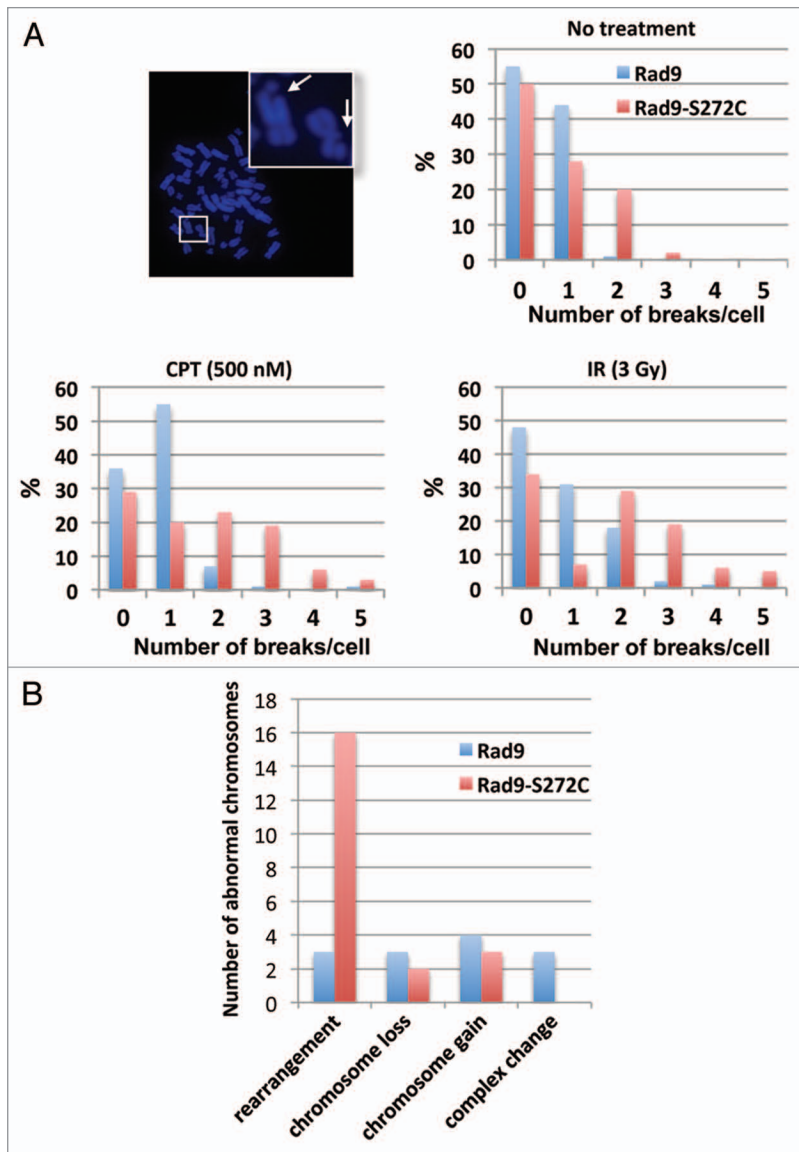


Figure 6. The Rad9-S272C mutant cells accumulate chromosome breakages and show chromosome instability phenotypes. (A) Chromosome breakages in Rad9 and Rad9-S272C cells. An example of metaphase spread of a Rad9-S272C cell (top, left). The Rad9-S272C cells induce a high frequency of chromosome breakages without damage (top, right), after IR treatment (3 Gy) (bottom, right), after CPT treatment (500 nM, 6 h) (bottom, left). (B) The Rad9-S272C mutant cells induce a high frequency of GCRs, chromosome rearrangements. Twenty cells for each cell line were karyotyped and chromosome abnormalities were detected.

The authors speculate that transient generation of DSBs during replication triggers a local activation of ATM on replication chromatin. The authors also showed that ATM slows the rate of replication by inhibiting Cdk2 kinase.^{44,45} Genomic DNA replicated in *Xenopus* extracts immunodepleted of X-MRE11 complex accumulate DSBs. Therefore, it is thought that the function of X-MRE11 complex is to repair DSBs that arise during normal DNA replication, although H2A phosphorylation was not detected in the cells. Rad9 is phosphorylated during unperturbed S to G₂ phase of the cell cycle in an ATM- and MRE11-dependent

manner. We speculate that this phosphorylation might be required for fast repair of DSBs and/or to protect the genome from replication fork collapse. Indeed, we observed elevated levels of chromosome breaks in the mutant cells without exogenous damage as in the case of X-MRE11-depleted cell extracts (Fig. 6).

Increased spontaneous intrachromosomal as well as extrachromosomal recombinations in ATM-deficient cells have been shown by several studies in references 46–48. Cytogenetic analysis revealed a higher spontaneous incidence of chromosome breaks, chromosome gaps, acentric fragments, dicentric chromosomes and aneuploidy in ATM patient cells.^{49,50} A recent study showed that ATM could be activated without DSBs by oxidative stress. Oxidation of ATM directly induces ATM activation, and this mechanism is Mre11-Rad50-Nbs1 (MRN) complex-independent.⁵¹ These results demonstrate important functions of ATM to prevent genomic instability during normal cell cycle.

In this study, we discovered a new ATM-dependent pathway to protect genome stability during unperturbed cell cycle. ATM phosphorylates a subset of its substrates for proper repair control without exogenous damage. It is yet unclear whether activation of this pathway requires spontaneous DSBs or not. Further studies are required to understand the mechanism of ATM activation during unperturbed cell cycle.

Materials and Methods

Materials, antibodies and cell lines. The Rad9-flag-expressing plasmids were created by inserting Rad9 cDNA into p3xFLAG-CMV-14 (Sigma-Aldrich) and pBMN vectors. The Rad9-S272C mutation was made using QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). Rad9 shRNA (V3LHS_401433) construct was obtained from OpenBio Systems. The Rad9 and Rad9.S272C cell lines (stably express exogenous wild-type or the mutant Rad9 proteins, and endogenous Rad9 expression is stably knocked down) were created first by selecting Rad9-flag-expressing single clones and then infecting a shRad9 construct in the DR-293 cell line. Finally, stable Rad9-knockdown cells were selected. The siATM, SI00604730 and SI00299299, siMRE11, SI02665173 and SI02665180 and negative control siRNAs were obtained from QIAGEN. The siATR, SignalSilence® ATR siRNA I (6288S), SignalSilence® ATR siRNA II (6289S) and negative control SignalSilence siRNA (6568S) were from Cell Signaling Technology. Nbs1 siRNA (h): sc-36061, Rad50 siRNA (h): sc-37397, DNA-PKCs siRNA (h): sc-35200 and control siRNA: sc-37007 were obtained from Santa Cruz Biotechnology. Five nM siRNAs were used for the experiments.

Anti-flag antibody was from Sigma-Aldrich. Anti-Chk1, phospho-Chk1, Chk2, phospho-Chk2, ATM, phospho-ATM (S1981), SMC1, phospho-SMC1 (S957), Nbs1, phospho-Nbs1 (S343), BRCA1, phospho-BRCA1 (S1524), 53BP1, phospho-53BP1 (S25/29), Rad50, Mre11 ATR and gamma-H2AX antibodies were obtained from Cell Signaling. Anti-phospho-Rad9-S272 antibody was from Abgent. Anti-Rad51 and antibody were from Calbiochem. Anti-Rad17, phospho-Rad17 (S645) and DNA-PKCs antibodies were from Abcam.

Cell synchronization and cell cycle analysis. HeLa cells were incubated with 2 mM thymidine for the first block for 18 h, followed by a 9 h release for the second block for 17 h then a release up to 18 h. Cells were trypsinized, washed in PBS, and resuspended in 70% ethanol for storage at -20°C until further analysis. Cells were stained with propidium iodide (50 µg/ml), and analyzed with a MoFlo MLS cytometer (Beckman Coulter).

Cell survival (proliferation) assay. DR293 cells (1 x 10⁴) cells, including control, Rad9 and Rad9-S272C cell lines, were plated on 96-well plates. After 4 h incubation, cells were exposed to UV, IR, CPT and HU and incubated for 24 h. 20 µl Cell Titer-Blue cell viability assay reagent (Promega) was added to each well, incubated for 1 h and measured with 96-well plate reader at 560_{Ex}/590_{Em}.

Clonogenic cell survival assay. DR293 cells, including control, Rad9 and Rad9-S272C cell lines, were plated (3 x 10³ cells) on a 10-cm plate, exposed to IR, UV, CPT and HU and incubated for 14 d. The resulting colonies were stained with 0.5% Crystal violet/50% Methanol and counted.

GFP-based recombination assay. *I-SceI* expression plasmids (100 µg) were electroporated (250 V, 950 µF) into DR293 cells (5 x 10⁶ cells).³² After electroporation, cells were plated in non-selective media for 72 h and were recovered and concentrated by centrifugation and resuspended in Opti-MEM media (Invitrogen) prior to flow cytometry. Cells were analyzed in FACSCalibur (BD Biosciences).

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Immunofluorescence. DR293 cells, Rad9 and Rad9-S272C cell lines were fixed in 4% paraformaldehyde for 20 min, washed three times in PBS and incubated for 45 min in blocking buffer containing 130 mM NaCl, 10 mM Na₂HPO₄, 3 mM NaH₂PO₄, 1% bovine serum albumin, 2% Triton-X-100, 0.5% Tween 20 and 5% donkey serum. Incubation with primary antibody was 1 h at room temperature with the following dilutions in blocking buffer: 1:100 for Anti-Rad9 antibody (BD Biosciences), 1:200 for Anti-phospho-Rad9-S272 antibody (Abgent). Following three washes with PBS, cells were incubated for 1 h with Fluorescein (FITC) or Rhodamine (TRITC) conjugated Donkey Anti-mouse or rabbit IgG (H + L) (Jackson ImmunoResearch Laboratories) diluted to 1:100 with blocking buffer. Cells were washed three times with PBS before observation and DAPI solution was applied to stain nuclei. Cells were visualized by Zeiss imager.Z1.

Cytogenetic analysis. Colcemid (0.1 µg/ml) was added to cell culture 3 h before harvesting by trypsinization. Cells were then swollen for 15 min at 37°C in 0.075 M KCl and fixed for 10 min in methanol:acetic acid (3:1; vol:vol). Fixed cells were spotted onto microscopic slides and stained by DAPI (4',6-diamidino-2-phenylindole). For each sample, chromosomal breakages in at least 100 well-spread metaphase cells were counted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental materials can be found at: www.landesbioscience.com/journals/cc/article/20161

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