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Functional Analysis of Histone H2AX for DNA Damage Responses in Cancer Cells

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Histone H2AX, a variant of histone H2A, is thought to be post-translationally controlled in response to DNA insults and to play diverse roles in DNA damage responses (DDR). Serine 139 and lysines 13 and 15 of H2AX were known to be phosphorylated and ubiquitylated upon DNA insults, respectively. However, the exact role of the post-translational modifications (PTMs) in DDR remains elusive. Here, in order to examine the role of these PTMs of H2AX in DDR, we generate $H2AX^{-/-}$ cells expressing various H2AX mutants, by substituting amino acids at each modification site with other amino acids including S139A and K13R/K15R, and analyze them in DDR. Recruitment of Mediator of DNA damage Checkpoint 1 (MDC1) to damaged chromatin is almost completely abolished in cells expressing H2AX S139A. In contrast, recruitment appears normal in cells expressing H2AX K13R/K15R. Recruitment of 53BP1 and Breast Cancer Susceptibility Gene 1 (BRCA1) to damaged chromatin is only partially impaired in cells expressing H2AX S139A. Activation of the G2 checkpoint and the sensitivity to DNA damage are comparable between wild-type cells and $H2AX^{-/-}$ cells expressing all mutant H2AX tested. Taken together, these results suggest that H2AX and its PTMs appear to be less important in DDR than previously expected.

Key words: DNA damage, H2AX, MDC1, 53BP1, BRCA1

INTRODUCTION

Response to endogenous and exogenous DNA damage[1)2)], DNA responses (DDR), are coordinated by the various post-translational modifications (PTMs) including phosphorylation and ubiquitination of involved proteins. Among them, histone H2AX, a variant of H2A[3)~5)], is thought to play crucial roles in DDR primarily through the phosphorylation of its C-terminal serine residue at 139 (S139) by the DDR kinase, ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR), or DNA-dependent protein kinase (DNA-PK). H2AX pS139 (γH2AX) then recruits the various pro-

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teins involved in DNA repair and cell cycle checkpoints to the damaged chromatin[6)~13)]. DNA-damage checkpoint protein 1 (MDC1) directly binds to H2AX pS139 via the BRCA1 C-Terminus (BRCT) repeat domain, leading to efficient accumulation of p53-Binding Protein 1 (53BP1), phosphorylated ATM, and BRCA1 at the damage lesions[14)~17)].

In addition to its phosphorylation, ubiquitination of H2AX has been reported to be involved in the accumulation of several DDR regulators, including 53BP1 and BRCA1, to the damage lesions[18)~21)]. Ring Finger Protein 8 and 168 (RNF8/RNF168)-dependent ubiquitination of H2A/H2AX at lysine 13 (K13)/lysine 15 (K15) was proposed to recruit 53BP1 to damaged foci through the interaction with a ubiquitination-dependent recruitment motif (UDR) in 53BP1[22)]. More recently, it was also shown that the RNF168 module recognizing RNF8-generated ubiquitination is a high-affinity reader of K63-ubiquitylated histone H1, which is essential for RNF8-dependent recruitment of RNF168 to DNA double strand breaks and ubiquitination of H2A/H2AX at K13/K15[23)]. Although the H2AX-independent recruitment of DDR factors and comprehensive screen for identifying functional residues that affect H2AX-PTMs have previously reported[11)24)25)], little is known about the relative contribution of the H2AX-PTMs to DDR. To address this important issue, we generated $H2AX^{-/-}$ cells expressing various mutant H2AX lacking DNA damage-induced PTMs and tested their DDR abilities.

MATERIALS AND METHODS

Plasmids

To construct the lentivirus constructs, the *Nhe*I/*Xho*I fragment of the PCR product containing cDNA for human H2AX-WT, K13R, K15R, K13R/K15R, or S139A fused to a 3xFLAG epitope at the N-terminal region, was inserted into a CSII-CMV-MCS-IRES2-Bsd vector (a gift of Hiroyuki Miyoshi, RIKEN) digested with *Dra*I (blunt filled)/*Not*I. Fragments amplified by PCR and the mutated site in these expression plasmids were sequenced to verify their DNA sequence fidelity.

Cell culture

The human cervical epithelial HeLa and the large-T antigen expressing human embryonic kidney HEK-293T cells were purchased from American Type Culture Collection (ATCC), and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37℃ under 5% CO_2. $H2AX^{-/-}$ HeLa cells were previously established using CRISPR/Cas9-mediated genome editing[26)27)]. The mutation of H2AX gene was confirmed by PCR analysis using a whole genome from $H2AX^{-/-}$ HeLa cells and specific primers for a CRISPR-targeting region in PCR products. A frameshift deletion was detected, but wild-type sequence was not in PCR products by direct sequence. $H2AX^{+/+}$ and $H2AX^{-/-}$ mouse embryonic fibroblasts (MEFs) were generously provided by Dr. A. Nussenzweig, and cultured in DMEM supplemented with 10% FBS.

Virus generation and infection

Lentiviruses expressing H2AX or its mutants were generated by co-transfection of 293T cells with pCMV-VSV-G-RSV-RevB, pCAG-HIVgp, and CS-II-CMV-IRES-Bsd-H2AX-WT and the respective mutants, using the calcium phosphate co-precipitation method as previously reported[28)]. Cells infected with the indicated viruses were treated with 10 μg/ml of blasticidin (Invitrogen) for 2–3 days.

Antibodies

The following antibodies were used: mouse anti-FLAG (F3165; Sigma), rabbit anti-FLAG (F7425; SIGMA), rabbit anti-Histone H2AX

(ab11175; Abcam), rabbit anti-phospho histone H2AX (Ser139) (GTX61796; Gene Tex), rabbit anti-53BP1 (sc-22760; Santa Cruz), rabbit anti-phospho 53BP1 (S1778) (2675; Cell Signaling), mouse anti-BRCA1 (sc-6954; Santa Cruz), mouse anti-Chk1 (C9358; SIGMA), rabbit anti-phospho Chk1 (Ser345) (#2348; Cell Signaling), rabbit anti-MDC1 (ab11169; Abcam), mouse anti-β actin (ab6276; Abcam), rabbit anti-Histone H3 (ab1791-100; Abcam), and goat anti-mouse or rabbit IgG conjugated with Alexa Fluor 594 or 488 (Life Technology).

Immunoblotting

Cells were directly lysed with Laemmli-buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, and 62.5 mM Tris HCl at pH 6.8). The cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) (Immobilon-P; Millipore) membrane, and then detected by immunoblotting with the indicated antibodies using the enhanced chemiluminescence (ECL) detection system. For chromatin-bound proteins, the chromatin-enriched fraction was prepared as previously described[29]. Briefly, chromatin fractionation was prepared as follows: 5×10^5 cells were suspended in 200 μl of solution A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 0.1% Triton X-100, and 1 mM DTT, protease and phosphatase inhibitors). The cells were incubated on ice for 5 min, and cytoplasmic fractions were harvested by centrifugation at 1,300xg for 4 min. The isolated nuclei were washed in solution A, lysed in 100 μl solution B (3 mM EDTA, 0.2 mM EGTA, and 1 mM DTT, protease and phosphatase inhibitors) and incubated on ice for 10 min. The soluble nuclear fractions were harvested by centrifugation at 1,700xg for 4 min. Insoluble chromatin was then washed in solution B and resuspended in the sample buffer.

Immunofluorescence

The cells grown on a glass slide were fixed with 2% paraformaldehyde for 20 min, incubated with Cytoskeleton (CSK) buffer (100 mM NaCl, 300 mM sucrose, 10 mM Piperazine-1,4-bis(2-ethanesulfonic Acid)(PIPES), 3 mM $MgCl_2$, and 1 mM EGTA; pH 6.8) for 3 min at room temperature and fixed with 2% paraformaldehyde for 15 min again. The fixed cells were treated with 0.5% Triton X-100 in PBS for 10 min and blocked with 5% BSA in PBST for over 20 min at 4℃. The cells were incubated for 1 hr with primary antibodies (1 : 500), incubated with Alexa Fluor-conjugated secondary antibodies (1 : 500) (Life Technologies) for 30 min, stained with Hoechst 33342 (1 : 1000), and observed using a confocal laser scanning microscope (A1Rsi, Nikon) equipped with a Plan Apo VC ×60 objective lens (NA 1.40, Nikon).

FACS analysis

The cells were fixed with 70% ethanol and permeabilized with 0.25% Triton X-100 in PBS for 15 min. The cells were stained with 0.1 mg/ml propidium iodide solution containing RNase for 30 min at 37℃ and observed using a FACSVerse (BD Biosciences).

Colony formation assay

HeLa cells were split into a 6 cm dish at 3.3×10^2 cells, and irradiated at the indicated doses. All cells were cultured for 14 days after IR, washed with PBS, and fixed with methanol/acetic acid (1 : 1) for 15 min. The fixed cells were stained with 0.4% trypan blue in PBS for 15 min. After extensive washing with water, colonies were counted. The experiments were repeated three times.

RESULTS

Reintroduction of H2AX mutants harboring a substitution of amino acids at sites targeted by post-translational modifications in H2AX$^{-/-}$ cells

Although post-translational modifications of H2AX have been proposed to play important roles in DDR, the relative contribution of each modification in DDR has not yet been determined experimentally. To address this important issue, we examined the abilities of mutants harboring an amino acid substitution in each modification site to rescue the impaired DDR observed in H2AX$^{-/-}$ HeLa cells. Loss of H2AX protein in H2AX$^{-/-}$ HeLa cells as well as H2AX$^{-/-}$ MEFs was confirmed by immunoblotting using its specific antibodies[9)26)] (Figure 1A), indicating that both wild-type alleles in H2AX$^{-/-}$ HeLa cells are mutated. RNF-mediated ubiquitination of H2AX at K13/K15 was reported to enforce DDR. ATM-mediated phosphorylation at S139 was also reported to function in the recruitment of proteins involved in DNA damage checkpoints. Therefore, we generated H2AX$^{-/-}$ HeLa cells stably expressing either FLAG-tagged H2AX-WT or K13R, K15R, K13R/K15R, or S139A. Immunocytochemical analysis revealed that γH2AX foci were readily detected in cells expressing wild-type H2AX and its K13R, K15R, K13R/K15R mutants at a similar level (over 60% were positive cells), whereas they were not detected in cells expressing S139A as well as in H2AX$^{-/-}$ cells (Figure 1B and 1C). The levels of wild-type H2AX or its mutants ectopically expressed were comparable to that of endogenous H2AX (Figure 1D). As expected, signals of γH2AX were markedly increased in cells expressing wild-type or K13R, K15R, or K13/K15R mutants 2 hrs after IR treatment (Figure 1D). Taken together, the results suggest that

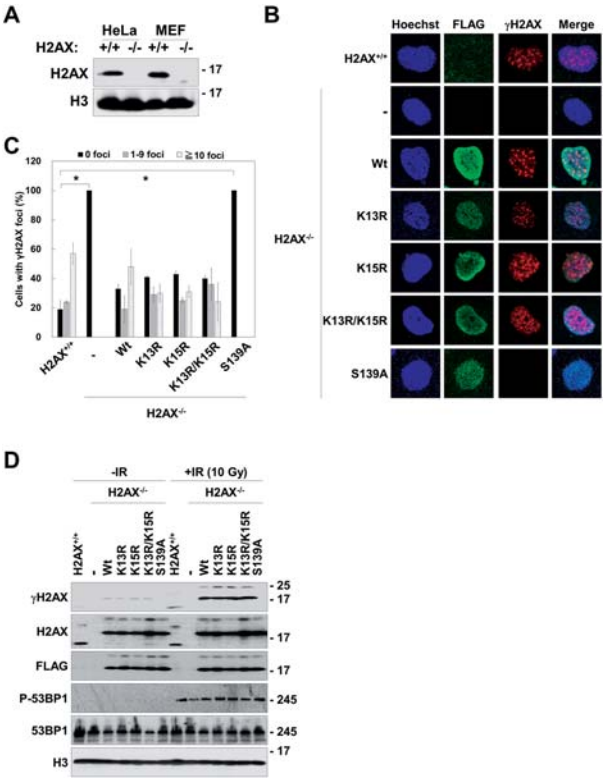


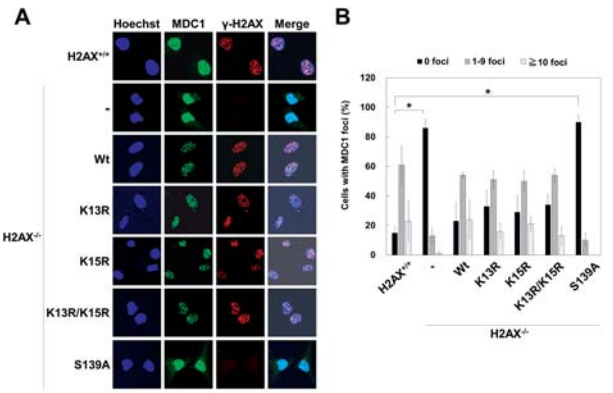
Figure 1. Ubiquitination of H2AX at K13 and/or K15 is dispensable for γH2AX upon IR treatment

(A) Lysates from asynchronous H2AX$^{+/+}$ or H2AX$^{-/-}$ HeLa cells or MEFs were subjected to immunoblotting using the antibodies specific to H2AX and H3. (B) Asynchronous H2AX$^{+/+}$, H2AX$^{-/-}$, H2AX$^{-/-}$ cells expressing 3xFLAG-H2AX-WT, K13R, K15R, K13R/K15R, or S139A mutants were immunostained using anti-FLAG or anti-γH2AX antibodies. DNA was counterstained with Hoechst 33342. (C) The portions of cells positive for γH2AX nuclear foci were determined by counting at least 200 cells per sample. Data are presented as the means±s.d. of at least three independent experiments. P-values were calculated using an ANOVA test. *$p < 0.003$ (D) Lysates from asynchronous H2AX$^{+/+}$, H2AX$^{-/-}$, H2AX$^{-/-}$ cells expressing 3xFLAG-H2AX-WT, K13R, K15R, K13R/K15R, or S139A mutant cells at 2 hrs after treatment with or without IR (10 Gy) were subjected to immunoblotting using the indicated antibodies.

reintroduction of H2AX mutants in H2AX$^{-/-}$ cells provides a useful tool to analyze the roles of the PTMs of H2AX in DDR.

Ubiquitination of H2AX at K13 and/or K15 is dispensable for the recruitment of MDC1 to damaged chromatin

Consistent with the previous reports[14)15)], loss



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	H2AX+/+		H2AX-/-		H2AX-/- Wt		H2AX-/- K13R		
	-	+	-	+	-	+	-	+	:IR
Sub-G1	9.0 ± 0.7	11.7 ± 0.3	7.2 ± 3.7	11.3 ± 6.6	5.8 ± 0.1.	7.7 ± 1.1	10.7 ± 0.1	12.7 ± 4.6	
G1	54.0 ± 14.1	40.9 ± 4.1	59.5 ± 5.3	49.8 ± 4.7	58.8 ± 7.3	53.2 ± 5.7	62.2 ± 7.7	51.5 ± 17.3	
S	11.7 ± 4.6	15.1 ± 2.9	12.4 ± 0.5	11.8 ± 1.6	11.9 ± 1.6	11.8 ± 1.5	9.0 ± 3.5	8.4 ± 2.8	
G2/M	15.3 ± 5.3	20.2 ± 2.2	12.3 ± 0.2	16.4 ± 4.8	14.2 ± 4.8	16.9 ± 0.8	11.2 ± 2.0	13.1 ± 1.4	

	H2AX-/- K15R		H2AX-/- K13R/K15R		H2AX-/- S139A		
	-	+	-	+	-	+	:IR
Sub-G1	10.6 ± 4.7	12.1 ± 6.1	15.4 ± 0.0	16.6 ± 4.5	4.4 ± 3.4	5.4 ± 3.1	
G1	59.4 ± 8.0	46.9 ± 17.4	53.5 ± 5.8	50.5 ± 8.2	58.3 ± 4.1	55.5 ± 4.2	
S	9.5 ± 0.7	9.9 ± 1.3	11.7 ± 1.3	10.5 ± 1.9	10.9 ± 0.9	11.6 ± 1.8	
G2/M	11.3 ± 1.4	12.2 ± 4.3	11.8 ± 2.0	12.9 ± 1.9	15.7 ± 0.8	16.4 ± 0.4	

Figure 2. γH2AX is required for the recruitment of MDC1 to damaged loci in HeLa cells
(A) Asynchronous $H2AX^{+/+}$, $H2AX^{-/-}$, $H2AX^{-/-}$ cells expressing 3xFLAG-H2AX-WT, K13R, K15R, K13R/K15R, or S139A mutants were treated with (+) or without IR (10 Gy). Two hours after irradiation, cells were immunostained using anti-MDC1 antibodies together with anti-γH2AX antibodies. DNA was counterstained with Hoechst 33342. (B) The portions of cells positive for MDC1 nuclear foci were determined by counting at least 200 cells per sample. Data are presented as the means ± s.d. of at least three independent experiments. P-values were calculated using an ANOVA test. *$p < 0.0003$ (C) Asynchronous $H2AX^{+/+}$, $H2AX^{-/-}$, $H2AX^{-/-}$ cells expressing 3xFLAG-H2AX-WT, K13R, K15R, K13R/K15R, or S139A mutants were treated with (+) or without IR (10 Gy). Two hours after irradiation, cells were stained with Hoechst 33342, and their cell cycle distributions were analyzed by FACScan.

of H2AX almost completely abolished the formation of MDC1 nuclear foci (less than 10% were positive cells) that used to be co-localized with γH2AX after IR treatment (Figure 2A). Reintroduction of K13R, K15R, and K13R/K15R mutants as well as wild-type H2AX into $H2AX^{-/-}$ HeLa cells effectively recovered the ability to form MDC1 nuclear foci (70–80% were positive cells), whereas reintroduction of the S139A mutant failed to do so (Figures 2A and 2B). Fluorescence-activated cell sorting (FACS) analysis revealed that the cell cycle profiles of these cells in the presence or absence of IR treatment did not vary although G1 populations were somewhat high in $H2AX^{-/-}$ cells, suggesting that loss of MDC1 foci formation was not due to alterations of their cell cycle progressions (Figure 2C). These results indicate that ubiquitination of H2AX at K13 and/or K15 is dispensable, but γH2AX is essential for recruitment of MDC1 to damaged chromatin.

Recruitment of 53BP1 and BRCA1 to damaged chromatin is partially γH2AX-dependent

MDC1 functions as a platform for the recruitment of 53BP1 and BRCA1 to damaged chromatin[14)15)]. We, therefore, examined the roles of the PTMs of H2AX in the nuclear foci formation of 53BP1 and BRCA1 after IR treatment. 53BP1 foci were still detectable in $H2AX^{-/-}$ cells after IR treatment (about 80% were positive cells), albeit the number of foci in $H2AX^{-/-}$ cells was less than that of control HeLa cells (Figure 3A) as reported previously[11)]. Reintroduction of K13R, K15R, and K13R/K15R mutants, but not the S139A mutant, into $H2AX^{-/-}$ HeLa cells recovered the ability of 53BP1 to form nuclear foci to a similar extent as control HeLa cells (Figures 3A and 3B). In addition, loss of H2AX moderately abolished the formation of BRCA1 foci after IR treatment (about 40% were positive cells) as compared to control HeLa cells (over 70% were positive cells). Reintroduction of K13R, K15R, and K13R/K15R mutants, but not S139A, into $H2AX^{-/-}$ HeLa cells recovered the ability of BRCA1 to form nuclear foci to a similar extent as control HeLa cells (Figures 3C and 3D). Taken together, these results suggest that 53BP1 and BRCA recruitment at damaged chromatin is γH2AX-dependent, but its effect is, at most, minimal. Ubiquitination of H2AX at

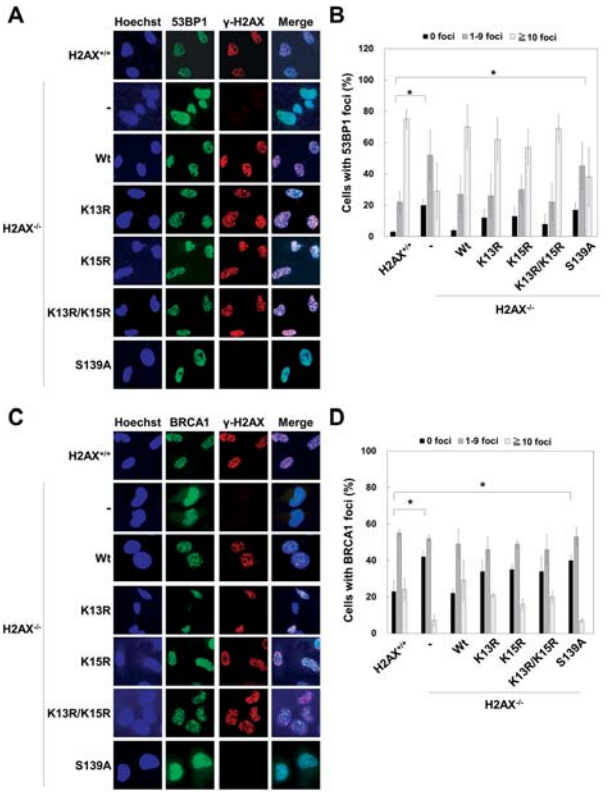

Figure 3. The recruitment of 53BP1 and BRCA1 to damaged loci in HeLa cells is partly dependent on γH2AX

(A and C) Asynchronous H2AX$^{+/+}$, H2AX$^{-/-}$, H2AX$^{-/-}$ cells expressing 3xFLAG-H2AX-WT, K13R, K15R, K13R/K15R, or S139A mutants were treated with (+) or without IR (10 Gy). Two hours after irradiation, cells were immunostained using anti-53BP1 (A) or anti-BRCA1 (C) antibodies together with anti-γH2AX antibodies. DNA was counterstained with Hoechst 33342. (B and D) The portions of cells positive for nuclear 53BP1 foci (B) or BRCA1 foci (D) were determined by counting at least 200 cells per sample. Data are presented as the means±s.d. of at least three independent experiments. P-values were calculated using ANOVA tests. *$p < 0.03$

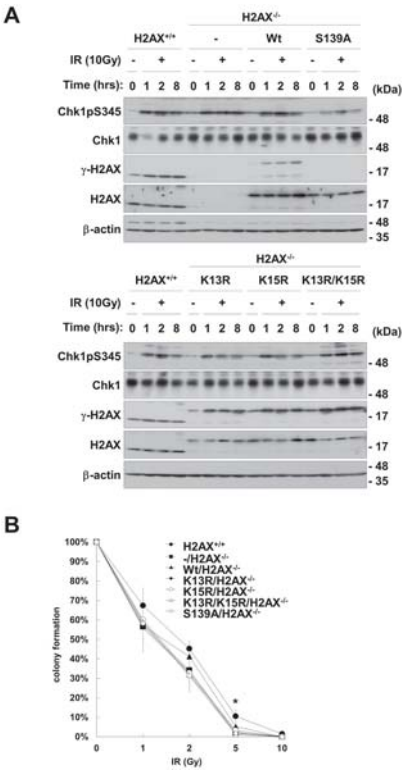

Figure 4. H2AX is dispensable for activation of the DNA damage checkpoint and is not involved in the determination of radio-sensitivity in HeLa cells

(A) Lysates from asynchronous H2AX$^{+/+}$, H2AX$^{-/-}$, H2AX$^{-/-}$ cells expressing 3xFLAG-H2AX-WT, K13R, K15R, K13R/K15R, or S139A mutants treated with (+) or without IR (10 Gy) at the indicated times were subjected to immunoblotting using the indicated antibodies. (B) A quantitative colony formation assay was performed using asynchronous H2AX$^{+/+}$, H2AX$^{-/-}$, H2AX$^{-/-}$ cells expressing 3xFLAG-H2AX-WT, K13R, K15R, K13R/K15R, or S139A mutants treated with IR at the indicated dose. Data are presented as the means±s.d. of at least three independent experiments. P-values were calculated using an ANOVA test. *$p < 0.05$

K13 and/or K15 did not appear to be involved in these recruitments.

H2AX is dispensable for the DNA damage G2 checkpoint and is not involved in radio-sensitivity

Given that γH2AX is essential for the recruitment of MDC1 to damaged chromatin, which mediates the DNA damage G2 checkpoint[14], we thus examined whether the PTMs of H2AX regulate G2 checkpoint activation after IR treatment. Activation of the G2 checkpoint is evaluated by phosphorylation of Checkpoint kinase 1 (Chk1) at S345[30]. Loss of H2AX as well as reintroduction of all H2AX mutants tested into H2AX$^{-/-}$ HeLa cells did not affect the level of Chk1 and its phosphorylation after IR treatment as compared to control HeLa cells (Figure 4A), indicating that H2AX is dispensable for Chk1-dependent G2 checkpoint activation.

H2AX$^{-/-}$ as well as MDC1$^{-/-}$ mice are highly sensitive to ionizing radiation in DSB repair[8)9)14)].

Therefore, a quantitative colony formation assay was used to determine the sensitivity of cells expressing H2AX mutants to DNA damage by IR. Loss of H2AX and reintroduction of all H2AX mutants tested into $H2AX^{-/-}$ HeLa cells did not affect the sensitivities of cells to IR as compared to control HeLa cells, except for reduced sensitivities at 5 Gy of IR (Figure 4B). These results suggest that H2AX and its PTMs are not involved in G2 checkpoint activation and radio-sensitivity.

DISCUSSION

Our current results have shown that H2AX and its PTMs is dispensable for the recruitment of 53BP1 and BRCA1 to DNA damage sites, both of which are critical events functioning downstream of ATM. In contrast, previous studies have shown that many events functioning downstream of ATM, including G2 checkpoint activation and recruitment of DNA damage repair machineries, are defective in mice lacking H2AX or MDC1 as well as in their embryonic fibroblasts[8)9)14)]. On the other hand, the H2AX independent recruitment of DDR factors during the initial phase as well as homologous recombination-mediated repair factors has also been reported[11)24)]. Thus, contribution of H2AX as well as MDC1 to the regulation of DDR pathways appears to vary depending on the experimental contexts. Given that our results were obtained using cancer cells, DDR pathways including H2AX and MDC1 in cancer cells and normal cells might be differently regulated. Indeed, the dysregulated expression and activity of DDR regulators have been reported in various cancer cells[31)~36)].

53BP1 was reported to bind to ubiquitinated H2A/H2AX at K13/K15 through its UDR motif, promoting its recruitment to damaged lesions[22)]. However, our results indicate that ubiquitination of H2AX at K13/K15 is dispensable for the recruitment of 53BP1 to damaged chromatin although the number of foci in $H2AX^{-/-}$ cells was less than that of control HeLa cells (Figure 3A and [11)]). This apparent dispensability of H2AX in 53BP1 recruitment may be explained by H2A ubiquitination at K13/K15. In addition, 53BP1 is also known to bind to methylated H4 at K20 through a tandem Tudor domain[18)].

Our results clearly demonstrated that MDC1 recruitment to DNA damage sites is severely impaired in $H2AX^{-/-}$ cells expressing H2AX S139A although these cells did not show a defect in G2 checkpoint activation and an increase in sensitivity to DNA damage. Thus, phosphorylation of H2AX at S139 as well as MDC1 recruitment appears not to play an essential role in DDR. In this respect, we have recently demonstrated that Aurora B-mediated phosphorylation of H2AX at S121 on centromeres during mitosis is essential for proper chromosome segregation, promoting Aurora B autophosphorylation[26)]. Other reports have also revealed that ATM phosphorylates H2AX at S139 at mitotic kinetochores, which may be required for MDC1 localization and formation with the mitotic checkpoint complex at kinetochores[37)38)]. Taken together, our present results suggest that H2AX phosphorylation and MDC1 recruitment mainly regulate mitotic progression but not DDR.

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Contributions

M.N. and Y.J designed this study. T.U., T.I, Y.J., M.S., K.K., T.S., S.I., T.A., S.K., M.M., T.S. performed experiments and analyzed the results. M.N. and Y.J. wrote the manuscript. All authors reviewed the manuscript.

Additional Information

Competing interests

The authors declare no competing financial interests.

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