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A role for the arginine methylation of Rad9 in checkpoint control and cellular sensitivity to DNA damage

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ABSTRACT

The genome stability is maintained by coordinated action of DNA repairs and checkpoints, which delay progression through the cell cycle in response to DNA damage. Rad9 is conserved from yeast to human and functions in cell cycle checkpoint controls. Here, a regulatory mechanism for Rad9 function is reported. In this study Rad9 has been found to interact with and be methylated by protein arginine methyltransferase 5 (PRMT5). Arginine methylation of Rad9 plays a critical role in S/M and G2/M cell cycle checkpoints. The activation of the Rad9 downstream checkpoint effector Chk1 is impaired in cells only expressing a mutant Rad9 that cannot be methylated. Additionally, Rad9 methylation is also required for cellular resistance to DNA damaging stresses. In summary, we uncovered that arginine methylation is important for regulation of Rad9 function, and thus is a major element for maintaining genome integrity.

INTRODUCTION

DNA repair and cell cycle checkpoint cooperate in minimizing the DNA damage constantly caused by intracellular and environmental genotoxic stresses and maintaining genomic integrity. Mutation in genes functioning in these two systems often leads to 'mutator' phenotype and enhances susceptibility to tumor development (1). Rad9 is conserved from yeast to human, and is critical for both DNA repair and cell cycle checkpoint control (2,3). Rad9 is required for homologous recombination, base excision and mismatch repairs (4–6), and for G2/M and S/M checkpoint activation (7,8).

Protein arginine methylation is a post-translational modification that results in symmetrical or asymmetrical dimethylarginines (9). Protein arginine methyltransferases (PRMTs) are classified as types I, II, III or IV enzymes. Types I, II and III PRMTs methylate terminal (or ω) guanidino nitrogen atoms. Both type I and type II enzymes catalyze the formation of a mono-methylated (MMA) intermediate, subsequently type I PRMTs (PRMT1, 3, 4, 6 and 8) further catalyze the generation of asymmetrical double-methylated arginine (aDMA), whereas type II PRMTs (PRMT5, PRMT7 and FBXO11) catalyze the formation of symmetrical double-methylated arginine (sDMA) (10). Both type I and II enzymes regulate gene transcription via methylating histones, and other cellular activities through methylating non-histone proteins.

Several proteins involved in DNA repair (MRE11, p53, DNA polymerase β) have been shown to be regulated by arginine methylation (11). In this study, we identified a few Rad9-associated proteins by combining immunoprecipitation and mass spectroscopy, one of these proteins was PRMT5 (12–14). It is of interest that both human and mouse Rad9 contains a methylation consensus amino acid sequence RGRR. We found that Rad9 form a complex with PRMT5, and PRMT5 can methylate Rad9 at the RGRR sequence. The methylation is critical for cellular resistance to hydroxyurea, and for S/M and G2/M checkpoint activation.

MATERIALS AND METHODS

Mass spectrometry

HEK 293T cells stably expressing FL-hRad9 at a level close to the endogenous hRad9 expression level were used for affinity-immunoprecipitation of hRad9-interacting proteins. The procedure for identifying these

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

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proteins by mass spectrometry has been described previously (13).

Antibodies, immunoprecipitation and western blotting

Anti-hRad9 polyclonal antibody was obtained by immunizing mice with purified MBP-hRad9 protein and anti-hRad9 monoclonal antibody (611324) was purchased from BD. Anti-PRMT5 rabbit polyclonal antibody (07-405) was from Millipore, and ab412 (anti-mono/dimethylarginine antibody) was from Abcam. Anti-FLAG M2 monoclonal antibody (F1804), anti-FLAG polyclonal antibody (F7425) and FLAG peptide were obtained from Sigma-Aldrich, and anti-HA antibody was obtained from Santa Cruz Technology. Phospho-Chk1 (P-Ser-345) and Chk1 (G-4) antibodies were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. Immunoprecipitation and western blotting were performed as described previously (15).

Gene subcloning

pFLAG-CMV2-*hRad9*, pFLAG-CMV2-*hRad1*, pET32 (+)-*hRad9* and pcDNA3-6HA-*hRad9* plasmids have been described previously (16). Full-length human PRMT5 sequence was amplified by PCR using cDNA from HEK 293T cells and cloned into pFLAG-CMV2 and pcDNA3-6HA, respectively. pGEX-6P-1-*PRMT5* was constructed by excising from pFLAG-CMV2-*PRMT5* and ligating into the EcoRI site of pGEX-6P-1. PCR site-directed mutagenesis was performed using the Quick-Change method (Stratagene). pFLAG-CMV2-*hRad9-3RK* was constructed by overlap PCR using pFLAG-CMV2-*hRad9*. All constructs generated using PCR were confirmed by sequencing. The primers and restriction sites used are shown in Supplementary Table S1.

RNA interference

To generate the PRMT5 ShRNA vector, oligonucleotides (5′-GATCCCC**GGCCATCTATAAATGTCTG**TTCAAG AGA**CAGACATTTATAGATGGCC**TTTTTA-3′ and 3′GGG**CCGGTAGATATTTACAGAC**AAGTTCTCT**GT CTGTAAATATCTACCGG**AAAAATTCGA-5′) were designed to target PRMT5 nucleotides 1016–1034 (shown in boldface). Oligonucleotides were annealed and cloned into the Bgl II and Hind III sites of the pSUPERpuro vector. HCT116 cells were transfected with the pSUPERpuro *PRMT5* vector or an empty pSUPERpuro vector. Cells were treated with 1 µg/ml puromycin for 3 days to eliminate cells without ShRNA.

Cell culture

Mouse ES cells, HeLa cells, HCT16 and HEK 293T cells were cultured according to previously published methods (7,13).

Expression of wild-type and mutant hRad9 in *mRad9*$^{-/-}$ ES cells

For R to A mutation analysis, ES cells were transfected using Lipofectamine with pZeoSV2-*hRad9* or pZeoSV2-*hRad9-3RA* according to the manufacturer's instructions (Invitrogen). Stable clones were selected in medium containing zeocin at a concentration of 100 µg/ml. Selected transfectants were subsequently cultured at 25 µg/ml zeocin to maintain the transfected genes within the cells. Multiple clones of each mutant expressing similar levels of protein were used for functional studies. For R to K mutation analysis, ES cells were transfected using Lipofectamine with pcDNA3.1-hygromycin (1 µg) and pFLAG-CVM2-*hRad9-3RK* (3 µg) or pFLAG-CVM2-*hRad9* according to the manufacturer's instructions (Invitrogen). Stable clones were selected in medium containing hygromycin at a concentration of 150 µg/ml. Selected cells were subsequently cultured at 50 µg/ml hygromycin to maintain the transfected genes within the cells. Multiple clones of each mutant expressing protein levels similar to the endogenous protein were used for functional studies.

GST pull-down

GST fusion proteins were expressed in cells of the *Escherichia coli* strain Rosetta (Invitrogen) using pGEX-6P-1 (GE Health). Purification of GST fusion proteins and *in vitro* GST pull-down test has been described previously (13).

ES cell survival and cell cycle checkpoint assays

Mouse ES cells were seeded in duplicate at designated numbers onto 60-mm gelatinized tissue culture dishes. Sensitivity to various doses of hydroxyurea (HU), and ^{60}Co γ rays was tested using a previously published procedure (7). Assays for detecting G2/M checkpoint control and S/M checkpoint function of mouse ES cells were performed as described previously (7,13).

In vitro methyltransferase assays

HEK 293T cells were transfected with the plasmid pFLAG-CMV2-*PRMT5*, and FL-PRMT5 protein was immunoprecipitated with anti-FLAG agarose beads 24 h after transfection. Purified GST-hRad9 or GST-hRad9-3RA (2 µg) were incubated with immunoprecipitated FL-PRMT5 in the presence of 0.55 µCi of [methyl-^{3}H] AdoMet (Amersham Biosciences) in 25 mM Tris–HCl at pH 7.5 in a final volume of 30 µl for 60 min at 37°C, histones (sigma) were introduced as a positive control. Reactions were stopped by adding 6 µl of 5× SDS–PAGE sample buffer, followed by heating at 100°C for 5 min. The samples were separated on 10% SDS–PAGE and stained with Coomassie blue. Destained gels were dried and exposed to X-ray film at −70°C for 14 days.

RESULTS

hRad9 associates with PRMT5 in cells

In the previous studies, using a strategy of immunoaffinity-Mass spectrometry, we found that hRad9 interacts with several proteins in HEK 293T cells, in which mismatch repair protein MLH1 was confirmed to be associated physically and functionally with hRad9 (13).

The analysis of Mass spectrometry shows that PRMT5, a key member of PRMTs (12–14), is also among the proteins associated with FL-hRad9 but not in the proteins associated with negative control FL-GFP (Figure 1A and Supplementary Table S2). Interestingly, hRad9 contains the glycine-arginine rich sequence (GAR) RGRR that exists in other proteins which have been shown in previous studies to be methylated by PRMTs (17). Therefore we chose to further characterize the interaction of hRad9 with PRMT5.

We used co-immunoprecipitation to confirm the interaction between hRad9 and PRMT5. The assay showed that overexpressed HA-tagged hRad9 (HA-hRad9) interacted with FLAG-tagged PRMT5 (FL-PRMT5) in HEK 293T cells, and did not interact with the negative control FL-GFP (Figure 1B). Importantly, endogenous PRMT5 and hRad9 were immunoprecipitated from HeLa cell extract by an anti-hRad9 polyclonal or anti-PRMT5 antibody, but not by pre-immune serum (Figure 1C D). The data above indicate that hRad9 associates with PRMT5 in cells.

hRad9 is arginine-methylated by PRMT5

The above data have established that hRad9 interacts with PRMT5. Interestingly the hRad9 protein harbors a sequence RGRR (Figure 2A), the typical methylation target for PRMTs (18–21). The RGRR sequence is conserved among humans, monkeys, mice and rats, but only GRR is conserved in horses, calves and dogs, and the sequence is not conserved in frogs and other lower animals. Here we examined whether hRad9 is arginine-methylated. A pcDNA3-6HA plasmid harboring wild-type hRad9 or mutated hRad9 in which Arg-172,174,175 were all mutated to Ala (hRad9-3RA) was transfected into HEK 293T cells. The transfected cell lysates were immunoprecipitated with anti-mono/dimethylarginine antibodies (ab412), the immunoprecipitated proteins were fractionated by SDS-PAGE and monitored with anti-HA antibody. As shown in Figure 2B, HA-hRad9 was clearly detected in proteins immunoprecipitated by ab412, while HA-hRad9-3RA could not be detected. These results indicate that hRad9 is methylated *in vivo*.

Having shown that hRad9 is methylated on the arginine residues in the arginine-rich motif, and that hRad9 interacts with PRMT5, we reasoned that PRMT5 might methylate hRad9. To test this hypothesis, an *in vitro* methylation assay was performed using [^{3}H]-AdoMet as a methyl donor. GST-hRad9 (arrow indicated) was clearly methylated by FL-PRMT5 immunoprecipitated from HEK 293T cells overexpressing FL-PRMT5 (lane 2 in Figure 2C). As a positive control, core histones were also methylated by FL-PRMT5 (22) (lane 1 in Figure 2C).

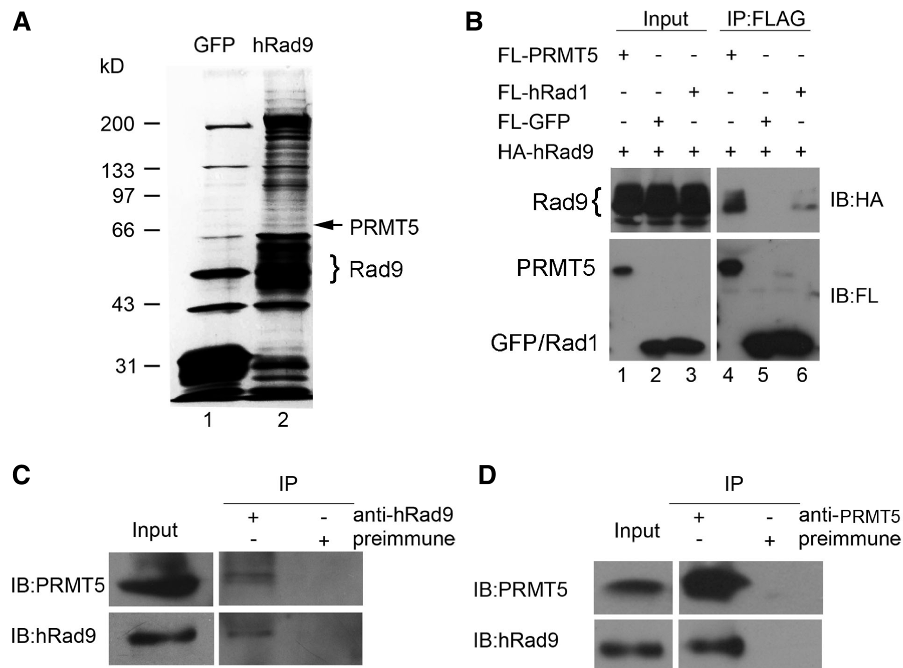

Figure 1. hRad9 associates with PRMT5. (**A**) Silver-staining of proteins associated with hRad9. HEK 293T cells stably expressing FL-hRad9 were immunoprecipitated with anti-FLAG antibody. Samples were resolved by SDS–PAGE and visualized by silver-staining (lane 2), lane 1 is a negative control. The bands of interest were cut out and analyzed by a mass spectrometer. (**B**) Co-immunoprecipitation of FL-PRMT5 and HA-hRad9. pcDNA3-6HA-*hRad9* was transfected into HEK 293T cells along with pFLAG-CMV2-*PRMT5* (lanes 1 and 4), pFLAG-CMV2-*GFP* (lanes 2 and 5: negative control) and pFLAG-CMV2-*hRad1* (lanes 3 and 6: positive control). The lysates were immunoprecipitated with anti-FLAG antibody, and blotting membrane was probed with antibodies against HA (upper) and against FLAG (lower). In the cell lysates over-expressing hRad9 labeled with antibody against tags (FLAG or HA), hRad9 appears as multiple protein bands, which are phosphoforms of hRad9; while the endogenous hRad9 appears only one band. In this figure and next figures, the over-expressed hRad9 (multiple bands) was marked with '}'. (**C**) Co-immunoprecipitation of endogenous hRad9 and PRMT5. In the upper panel, endogenous PRMT5 was immunoprecipitated with anti-hRad9 polyclonal antibodies from HeLa cell lysate (lane 2), but not with pre-immune antiserum (lane 3). (**D**) Co-immunoprecipitation of endogenous hRad9 and PRMT5, immunoprecipitated with anti-PRMT5 polyclonal antibody.

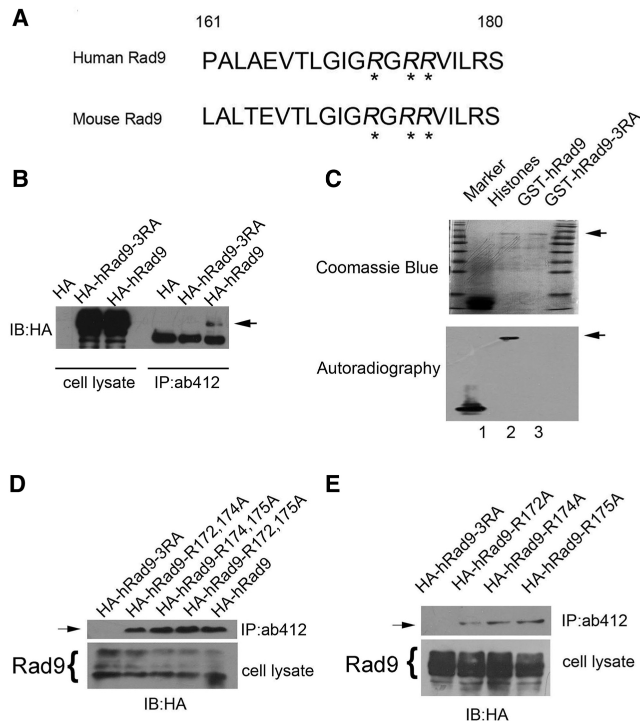

Figure 2. hRad9 is methylated *in vivo* and *in vitro* by PRMT5. (**A**) Potential arginine methylation sites of hRad9 protein. The arginine-rich motifs in the hRad9 and mRad9 proteins are indicated in italic type and stars. (**B**) hRad9 is arginine methylated *in vivo*. Plasmids of pcDNA3-6HA harboring wild-type hRad9 (lanes 3 and 6), hRad9-3RA (lanes 2 and 5) or empty vector (lanes 1 and 4) were transfected into HEK 293T cells. Ten percent of the lysate was used for the control (left) and the remaining 90% (right) was immunoprecipitated with anti-mono/dimethylarginine antibodies (ab412), the methylated hRad9 band is indicated by arrow. (**C**) hRad9 is methylated by PRMT5 *in vitro*. The protein of FL-PRMT5 that was immunoprecipitated from HEK 293T cells overexpressing FL-PRMT5 was incubated with commercial core histones (lane 1), recombinant GST-hRad9 (lane 2) or GST-hRad9-3RA (lane 3) in the presence of [^{3}H]-AdoMet as the methyl donor. Proteins were visualized by Coomassie staining (upper) and ^{3}H-labeled proteins were visualized by fluorography (lower), the GST-hRad9 and GST-hRad9-3RA protein bands are indicated by arrows. (**D**) Single mutations of the three arginines to alanines in the arginine-rich motif of hRad9 influence the hRad9 methylation only slightly. The Arg172, Arg174 and Arg175 residues were mutated into alanine separately. Each mutated hRad9 was cloned into pcDNA3-6HA plasmid and transfected into HEK 293T cells. Cell lysates were immunoprecipitated with anti-mono/dimethylarginine antibodies (ab412) and analyzed by western blotting with anti-HA antibody. The methylated proteins are indicated by arrows. (**E**) Double mutations of Arg172, Arg174 and Arg175 to alanines do not eliminate the methylation of hRad9. The procedure was the same as that in Figure 2D.

To determine whether the arginines in the arginine-rich motif were indeed the target for methylation by PRMT5, we used a GST-hRad9 mutant (GST-hRad9-3RA) as a substrate in which all Arg-172,174,175 were mutated to Ala. As expected, this mutation resulted in the complete loss of the methylation of the fusion protein by FL-PRMT5 (lane 3 in Figure 2C). Taken together, our results demonstrate that hRad9 is arginine-methylated within the arginine-rich consensus sequence by PRMT5.

Each of the three arginine residues in the arginine-rich stretch is a potential target of methyltransferase enzymes. To evaluate the arginine residues responsible for hRad9 methylation, we generated a series of mutants within the arginine-rich stretch by substituting the corresponding arginines with alanine. The mutated hRad9s were cloned into the pcDNA3-6HA plasmid and tested for their ability to serve as methyltransferase substrates in the *in vivo* assay system. None of the single arginine mutations or double-arginine mutations combinations significantly reduced hRad9 methylation, while the mutation of all three arginines abolished methylation of hRad9 (Figure 2D and E). We conclude that the three arginine residues (Arg172, Arg174 and Arg175) within the arginine-rich region of hRad9 are all methylated *in vivo*.

We have found that hRad9 is methylated *in vivo* and can be methylated by PRMT5 *in vitro*. To determine whether PRMT5 is the physiological enzyme methylating hRad9, we knocked down PRMT5 in HCT116 cells. Transfection of PRMT5 ShRNA reduced its protein level by ~80% (Figure 3A), and this lower level of PRMT5 correlated with a dramatic reduction in the hRad9 methylation level (Figure 3B), suggesting that PRMT5 is the main enzyme for hRad9 methylation in cells.

As hRad9 is critical for DNA damage repair and cell cycle control, we want to know whether DNA damage affects hRad9 methylation. To test this, HeLa cells were mock- or HU-treated for 24 h, then the cells were lysed and immunoprecipitated with ab412, and probed with anti-hRad9 monoclonal antibody. In HeLa cells, endogenously methylated hRad9 was increased after cells were treated with HU (Figure 3C), indicating that methylation of hRad9 is DNA damage dependent.

Methylation of hRad9 regulates cellular sensitivity to DNA damage

It has been reported that phosphorylation of hRad9 at multiple amino acid residues influences cell sensitivity to the replication inhibitor HU, and S/M and G2/M checkpoint controls following genotoxin treatment (23–25). Since hRad9 is methylated on its arginine-rich motif, and methylation of hRad9 is DNA damage-dependent, we asked whether the arginine-rich domain is important for the known functions of hRad9 in DNA damage. We therefore established $mRad9^{-/-}$ ES cell clones stably expressing wild-type hRad9 and hRad9-3RA at levels equivalent to the endogenous mRad9 level in $mRad9^{+/+}$ cells (Supplementary Figure S2). First, we tested the influence of hRad9 methylation on cell survival against HU and γ rays. The $mRad9^{-/-}$ ES cells expressing hRad9-3RA were significantly more sensitive to HU than $mRad9^{+/+}$ cells and the $mRad9^{-/-}$ ES cells ectopically expressing hRad9. The hRad9-3RA-expressing $mRad9^{-/-}$ ES cells were equally sensitive to HU as the $mRad9^{-/-}$ ES cells at a low dose (100 μM), and were slightly more sensitive than the $mRad9^{-/-}$ ES cells at higher doses (250 and 500 μM) (Figure 4A). In contrast, $mRad9^{-/-}$ cells expressing hRad9-3RA exhibited equal sensitivity to γ rays at low doses (≤6 Gy) and only moderately higher sensitivity to 8 Gy compared to $mRad9^{+/+}$ and $mRad9^{-/-}$ cells

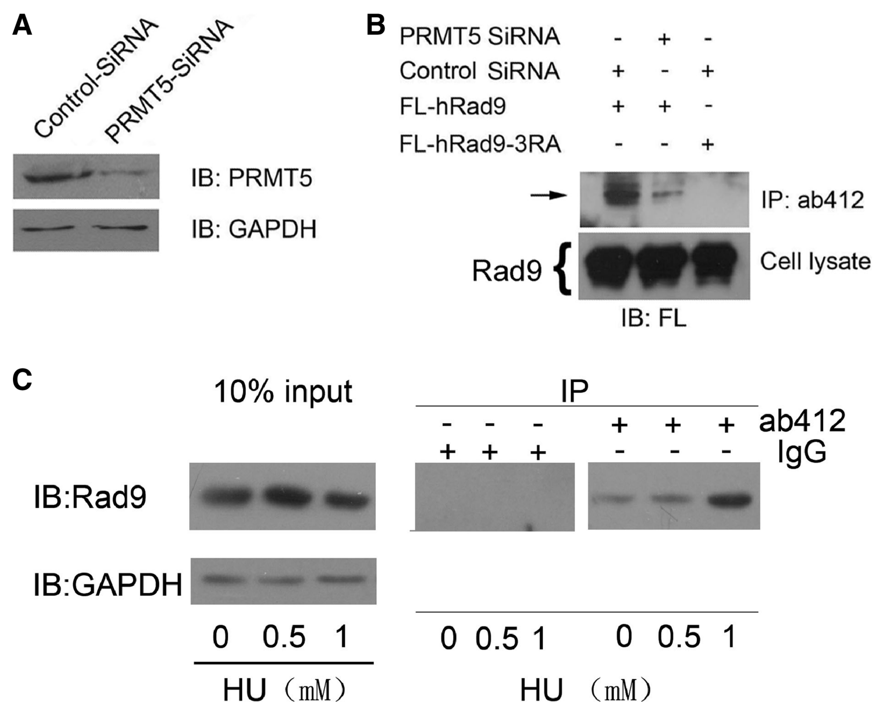

Figure 3. Knock down of PRMT5 influences the arginine methylation of hRad9. (**A**) PRMT5 is knocked down in HCT 116 cells transiently expressing PRMT5 ShRNA. Levels of PRMT5 and GAPDH were assayed in HCT116 cells expressing PRMT5 ShRNA or control ShRNA. (**B**) Knockdown of PRMT5 reduces the arginine methylation of hRad9. HCT116 cells expressing PRMT5 ShRNA or control ShRNA were transfected with pFLAG-CMV2-*hRad9* and arginine methylation of hRad9 was detected in these cells. HCT116 cells expressing FL-*hRad9-3RA* were used as a negative control. (**C**) Methylation of hRad9 is DNA damage dependent. HeLa cells were mock treated or treated with 0.5 mM or 1 mM HU for 24 h. Ten percent cells were lysed as input, the rest cells were lysed and immunoprecipitated with ab412 antibody and then immunoblotted with anti-hRad9 monoclonal antibody.

expressing wild-type hRad9 (Figure 4B). Collectively, our results indicate that hRad9 methylation plays important roles in the cellular response to HU, but a minor role in cellular resistance to ionizing radiation. Next, we performed a colony-formation assay to determine the effect of PRMT5 knock-down on cellular sensitivity to HU. HCT116 cells in which PRMT5 is knocked down are more sensitive than cells expressing control ShRNA (Figure 4C), suggesting that PRMT5 methylation of the three arginines in hRad9 is required for cellular resistance to DNA damage.

Loss of hRad9 methylation leads to S/M and G2/M checkpoint defects

It has been reported that hRad9 is critical in S/M and G2/M checkpoint controls (7,25). Here, we tested whether hRad9 methylation plays roles in these checkpoints. The classical cell cycle checkpoint analysis was introduced to test S/M and G2/M checkpoint controls (7,13). To test the G2/M cell cycle checkpoint, four types of cells (wild-type, $mRad9^{-/-}$, $mRad9^{-/-}$ cells expressing wild-type hRad9 and $mRad9^{-/-}$ cells expressing hRad9-3RA) were mock irradiated or exposed to 6 Gy of γ rays. At various post-irradiation times, the cells were fixed and examined with flow cytometry. Another set of cells was treated with colcemid immediately after radiation exposure and harvested 12 h after irradiation. As shown in Figure 5A and B, at 8 h and 12 h after exposure to 6-Gy γ rays, more cells expressing hRad9-3RA accumulated in the G1 and S phase (arrow) than cells expressing wild-type hRad9, and difference between the two types of cells above in G1 phase is statistically significant (Figure 5B). $mRad9^{-/-}$ and $mRad9^{+/+}$ cells were used as negative and positive controls, respectively. A similar G2/M checkpoint deficient result of *mRad9* knockout was reported previously by Hopkins *et al*. (7,26). As all the cells treated with colcemid were blocked in G2/M at 12 h after radiation exposure, these results suggest that unmethylatable hRad9-3RA leads to G2/M checkpoint deficiency.

To test the effect of the Rad9 methylation on S/M checkpoint, the four types of cells used above were mock-treated or treated with 1 mM HU for 8 h to monitor S/M cell cycle checkpoint status. HU treatment activated S/M checkpoint and arrested $mRad9^{+/+}$ cells in the S phase, while cells with the Rad9 deletion exhibited S/M checkpoint deficiency and were not blocked in the S phase (4.99% cells entered the M phase without completing DNA replication). S/M checkpoint deficiency was also observed in $mRad9^{-/-}$ cells expressing hRad9-3RA; a higher percentage of cells (0.78%) expressing the mutant hRad9 entered the M phase without DNA replication than was the case for normal cells (0.26%) (Figure 5C). A statistical analysis shows that the difference is significant (Figure 5D), demonstrating that methylation of hRad9 plays an important role in S/M checkpoint control. The effect of mutation of the three arginine residues on the S/M checkpoint, although

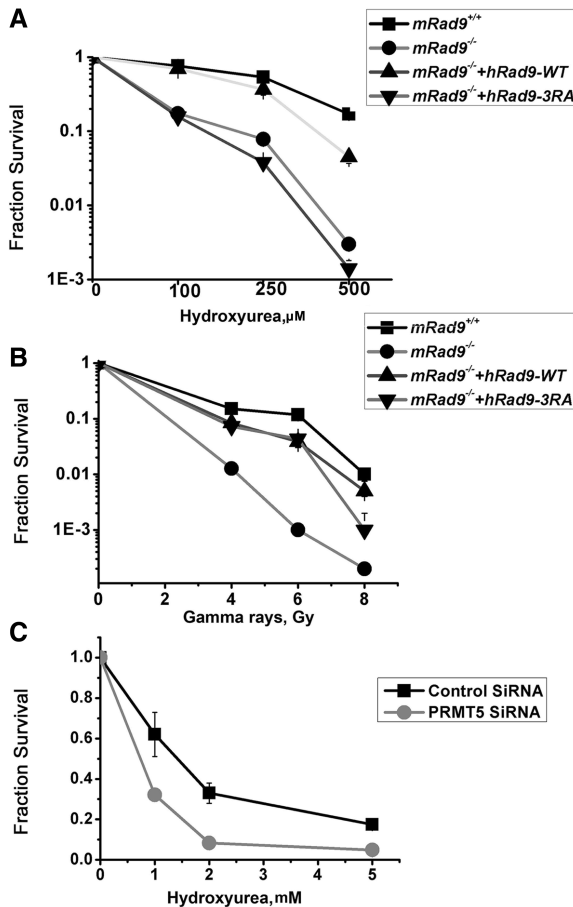

Figure 4. Methylation of hRad9 plays an important role in cell sensitivity to HU, but a minor role in the sensitivity to γ rays. (**A**) Sensitivity of four types of cells (*mRad9*$^{+/+}$ ES cells, *mRad9*$^{-/-}$ ES cells and the two *mRad9*$^{-/-}$ ES cells that stably express hRad9−WT and hRad9-3RA, respectively) to HU. The cells were treated with various concentrations of HU or mock-treated for 24 h. After washing with PBS and addition of fresh medium, cells were allowed to grow for 2 weeks to form colonies. (**B**) Sensitivity of the four types of cells to γ rays. The cells were exposed to various doses of γ rays and were allowed to grow for 2 weeks to form colonies. (**C**) PRMT5 knockdown influences cell sensitivity to HU. Cell sensitivity of HCT116 cells expressing PRMT5 ShRNA or control ShRNA to various doses of HU were measured as in Figure 3A.

statistically significant (Figure 5C and D), is much weaker than that on G2/M checkpoint (Figure 5A and B), suggesting that the mechanisms by which Rad9 regulates the two checkpoints might be different.

hRad9 methylation is required for genotoxin-induced Chk1 activation

It has been reported that both replication inhibitors and γ-rays irradiation trigger ATR-dependent Chk1 phosphorylation on Ser-345, which is essential for Chk1 activation (27,28), and that hRad9 phosphorylation is required for Chk1 activation (25). In order to test whether hRad9 methylation also plays a role in Chk1 activation, we treated *mRad9*$^{+/+}$, *mRad9*$^{-/-}$, *mRad9*$^{-/-}$ expressing wild-type hRad9, and *mRad9*$^{-/-}$ expressing hRad9-3RA with 1 mM HU for 12 h or 10 Gy of γ rays. Both treatments resulted in Chk1 Ser-345 phosphorylation in *mRad9*$^{+/+}$ cells and *mRad9*$^{-/-}$ cells which express wild-type hRad9. However, the phosphorylation induced was eliminated in *mRad9*$^{-/-}$ cells and dramatically reduced in *mRad9*$^{-/-}$ cells expressing hRad9-3RA (Figure 6A and B), indicating that hRad9 methylation on the arginine-rich motif is required for downstream signaling from hRad9 to Chk1 in response to genotoxic stresses. Our results on IR sensitivity (Figure 4B) and Chk1 phosphorylation (Figure 6A and B) indicate that Chk1 activation (a strong effect) by Rad9 is largely unrelated to IR sensitivity (a weak effect), consistent with a previous report (25).

Mutation of Arg-172,174,175 on hRad9 to Lys (hRad9-3RK) shows the similar phenotype as to Ala

As arginine carries positive charge and the charge of Arg 175 is completely conserved (Arg or Lys) across the species from tunicates to humans, suggesting the importance of this arginine on hRad9 function. To avoid changing the positive charge in the study of the methylation role on hRad9 function, we mutated the three Args to Lys and introduced the mutated hRad9 (hRad9-3RK) into *mRad9*$^{-/-}$ cells to study the known function of hRad9 in DNA damage resistance and cell cycle checkpoint activation. As seen in Supplementary Figure S4, the phenotypes (cell cycle checkpoint control, cell sensitivity to DNA damage and Chk1 activation) are similar to those of hRad9-3RA. Therefore, we conclude that methylation, but not the charge, is important for Chk1 activation and resistance to DNA damage.

DISCUSSION

In this study we have documented that Rad9 is methylated on arginines of its RGRR amino acid sequence stretch by PRMT5 (Figure 2), and the methylation is critical for cellular resistance to HU (Figure 4A), and for S/M and G2/M (Figure 5) cell cycle checkpoint activation.

Human hRad9 is highly phosphorylated constitutively and inductively (29). Both Tyr28 and Ser387 are required for checkpoint activation (26). Tyr28 phosphorylation is carried out by c-Abl tyrosine kinase in response to DNA damage, and required for the interaction between hRad9 and Bcl-2 (30). hRad9 Ser272 was reported to be phosphorylated by ATM and it was shown that the overexpression of hRad9 (Ser272Ala) sensitized cells to γ rays slightly. However, a more detailed characterization later did not confirm the sensitization by the mutation. The Ser328 of hRad9 can be phosphorylated by Tousled-like kinase TLKB and the Ser328Ala mutation leads to slight but statistically significant enhancement of sensitivity to γ rays (31). hRad9 being involved in S/M and G2/M checkpoint controls, and three different forms of DNA repair (homologous recombination, base excision and mismatch repairs) as well as apoptosis (1,13,32,33), it is conceivable that the various functions of hRad9 are regulated through phosphorylation on multiple sites by multiple kinases. It is also not surprising that its activities

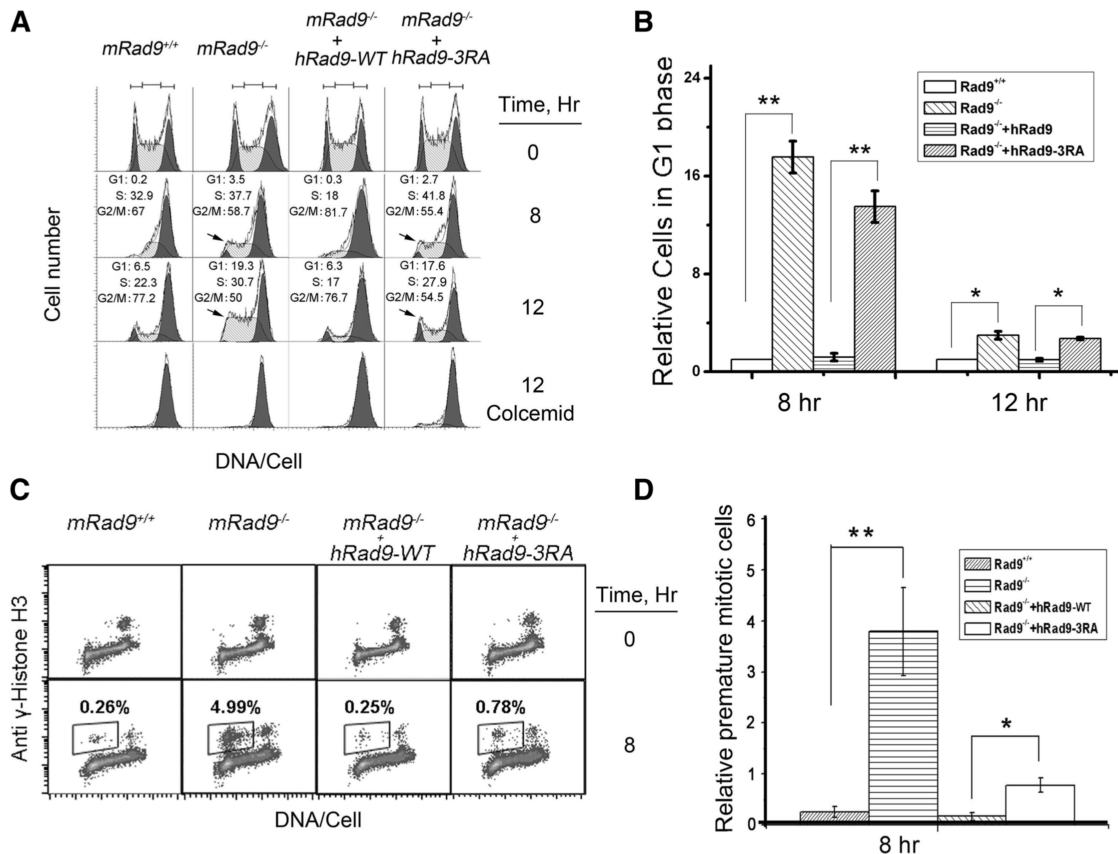

Figure 5. Deficiency of hRad9 methylation leads to S/M and G2/M checkpoint control defects. (**A**) The lack of hRad9 methylation affects ionizing radiation-induced G2 arrest. The four types of mouse ES cells (*mRad9*$^{+/+}$, *mRad9*$^{-/-}$, *mRad9*$^{-/-}$ cells expressing wild-type hRad9 and *mRad9*$^{-/-}$ cells expressing hRad9-3RA, respectively) were mock-treated or irradiated with 6 Gy of γ rays in the absence or presence of colcemid. Regions of the profiles corresponding to G1, S or G2/M are delineated above the first row of graphs, and the ratio of cells in G1, S or G2/M phase were shown. (**B**) Statistics analysis of the relative cell number in G1 phase out of three independent experiments in (A). Double asterisks indicate extremely significant difference ($P < 0.01$) and asterisk indicates significant ($P < 0.05$). (**C**) Lack of hRad9-methylation results in the S/M checkpoint control defect. The four types of cells were treated or mock-treated with 1 mM HU for 8 h. Cells were collected and labeled with the mitotic marker phosphor-histone H3 antibody, stained with propidium iodide, and analyzed by flow cytometry. Staining intensity for PI (*x*-axis) is plotted versus that for phosphor-histone H3 (*y*-axis). The cells in the boxed region are premature mitotic cells. Numbers above the box are the percentage of the cells boxed in the total cells. (**D**) Statistics analysis of relative premature mitotic cells derived from three independent experiments descried in (C). In (B) and (D), Double asterisks indicate extremely significant difference ($P < 0.01$) and asterisk indicates significance ($P < 0.05$).

are modulated by other post-translational modifications such as methylation shown in this study.

Cellular sensitivity to HU is most severely affected by mutating the three arginines in RGRR methylation site into alanines (Figure 4A), but only moderately changed cellular sensitivity to γ rays at high dose (Figure 4B). Similar phenotypes were also observed when all the eight phosphorylation sites of the hRad9 C-terminus are altered (25). The hRad9 C-terminus stretches out of the ring formed by Rad9, Rad1 and Hus1. The RGRR methylation site (amino acids 172–175) is located on the ring. The hRad9 Tyr28 is also situated on the 9-1-1 ring (Figure 6D) and critical for HU induced S/M checkpoint activation. That the three separate sites are all needed in response to HU treatment suggests that different portions of hRad9 coordinate the events critical for managing the disturbance caused by HU. In contrast to the response to HU treatment, the mutations on the C-terminus and RGRR cause very slight changes in cellular resistance to γ rays suggest that the mechanism of the response to γ rays irradiation is not overlapped with those of the response to HU treatment. The γ rays cause DNA double-strand breaks, and two studies demonstrated that Rad9 and its partner Hus1 in the 9-1-1 complex repaired double-strand breaks by homologous recombination (4,34). Further study will be needed to map the region(s) of the Rad9 protein that is responsible for the recombination repair function.

To understand the functional mechanism of Rad9 methylation in cell cycle checkpoint activation and DNA damage response, we have investigated the roles of Rad9 methylation in the formation of the 9-1-1 complex, DNA damage induced Rad9 association onto chromatin, but Rad9 with mutations on all the three arginines in the RGRR sequence showed no effect on the 9-1-1 complex formation (Supplementary Figure S1) and DNA damage induced Rad9 association onto chromatin (Supplementary Figure S3). It is worth noting that we used overexpressed, instead of endogenous, Rad1 and Hus1 to test the effect of the arginines methylation on the 9-1-1 complex formation

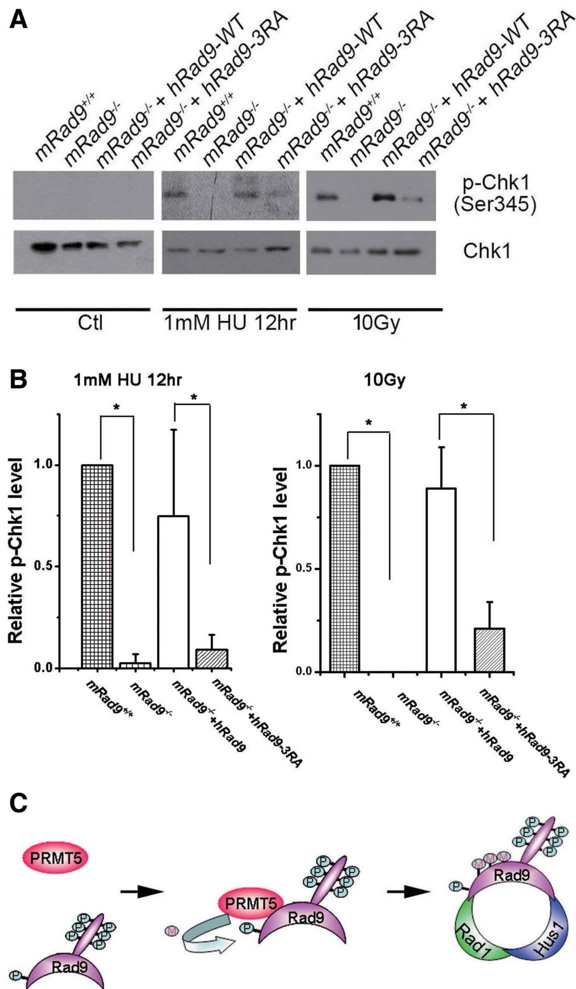

Figure 6. hRad9 methylation is required for genotoxin-induced Chk1 phosphorylation. (**A**) Methylation of hRad9 is important for activation of Chk1. Mouse ES cells were mock-treated or treated with 1 mM HU for 12 h or 10 Gy γ rays. Then, cell lysates were sequentially immunoblotted with antibody against phosphorylated Chk1 (p-Ser-345) and antibody against Chk1. (**B**) Statistic analysis of influence of phosphorylation of Chk1 by hRad9 methylation. Data were derived from three independent experiments as in Figure 6A. Asterisk indicates the statistical significance ($P < 0.01$) between the two group of data. (**C**)This modification is required for hRad9 functions in cell cycle control and DNA repair. hRad9 interacts with and is methylated by PRMT5. The phosphorylated and methylated hRad9 forms a complex with hRad1 and hHus1, and plays a significant role in cell cycle control and DNA damage repair. The location of phosphorylation and methylation sites in hRad9 are shown as the published structure data of hRad9 (40).

because proper anti-Rad1 and anti-Hus1 are not available, thus small effect of the Rad9 methethylation on the 9-1-1 complex formation is still possible.

We noticed that knocking down PRMT5 did sensitize HCT116 to HU but the sensitized extent was less than the mutations of the arginines on Rad9 using mouse ES cells (Figure 4A and C). We also found that knocking down PRMT5 did not impair S/M checkpoint activation in HCT116 cells (data not shown). These differences may reflect the differences of these two cell types. Indeed, untreated HCT116 cells were much more resistant to HU than wild-type mouse ES cells (Figure 4A and C).

A large body of evidence demonstrates that the phosphorylation/dephosphorylation of proteins functioning in cell cycle checkpoint controls and DNA damage repair plays an essential role in orchestrating molecular events required for maintaining genome integrity (35,36). Arginine methylation of histones has been demonstrated to be critical in the regulation of transcription induction/repression and chromatin remodeling, and arginine methylation of other cellular proteins is also emerging to play important roles in other cellular events including DNA damage repair and cell cycle checkpoint controls (10,37). DNA polymerase β methylation by PRMT6 strongly stimulates the activity of this enzyme and is required for efficient DNA base excision repair (22). MRE11 methylation by PRMT1 and p53 methylation by PRMT5 play important roles in G1/S and intra-S phase checkpoint controls, respectively (18,38). In this study we have documented that Rad9 is methylated on the arginines in its RGRR amino acid sequence stretch by PRMT5, and that this methylation is critical for cellular resistance to DNA damage caused by HU, and for S/M and G2/M cell cycle checkpoints activation. Therefore, protein arginine methylation is important for the activation of at least four of the five major cell cycle checkpoints; the role of arginine methylation in spindle checkpoint control remains to be tested. Although the DNA repairs behind the hRad9 methylation-conferred cellular resistance to HU treatment have not been identified, hRad9 has been shown to play important roles in DNA base excision, mismatch and homologous recombination repairs, and these repairs have been shown to be at least partially responsible for resistance to HU treatment (1,39). In summary (Figure 6C), in addition to protein phosphorylation, protein arginine methylation has emerged as another major factor critical for cell cycle checkpoint controls and DNA damage repairs, and thus for maintaining genome integrity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Sancar,A., Lindsey-Boltz,L.A., Unsal-Kacmaz,K. and Linn,S. (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Ann. Rev. Biochem.*, **73**, 39–85.

2. Parrilla-Castellar,E.R., Arlander,S.J. and Karnitz,L. (2004) Dial 9-1-1 for DNA damage: the Rad9-Hus1-Rad1 (9-1-1) clamp complex. *DNA Repair*, **3**, 1009–1014.
3. Lieberman,H.B. (2006) Rad9, an evolutionarily conserved gene with multiple functions for preserving genomic integrity. *J. Cell Biochem.*, **97**, 690–697.
4. Pandita,R.K., Sharma,G.G., Laszlo,A., Hopkins,K.M., Davey,S., Chakhparonian,M., Gupta,A., Wellinger,R.J., Zhang,J., Powell,S.N. *et al.* (2006) Mammalian Rad9 plays a role in telomere stability, S- and G2-phase-specific cell survival, and homologous recombinational repair. *Mol. Cell. Biol.*, **26**, 1850–1864.
5. Guan,X., Madabushi,A., Chang,D.Y., Fitzgerald,M.E., Shi,G., Drohat,A.C. and Lu,A.L. (2007) The human checkpoint sensor Rad9-Rad1-Hus1 interacts with and stimulates DNA repair enzyme TDG glycosylase. *Nucleic Acids Res.*, **35**, 6207–6218.
6. Shi,G., Chang,D.Y., Cheng,C.C., Guan,X., Venclovas,C. and Lu,A.L. (2006) Physical and functional interactions between MutY glycosylase homologue (MYH) and checkpoint proteins Rad9-Rad1-Hus1. *Biochem. J.*, **400**, 53–62.
7. Hopkins,K.M., Auerbach,W., Wang,X.Y., Hande,M.P., Hang,H., Wolgemuth,D.J., Joyner,A.L. and Lieberman,H.B. (2004) Deletion of mouse rad9 causes abnormal cellular responses to DNA damage, genomic instability, and embryonic lethality. *Mol. Cell. Biol.*, **24**, 7235–7248.
8. Chunbo Zhang,C.X.Z., He,Y. and Hang,H. (2008) Phosphorylation sites on Tyr28 and the C-terminus of Rad9 are required for inhibition of premature chromosomal condensation across the entire s phase. *Cell. Physiol. Biochem.*, **22**, 295–306.
9. Gary,J.D. and Clarke,S. (1998) RNA and protein interactions modulated by protein arginine methylation. *Prog. Nucleic Acid Res. Mol. Biol.*, **61**, 65–131.
10. Bedford,M.T. (2007) Arginine methylation at a glance. *J. Cell Sci.*, **120**, 4243–4246.
11. Bedford,M.T. and Clarke,S.G. (2009) Protein arginine methylation in mammals: who, what, and why. *Mol. Cell*, **33**, 1–13.
12. Friesen,W.J., Wyce,A., Paushkin,S., Abel,L., Rappsilber,J., Mann,M. and Dreyfuss,G. (2002) A novel WD repeat protein component of the methylosome binds Sm proteins. *J. Biol. Chem.*, **277**, 8243–8247.
13. He,W., Zhao,Y., Zhang,C., An,L., Hu,Z., Liu,Y., Han,L., Bi,L., Xie,Z., Xue,P. *et al.* (2008) Rad9 plays an important role in DNA mismatch repair through physical interaction with MLH1. *Nucleic Acids Res.*, **36**, 6406–6417.
14. Friesen,W.J., Paushkin,S., Wyce,A., Massenet,S., Pesiridis,G.S., Van Duyne,G., Rappsilber,J., Mann,M. and Dreyfuss,G. (2001) The methylosome, a 20S complex containing JBP1 and pICln, produces dimethylarginine-modified Sm proteins. *Mol. Cell. Biol.*, **21**, 8289–8300.
15. Hang,H., Zhang,Y., Dunbrack,R.L. Jr, Wang,C. and Lieberman,H.B. (2002) Identification and characterization of a paralog of human cell cycle checkpoint gene HUS1. *Genomics*, **79**, 487–492.
16. Hang,H. and Lieberman,H.B. (2000) Physical interactions among human checkpoint control proteins HUS1p, RAD1p, and RAD9p, and implications for the regulation of cell cycle progression. *Genomics*, **65**, 24–33.
17. Pahlich,S., Zakaryan,R.P. and Gehring,H. (2006) Protein arginine methylation: cellular functions and methods of analysis. *Biochim. Biophys. Acta*, **1764**, 1890–1903.
18. Boisvert,F.M., Dery,U., Masson,J.Y. and Richard,S. (2005) Arginine methylation of MRE11 by PRMT1 is required for DNA damage checkpoint control. *Genes Dev.*, **19**, 671–676.
19. Dery,U., Coulombe,Y., Rodrigue,A., Stasiak,A., Richard,S. and Masson,J.Y. (2008) A glycine-arginine domain in control of the human MRE11 DNA repair protein. *Mol. Cell. Biol.*, **28**, 3058–3069.
20. Bedford,M.T. and Richard,S. (2005) Arginine methylation an emerging regulator of protein function. *Mol. Cell*, **18**, 263–272.
21. Najbauer,J., Johnson,B.A., Young,A.L. and Aswad,D.W. (1993) Peptides with sequences similar to glycine, arginine-rich motifs in proteins interacting with RNA are efficiently recognized by methyltransferase(s) modifying arginine in numerous proteins. *J. Biol. Chem.*, **268**, 10501–10509.
22. El-Andaloussi,N., Valovka,T., Toueille,M., Steinacher,R., Focke,F., Gehrig,P., Covic,M., Hassa,P.O., Schar,P., Hubscher,U. *et al.* (2006) Arginine methylation regulates DNA polymerase beta. *Mol. Cell*, **22**, 51–62.
23. St Onge,R.P., Besley,B.D., Pelley,J.L. and Davey,S. (2003) A role for the phosphorylation of hRad9 in checkpoint signaling. *J. Biol. Chem.*, **278**, 26620–26628.
24. Chen,M.J., Lin,Y.T., Lieberman,H.B., Chen,G. and Lee,E.Y. (2001) ATM-dependent phosphorylation of human Rad9 is required for ionizing radiation-induced checkpoint activation. *J. Biol. Chem.*, **276**, 16580–16586.
25. Roos-Mattjus,P., Hopkins,K.M., Oestreich,A.J., Vroman,B.T., Johnson,K.L., Naylor,S., Lieberman,H.B. and Karnitz,L.M. (2003) Phosphorylation of human Rad9 is required for genotoxin-activated checkpoint signaling. *J. Biol. Chem.*, **278**, 24428–24437.
26. Zhang,C., Zhang,C.X., He,Y. and Hang,H. (2008) Phosphorylation sites on Tyr28 and the C-terminus of Rad9 are required for inhibition of premature chromosomal condensation across the entire S phase. *Cell Physiol. Biochem.*, **22**, 295–306.
27. Zhou,B.B. and Elledge,S.J. (2000) The DNA damage response: putting checkpoints in perspective. *Nature*, **408**, 433–439.
28. Liu,Q., Guntuku,S., Cui,X.S., Matsuoka,S., Cortez,D., Tamai,K., Luo,G., Carattini-Rivera,S., DeMayo,F., Bradley,A. *et al.* (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev.*, **14**, 1448–1459.
29. St Onge,R.P., Besley,B.D., Park,M., Casselman,R. and Davey,S. (2001) DNA damage-dependent and -independent phosphorylation of the hRad9 checkpoint protein. *J. Biol. Chem.*, **276**, 41898–41905.
30. Yoshida,K., Komatsu,K., Wang,H.G. and Kufe,D. (2002) c-Abl tyrosine kinase regulates the human Rad9 checkpoint protein in response to DNA damage. *Mol. Cell. Biol.*, **22**, 3292–3300.
31. Sunavala-Dossabhoy,G. and De Benedetti,A. (2009) Tousled homolog, TLK1, binds and phosphorylates Rad9; TLK1 acts as a molecular chaperone in DNA repair. *DNA Repair*, **8**, 87–102.
32. Hartwell,L.H. and Weinert,T.A. (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science*, **246**, 629–634.
33. Paulovich,A.G. and Hartwell,L.H. (1995) A checkpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. *Cell*, **82**, 841–847.
34. Wang,X., Hu,B., Weiss,R.S. and Wang,Y. (2006) The effect of Hus1 on ionizing radiation sensitivity is associated with homologous recombination repair but is independent of nonhomologous end-joining. *Oncogene*, **25**, 1980–1983.
35. Henneke,G., Koundrioukoff,S. and Hubscher,U. (2003) Multiple roles for kinases in DNA replication. *EMBO Rep.*, **4**, 252–256.
36. McGowan,C.H. (2003) Regulation of the eukaryotic cell cycle. *Prog. Cell Cycle Res.*, **5**, 1–4.
37. Pal,S. and Sif,S. (2007) Interplay between chromatin remodelers and protein arginine methyltransferases. *J. Cell Physiol.*, **213**, 306–315.
38. Jansson,M., Durant,S.T., Cho,E.C., Sheahan,S., Edelmann,M., Kessler,B. and La Thangue,N.B. (2008) Arginine methylation regulates the p53 response. *Nat. Cell Biol.*, **10**, 1431–1439.
39. Sarkaria,J.N., Kitange,G.J., James,C.D., Plummer,R., Calvert,H., Weller,M. and Wick,W. (2008) Mechanisms of chemoresistance to alkylating agents in malignant glioma. *Clin. Cancer Res.*, **14**, 2900–2908.
40. Xu,M., Bai,L., Gong,Y., Xie,W., Hang,H. and Jiang,T. (2009) Structure and functional implications of the human rad9-hus1-rad1 cell cycle checkpoint complex. *J. Biol. Chem.*, **284**, 20457–20461.