

H2AX post-translational modifications in the ionizing radiation response and homologous recombination

Anyong Xie, Shobu Odate,[†] Gurushankar Chandramouly and Ralph Scully*

[†]Department of Medicine; Harvard Medical School and Beth Israel Deaconess Medical Center; Boston, MA USA

[†]Current address: Constellation Pharmaceuticals; Cambridge, MA USA

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Abbreviations: 53BP1, p53-binding protein 1; ATM, ataxia and telangiectasia mutated; BARD1, BRCA1-associated RING domain protein 1; BRCA1, breast cancer predisposition gene 1; BRCT, BRCA1 C-terminal domain; CSR, class switch recombination; DSB, double strand break; ES cells, embryonic stem cells; HA, influenza hemagglutinin peptide; HR, homologous recombination; IP, immunoprecipitate; IR, ionizing radiation; MDC1, mediator of DNA damage checkpoint 1; MRN, Mre11/Rad50/NBS1 complex; MS, mass spectroscopy; NHEJ, non-homologous end joining; RNF8, RING finger protein 8; RNF168, RING finger protein 168; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Ub, ubiquitin; wt, wild type

Histone H2AX phosphorylation on a C-terminal serine residue to form “ γ -H2AX” is a critical early event in the chromatin response to chromosomal DNA double strand breaks in eukaryotes. In mammalian cells, γ -H2AX is formed when H2AX is phosphorylated on serine 139 by ATM or by other DNA damage response kinases. H2AX prevents genomic instability and tumorigenesis, and supports class-switch recombination at immunoglobulin heavy chain loci in mammals. We showed previously that H2AX controls double strand break repair by homologous recombination (HR) between sister chromatids. The HR functions of H2AX are mediated by interaction of γ -H2AX with the chromatin-associated adaptor protein MDC1. H2AX is potentially subject to additional post-translational modifications associated with the DNA damage response and with other chromatin functions. To test this idea, we used mass spectroscopy to identify H2AX residues additional to serine 139 that are post-translationally modified following exposure of cells to ionizing radiation (IR) and identified several new IR-responsive residues of H2AX. We determined the impact of IR-responsive H2AX residues on cellular resistance to IR and on H2AX-dependent HR, and also analyzed the contribution to HR of other known or potential post-translationally modified residues of H2AX. The results suggest that the HR and IR-resistance functions of H2AX are controlled in large part by specific MDC1-interacting residues of H2AX, but that additional H2AX residues modulate these core functions of H2AX.

Introduction

Chromosomal DNA double strand breaks (DSBs) provoke an extensive reaction in neighboring chromatin, characterized by phosphorylation of histone H2AX on serine 139, forming “ γ -H2AX”.^{1,2} The major kinase responsible for H2AX phosphorylation is ATM, but other DNA damage response kinases can substitute for ATM.³⁻⁶ γ -H2AX, in turn, recruits the chromatin-associated adaptor protein, MDC1,^{7,8} which interacts with multiple partners, including the Mre11/Rad50/NBS1 (MRN) complex,⁹ the ATM kinase, and the E3 ubiquitin ligase, RNF8.¹⁰⁻¹³ RNF8 plays a critical role with RNF168,^{14,15} in recruiting additional DNA damage response factors to chromatin marked by γ -H2AX.

RNF8/RNF168-dependent elements of the γ -H2AX response include 53BP1 and the breast/ovarian cancer predisposition gene products, BRCA1 and BARD1.¹⁰⁻¹⁵ Important intermediates in the recruitment of these later factors are the generation of ubiquitinated chromatin species, including ubiquitinated histones H2A and H2AX. Although BRCA1/BARD1 and 53BP1 have known functions in DSB repair—in HR and non-homologous end joining, respectively—each of these exerts its DSB repair functions independently of H2AX.¹⁶⁻¹⁹ Indeed, although RNF8 contributes to HR,^{10,12,13} a major HR function of MDC1 is intact in MDC1 mutants defective for RNF8 binding.¹⁸ Further, a recent report indicates that the RNF8/RNF168 pathway contributes to transcriptional silencing at the DSB.²⁰ For these reasons, the

*Correspondence to: Ralph Scully; Email: rscully@bidmc.harvard.edu

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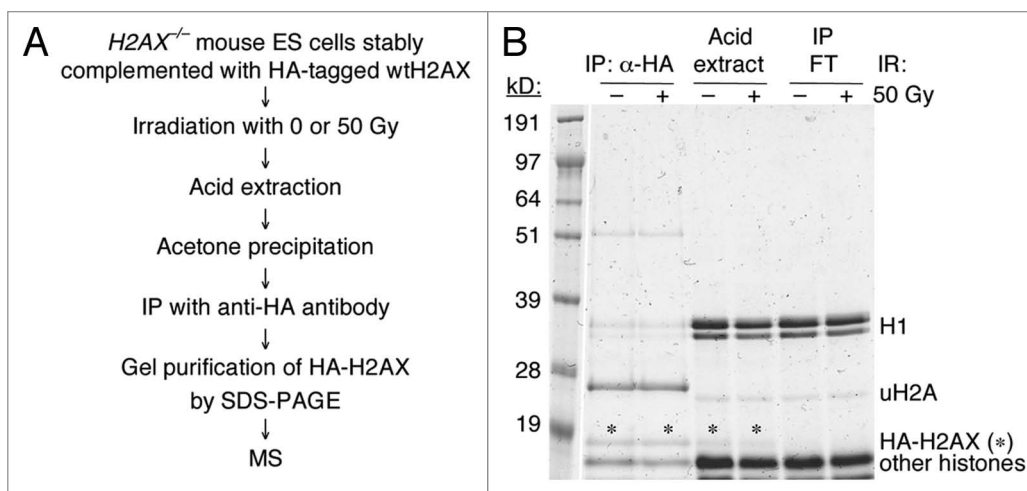


Figure 1. Strategy for identification of IR-induced post-translational modifications of H2AX. (A) Outline of protocol for identifying IR-induced post-translational modifications of H2AX. IP: immunoprecipitation; HA: influenza hemagglutinin peptide; MS: mass spectroscopy. (B) Coomassie blue staining of immunoprecipitates after SDS-PAGE analysis. Several histone species are shown. FT: flow-through. uH2A: monoubiquitinated histone H2A. "*" indicates the HA-H2AX bands, which were cut from the gel and purified for analysis by MS.

biochemical mechanisms of action of γ -H2AX/MDC1 in HR are not yet well understood.

We have taken a genetic approach to the study of γ -H2AX-mediated HR. *H2AX* null mouse embryonic stem (ES) cells reveal a defect in HR and, specifically, in sister chromatid recombination, that is corrected by either stable or transient expression of wild type (wt) *H2AX*, but not by expression of *H2AX* alleles encoding H2AX serine 139 mutants.¹⁹ This points to a specific role for γ -H2AX in HR. H2AX tyrosine 142 to alanine (Y142A) mutants, known to be defective for binding to the MDC1 tandem BRCT repeat, were also found to be defective for HR.¹⁸ Indeed, the ability of different H2AX Y142 mutants to rescue HR in *H2AX* null cells correlated with their ability to bind MDC1.¹⁸

γ -H2AX is formed in situ in chromatin flanking the chromosomal DSB, and this histone is likely subject to the complex signals that are normally integrated within the chromatin fiber in the form of a "histone code".^{21,22} Indeed, ubiquitination of H2A(X) on lysine 118/119, controlled by RNF8 and RNF168, have been implicated in the later stages of the γ -H2AX response, contributing to recruitment of BRCA1/BARD1 (in complex with the Abraxas/Rap80 complex) and 53BP1.^{10,13,23} Further, the *Drosophila melanogaster* homolog of H2AX, H2Av, undergoes dTip60-mediated acetylation on lysine 5 as a potential trigger to replace γ -H2Av with unmodified H2Av following DNA repair.²⁴ This points to an intramolecular integration of function within H2Av that controls the dynamics of " γ -H2Av" turnover. Related phenomena have been described in mammalian cells.²⁵ In addition to these DNA damage-specific alterations, H2AX has almost 100% identity with histone H2A throughout the N-terminal tail and core histone fold domains, diverging significantly only at the C-terminal tail. Therefore, it is likely that H2AX is subject to additional non-DNA damage-related post-translational modifications in concert with other H2A species during other chromatin processes.

These observations raised the possibility that mammalian H2AX, like *D. melanogaster* H2Av, might undergo additional DNA damage-specific post-translational modifications to coordinate its function. Second, it suggested that non-DNA damage related modifications on H2AX might interact functionally with γ -H2AX to modulate DSB repair and HR in particular. To test these ideas, we have used mass spectroscopy to characterize additional IR-induced modifications of H2AX, and have examined the contribution of other post-translationally modified residues of H2AX to *H2AX*-dependent IR resistance and HR.

Results

IR-induced post-translational modifications of H2AX. In order to identify additional IR-induced post-translational modifications of histone H2AX, we stably expressed HA-tagged wtH2AX in *H2AX*^{-/-} mouse embryonic stem (ES) cells, treated these cells with either 0 or 50 Gy of IR, extracted histones with acid, precipitated them with acetone and immunoprecipitated HA-tagged H2AX from the histone extract (Fig. 1A). After SDS PAGE (Fig. 1B), we used mass spectroscopy to sequence peptides from the anti-HA immunoprecipitates. This revealed several new IR-induced modifications of purified histone H2AX in addition to S139 phosphorylation, as well as certain constitutive post-translational modifications of H2AX. The IR-induced changes included acetylation of K5 and K36 and phosphorylation of T101 (Table 1). In addition, T120 and S121 were phosphorylated constitutively (Table 1). We did not detect constitutive or IR-induced phosphorylation of Y142, a modification recently identified and associated with apoptosis.^{26,27} Ubiquitination of K119 of histone H2A and H2AX is well established,^{23,28} and was again noted (Fig. 1B).

Based on previous studies of yeast and mammalian histone H2A, many other residues of histone H2AX are also potential sites for post-translational modifications (Table 1 and Suppl. Fig. 1), including S1,²⁹ T123 and T136 phosphorylation,³⁰ K9

acetylation,^{31,32} and K74, K75 and R76 methylation.³³ However, we did not detect these modifications on histone H2AX in this experiment.

IR sensitivity of H2AX mutant cells. To test whether the above-noted H2AX modifications affect H2AX DSB repair functions, we generated H2AX mutants carrying mutations at relevant residues and expressed each of them in mouse *H2AX*^{-/-} ES cells. Because *H2AX*^{-/-} cells show increased IR sensitivity,^{34,35} we first analyzed whether expression of these H2AX mutants affects IR sensitivity of mouse *H2AX*^{-/-} ES cells. As expected, complementation with wtH2AX reduced the IR sensitivity of mouse *H2AX*^{-/-} ES cells (Fig. 2A). Like wtH2AX, expression of H2AX mutants K5R, K9R and T136V also reduced IR sensitivity in mouse *H2AX*^{-/-} ES cells (Fig. 2A). This indicates that K5 or K9 acetylation and T136 phosphorylation are dispensable for H2AX-dependent IR resistance, despite the fact that K5 acetylation of H2Av is suggested in the DNA damage response in *D melanogaster*.²⁴ Recent evidence implicated a role for ubiquitination of H2A and H2AX in the DSB response.²³ However, H2AX ubiquitination mutant 5KR (Lysine 118, 119, 127, 133 and 134 to arginine), which cannot be ubiquitinated (Fig. 2B), complemented the IR sensitivity of mouse *H2AX*^{-/-} ES cells as effectively as wtH2AX (Fig. 2A). This argues against a unique role for H2AX ubiquitination in IR resistance. In contrast, as expected, mouse *H2AX*^{-/-} ES cells expressing the S139A and Y142A were as sensitive to IR as those expressing the empty vector control. This is consistent with the known roles of S139 phosphorylation and Y142 in recruiting MDC1 during the DSB response.^{7,8,18} Expression of K36R or T101A did not reverse the IR sensitivity of mouse *H2AX*^{-/-} ES cells, suggesting that K36 and T101 and, by extension, acetylation of K36 and phosphorylation of T101, participate in H2AX-mediated IR resistance. The steady state abundance of each of these *H2AX* mutant gene products was comparable with that of wtH2AX (Fig. 2B).

S139 phosphorylation of H2AX mutants. To determine whether the failure of H2AX mutants of K36, T101 and Y142 to reverse IR sensitivity is due to a failure to support S139 phosphorylation of histone H2AX, we transiently transfected mouse *H2AX*^{-/-} ES cells with *H2AX* mutants encoding K36R, T101A, Y142A, Y142W or Y142F, in parallel with wtH2AX and empty vector control and analyzed IR-induced S139 phosphorylation for each histone H2AX mutant protein (Fig. 3A–C). With the exception of S139 mutation itself, all of these H2AX mutants displayed appropriate S139 phosphorylation following IR treatment (Fig. 3A). S139 phosphorylation of Y142 mutants appeared to be diminished (Fig. 3B and C). However, this likely reflects diminished binding of anti-γ-H2AX antibody to Y142 mutants, as this antibody was developed to target the phosphorylated SQEY peptide and cannot fully recognize the SQE-tail in which Y142 is mutated (unpublished observations). Of note, the ubiquitination levels of these H2AX mutants were not affected (Fig. 3B and C; data not shown), suggesting that these residues do not regulate H2AX ubiquitination.

To determine whether ubiquitination of H2AX regulates S139 phosphorylation or vice versa, we transiently expressed H2AX ubiquitination mutants KK118RR (in which the major

Table 1. Identification of post-translational modifications of HA-H2AX by MS

H2AX residues	Potential modifications	Modifications identified by MS	IR-induced modifications
S1	Phosphorylation	ND	/
K5	Acetylation	Acetylation	YES
K9	Acetylation	ND	/
K36	Acetylation	Acetylation	YES
K74, K75 or R76	Methylation	ND	/
T101	Phosphorylation	Phosphorylation	YES
T120	Phosphorylation	Phosphorylation	NO
S121	Phosphorylation	Phosphorylation	NO
K119	Ubiquitylation	NA	/
S139	Phosphorylation	Phosphorylation	YES
Y142	Phosphorylation	ND	/

Potential modifications are indicated, as are the experimentally observed constitutive and IR-induced modifications. ND, not detected; NA, not applicable.

ubiquitination target sites, K118 and K119 were each mutated to arginine) and 5KR (in which the five above-noted lysine residues were mutated to arginine) as well as S139A mutant, in parallel, in mouse *H2AX*^{-/-} ES cells. We found that KK118RR and 5KR were phosphorylated normally on S139 in response to IR (Fig. 3C). Further, the S139A mutant showed normal levels of H2AX ubiquitination (Fig. 3C). This data suggests that H2AX monoubiquitination and S139 phosphorylation are regulated independently.

We also analyzed monoubiquitination and IR-induced S139 phosphorylation of the following additional H2AX mutants, reflecting sites of known or potential post-translational modification: S1A, K5R, K9R, K74Q, KR75RQ (combined mutations of K75R and R76Q), TS120AV (combined mutations of T120A and S121V) and T136V. These mutants underwent IR-induced S139 phosphorylation and K119 ubiquitination in a manner indistinguishable from wtH2AX (Fig. 3A and B and data not shown), arguing against a role of these residues in regulating these two modifications.

IR-induced focus formation by downstream factors in H2AX mutant cells. H2AX is required for efficient IR-induced focus formation by MDC1 and by other factors such as 53BP1 and BRCA1/BARD1.³⁶ To determine whether K36, T101, Y142 and ubiquitination of H2AX are required for this H2AX function, we stably expressed K36R, T101A, Y142A, Y142W, Y142F and 5KR, in parallel, in mouse *H2AX*^{-/-} ES cells and analyzed IR-induced focus formation of MDC1 and 53BP1. As expected, in cells containing S139A and Y142A or empty vector control, no IR-induced MDC1 and 53BP1 nuclear foci were observed (Fig. 4). This is consistent with the known inability of H2AX S139A or Y142A mutants to interact with MDC1.⁷ Unlike Y142A, Y142W and Y142F were able to restore IR-induced MDC1 and 53BP1 nuclear focus formation to mouse *H2AX*^{-/-} ES cells (Fig. 4). Because neither tryptophan nor phenylalanine residues can be phosphorylated, this indicates that Y142 phosphorylation, which

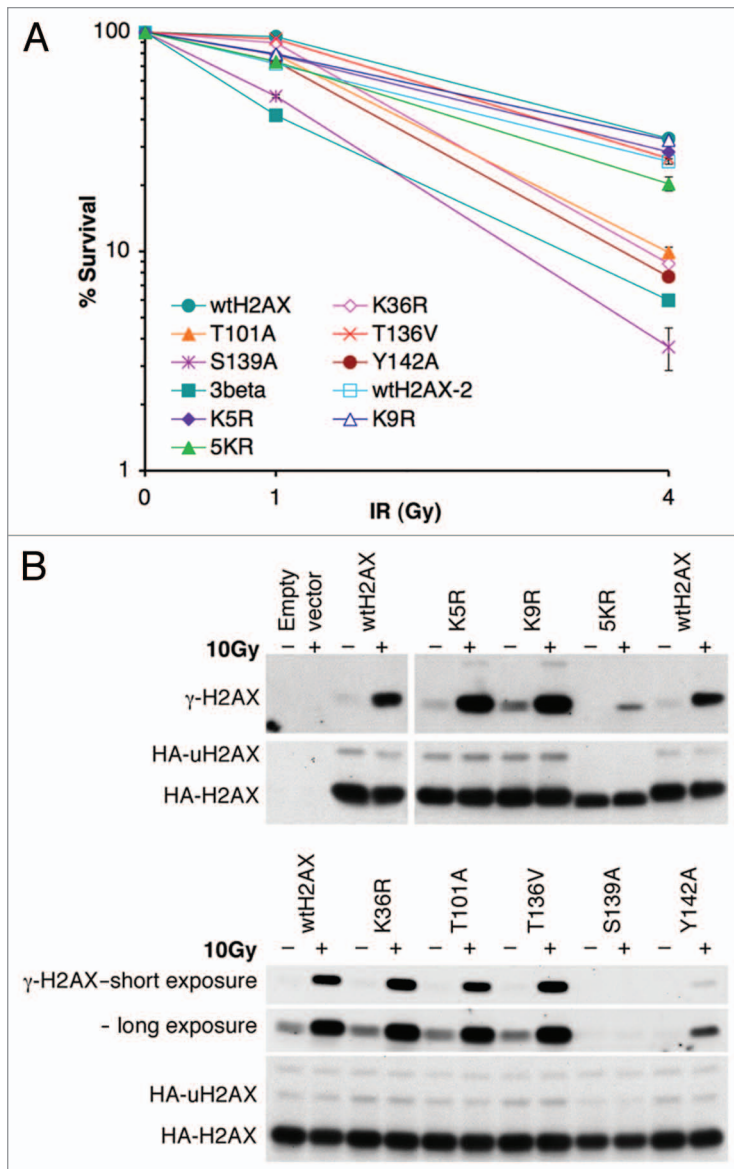


Figure 2. IR-sensitivity of *H2AX*^{-/-} ES cells expressing H2AX mutants. (A) Survival rate of *H2AX*^{-/-} ES cells expressing individual H2AX mutants after exposure to IR. Each survival rate, normalized to transfection efficiency and plating efficiency, represents mean of triplicate samples. Error bars indicate standard error of the mean (s.e.m.). (B) Steady-state level of HA-tagged H2AX mutants expressed in *H2AX*^{-/-} ES cells. Treatment with 10 Gy of IR is indicated. γ -H2AX, HA-H2AX and HA-uH2AX as shown are detected by anti- γ -H2AX and anti-HA antibodies respectively.

has been observed by several groups,^{26,27} is not required for the recruitment of MDC1 to damaged chromatin. Other H2AX mutants K36R, T101A and 5KR effectively rescued IR-induced focus formation by MDC1 and 53BP1 in mouse *H2AX*^{-/-} ES cells (Fig. 4; data not shown), suggesting that H2AX K36 acetylation, T101 phosphorylation and ubiquitination of H2AX are not required to set up focus formation of downstream factors in response to IR.

DSB repair by homologous recombination in H2AX mutant cells. We reported previously that H2AX Y142 as well as S139

are important for its HR function, since H2AX Y142A and S139A are each unable to restore HR to *H2AX*^{-/-} ES cells.^{18,19} In contrast, H2AX Y142F and Y142W were shown previously to rescue HR as efficiently as wtH2AX.¹⁸ We extended this study to determine whether H2AX K36 acetylation, T101 phosphorylation or ubiquitination of H2AX are important for H2AX-mediated HR. To evaluate the role of K36, T101 and ubiquitination of H2AX in HR, we transiently expressed, in parallel, K36R, T101A, KK118RR and 5KR in mouse *H2AX*^{-/-} ES cells containing a single copy of an HR/SCR reporter targeted to the *ROSA26* locus, with parallel expression of wtH2AX (positive control) and S139A (negative control) and analyzed I-SceI induced HR as described in Materials and Methods. As shown previously,¹⁹ HR is less efficient in mouse *H2AX*^{-/-} ES cells (Fig. 5A) and wtH2AX, but not H2AX S139A, rescued HR in these cells (Fig. 5B). Notably, K36R, T101A and ubiquitination mutants KK118RR and 5KR rescued HR as efficiently as wtH2AX (Fig. 5C and D), suggesting that post-translational modifications of these residues are not required for H2AX-mediated HR. Stable expression of K36R and T101A also rescued HR as effectively as wtH2AX in mouse *H2AX*^{-/-} HR/SCR reporter ES cells (Suppl. Fig. 2), further confirming that modifications of these two residues are not required for H2AX-dependent HR. Interestingly, stable expression of the H2AX ubiquitination-defective mutant 5KR slightly stimulated HR when compared to wtH2AX, for reasons that are not yet clear (Suppl. Fig. 2).

We further analyzed the ability of H2AX mutants S1A, K5R, K9R, K74R, KR75RQ, T120A, TS120AV and T136V to rescue HR in mouse *H2AX*^{-/-} HR/SCR reporter ES cells. All of these mutants rescued HR as efficiently as wtH2AX (Fig. 5B, D and E), indicating that these residues or modifications occurring at these sites, are not required for H2AX-mediated HR. The effect of Y142 mutation was as noted previously (Fig. 5D and E).

DSB repair by NHEJ in H2AX mutant cells. We previously developed a rapid reporter for measuring I-SceI-induced NHEJ in mammalian cells.³⁷ This reporter scores positive when a DNA break is induced at two closely positioned, tandem I-SceI sites and repaired by NHEJ. We found that *H2AX* is dispensable for NHEJ of two closely apposed DNA ends.³⁷ However, modifications on different residues of H2AX might, in theory, offset one another in regulating the action of H2AX in NHEJ, thus possibly masking a role for H2AX in NHEJ. Such interactions could be revealed by specific loss of one NHEJ-regulatory modification, while leaving other modifications intact. To test this possibility, we transiently expressed a panel of H2AX mutants, in parallel, in mouse *H2AX*^{-/-} ES cells containing a single copy of the NHEJ reporter and analyzed I-SceI-induced NHEJ as described in Materials and Methods. As before, wtH2AX and H2AX S139A served as controls. Consistent with our previous findings, deletion of *H2AX* had no effect on I-SceI-induced NHEJ (Fig. 6A) and this was not altered by expression of wtH2AX. Similarly,

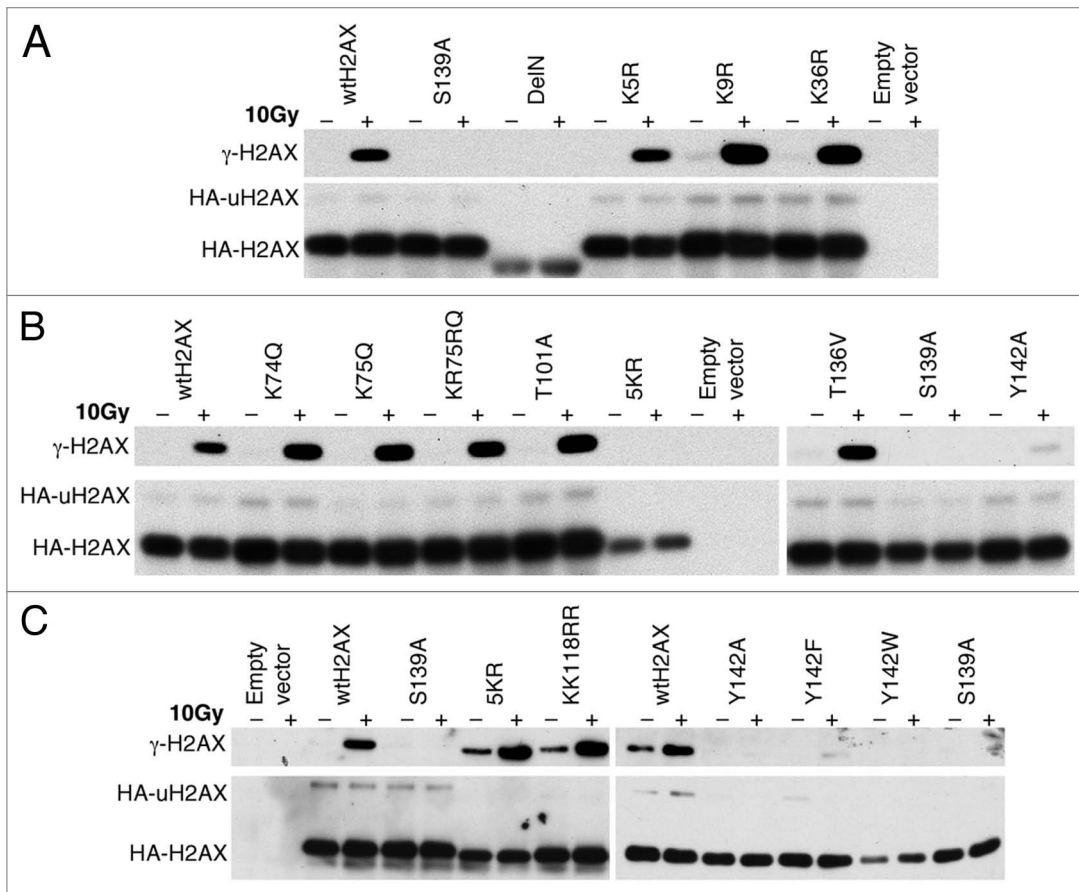


Figure 3. S139 phosphorylation and K119 monoubiquitination of H2AX mutants. *H2AX*^{-/-} ES cells were transiently transfected with mammalian expression vectors encoding H2AX mutants shown and treated with IR as indicated 3 days after transfection. 30 minutes post-IR, histones were extracted for analysis by western blotting. γ -H2AX, HA-H2AX and HA-uH2AX are shown. Wild type H2AX (wtH2AX), S139A and empty vector were used as controls in each experiment. H2AX mutants DelN (deletion of N-terminal 15 residues of H2AX), K5R, K9R and K36R are grouped in (A). K74Q, K75Q, KR75RQ (K75 to R and R76 to Q), T101A, 5KR (K118, 119, 127, 133 and 134 to R) and T136V as well as Y142A are grouped in (B). KK118RR (K118 and 119 to R), Y142F and Y142W as well as 5KR and Y142A are grouped in (C).

expression of almost all H2AX mutants did not change NHEJ quantitatively in mouse *H2AX*^{-/-} ES NHEJ reporter cells (Fig. 6B; data not shown). An exception was H2AX Y142A, which appeared to modestly stimulate NHEJ in *H2AX*^{-/-} ES cells, although the biological significance of this stimulation is not yet clear.

Discussion

The work described here identifies new IR-induced post-translational modifications of H2AX. In addition to serine 139 phosphorylation of H2AX, we identified specific IR-induced acetylation of K5 and K36 and phosphorylation of T101. Interestingly, the K36 mutation, rendering the site unavailable for acetylation, abrogated the ability to reverse IR sensitivity in *H2AX*^{-/-} cells. Similarly, H2AX T101A mutation, resulting in a site that cannot be phosphorylated on this residue, abolished IR resistance. In contrast, the acetylation defective mutant, K5R, performed equivalently to wtH2AX in reversing IR sensitivity. These results suggest that two of the new modifications identified—K36 acetylation and T101 phosphorylation—contribute

to H2AX-mediated IR resistance. Each of these H2AX mutant proteins was incorporated into chromatin and was competent for S139 phosphorylation and for monoubiquitination, performing indistinguishably to wtH2AX in these two regards. Importantly, the ability of each mutant to restore HR to *H2AX*^{-/-} ES HR reporter cells was also equivalent to that of wtH2AX. This suggests that H2AX residues K36 and T101 contribute to IR resistance by a new, as yet undefined mechanism, which is distinct from the established function of H2AX in HR.^{18,19} We considered the possibility that the K36R or T101A mutations might have inactivated a previously uncharacterized NHEJ function of H2AX. However, these mutants did not appear to perturb NHEJ of two closely positioned, easily rejoined DSBs. Conceivably, the defect in these mutants has an impact on an IR-resistance function of H2AX that is unrelated to DSB repair or is related to another DSB repair pathway that we have not yet assayed.

The role of H2AX ubiquitination has received significant attention, in view of the coordination of the γ -H2AX/MDC1 response with the recruitment and activation of the E3 ubiquitin ligases, RNF8 and RNF168.^{10,12-15,20,23,38} Indeed, H2AX

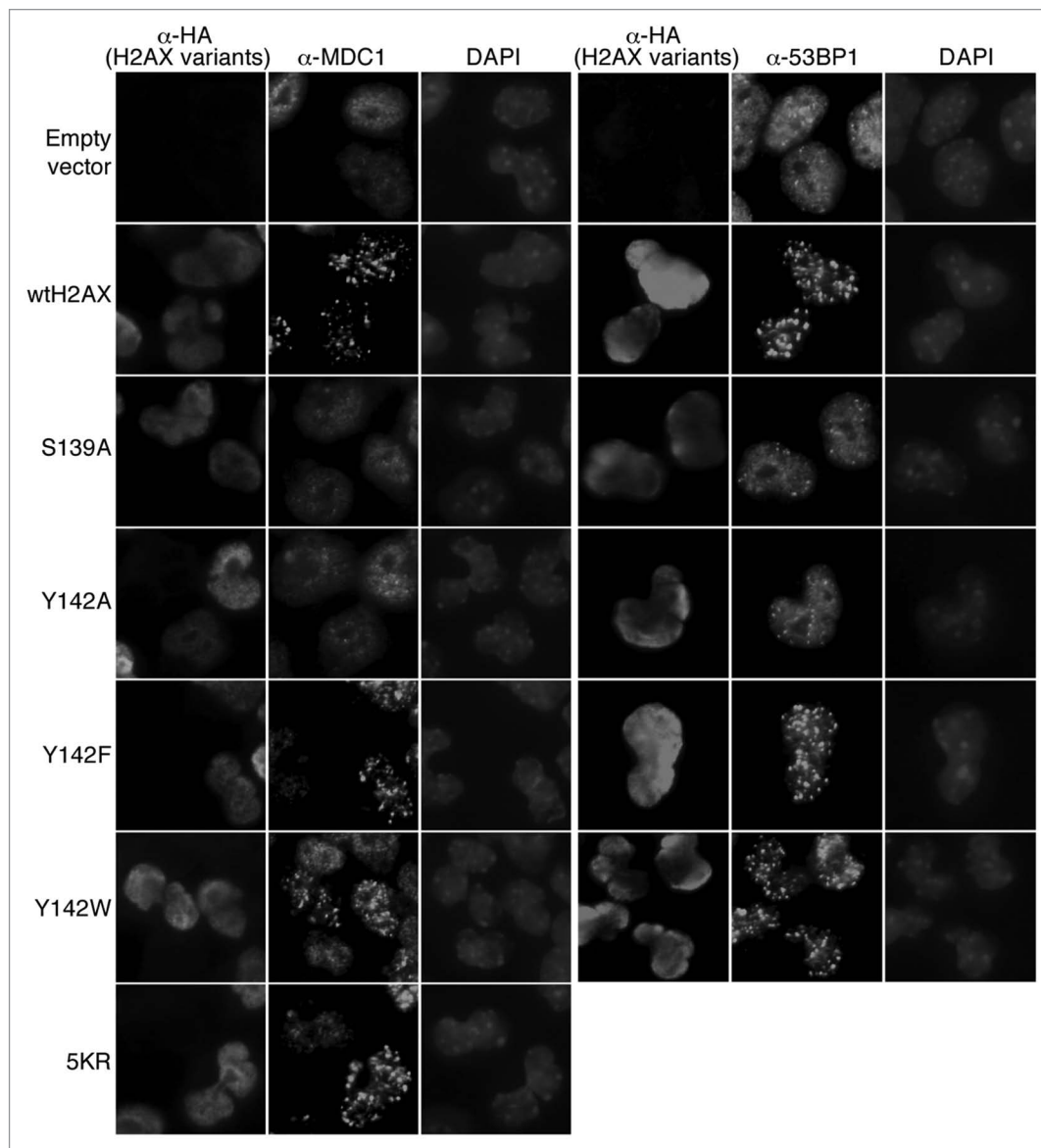


Figure 4. Effect of *H2AX* mutations on IR-induced focus formation of MDC1 and 53BP1. *H2AX*^{-/-} ES cells were transiently transfected, in parallel, with mammalian expression vectors encoding H2AX Y142 mutants or ubiquitination mutant 5KR and then treated with 10 Gy of IR. Cells were immunostained for HA-H2AX species, MDC1 and 53BP1. DAPI staining is also shown.

ubiquitination might serve as part of the substrate on chromatin that is extensively ubiquitinated during the DNA damage response. We found that mutation of the critical H2AX ubiquitin target lysines, K118 and K119, although it effectively abolished ubiquitination of H2AX, failed to impair the IR resistance function or HR function of H2AX. These results suggest that H2AX ubiquitination is not required for either H2AX-mediated IR resistance or H2AX-mediated HR. Notably, chromatin-associated histone H2A species other than H2AX will retain the capacity to undergo ubiquitination on K118/119. Therefore, our data does not address the recently described role for general H2A ubiquitination in the DNA damage response.²³

We have previously analyzed the role of H2AX Y142 in HR and observed a correlation between the ability of specific

H2AX Y142 mutants to support HR and their ability to interact with the MDC1 tandem BRCT repeat.¹⁸ Consistent with this, H2AX Y142F and Y142W mutants were fully functional for IR resistance and HR in the experiments described here. We were unable to efficiently detect γ -H2AX products of these mutants, likely reflecting loss of affinity of the anti- γ -H2AX antibody for the H2AX C-terminus when it lacks Y142. However, the functionality of the Y142F and Y142W mutants is underscored by their ability to recruit MDC1, 53BP1 and BRCA1 to chromatin in the IR response. The solved crystal structure of the γ -H2AX peptide interacting with the MDC1 tandem BRCT repeat reveals Y142 interacting with a hydrophobic pocket on MDC1.^{7,8} Presumably, the replacement of Y142 with either F or W residues results in a peptide in which the replacement

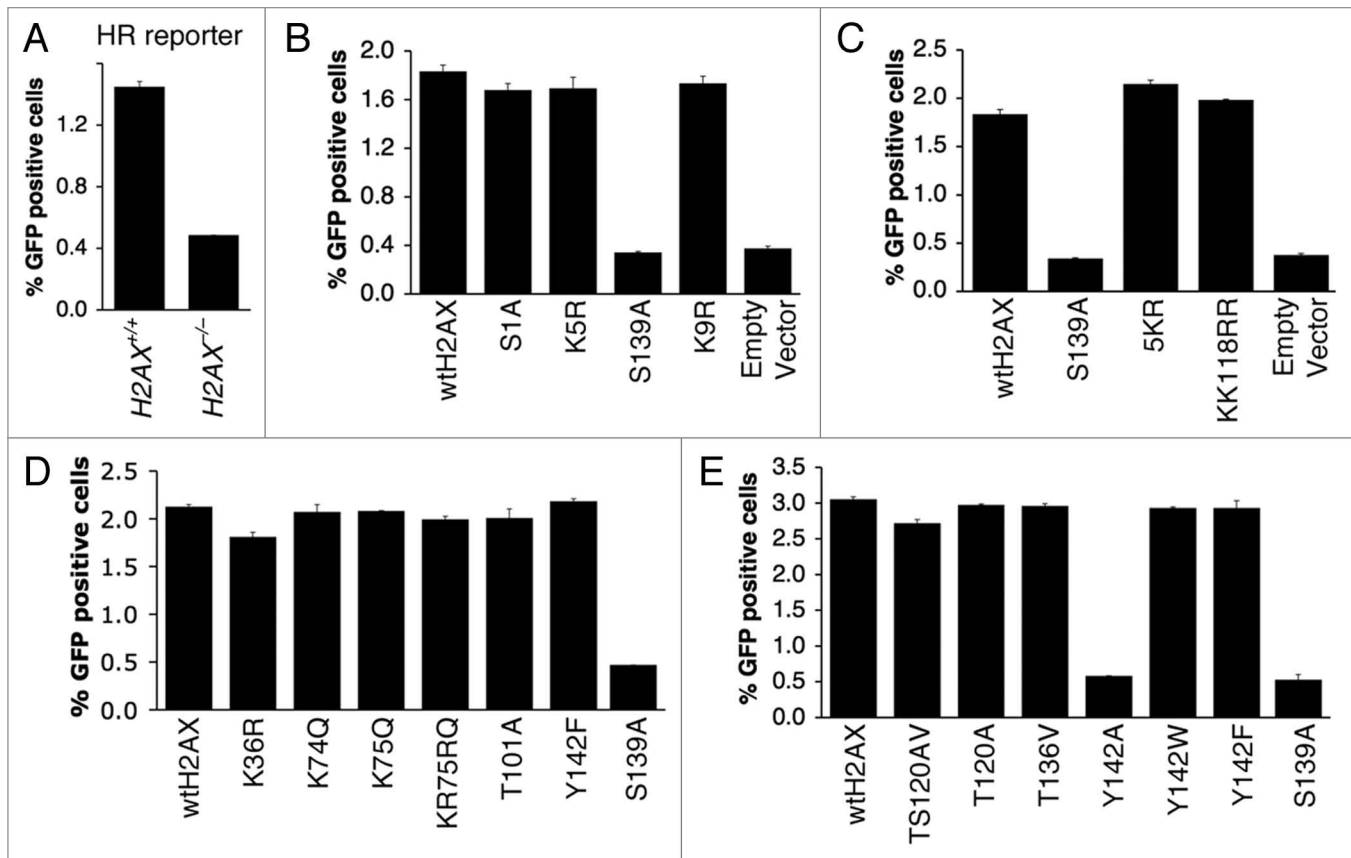
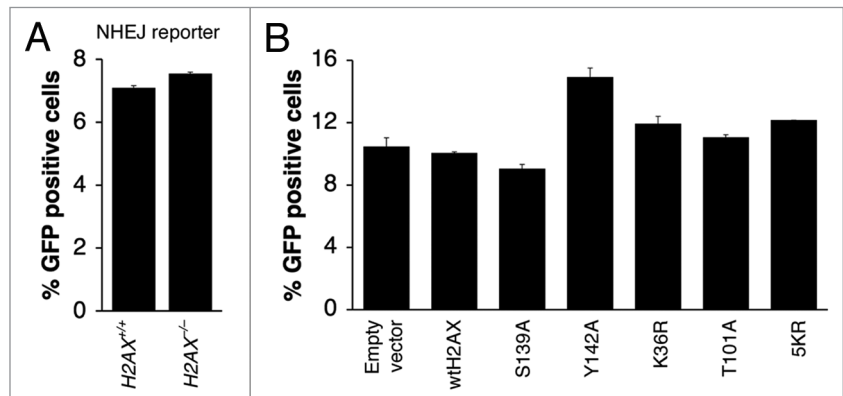


Figure 5. Effect of H2AX mutations on H2AX-dependent HR. Percentage of I-SceI-induced GFP⁺ cells from H2AX^{+/+} and H2AX^{-/-} HR reporter mouse ES cells (A) or H2AX^{-/-} HR reporter mouse ES cells transiently transfected with mammalian expression vectors encoding H2AX mutants as indicated in (B–E). See Materials and Methods for details. Bars represent mean of triplicates. Error bars indicate s.e.m.

Figure 6. Effect of H2AX mutations on I-SceI-induced NHEJ. Percentage of I-SceI-induced GFP⁺ cells from H2AX^{+/+} and H2AX^{-/-} NHEJ reporter mouse ES cells (A) or H2AX^{-/-} HR reporter mouse ES cells transiently transfected with mammalian expression vectors encoding H2AX mutants as indicated in (B). Bars represent mean of triplicates. Error bars indicate s.e.m.



residues (F or W), which are similarly bulky and hydrophobic, interact with the same hydrophobic pocket. Several recent papers report that H2AX can be phosphorylated on Y142.^{26,27} We found that the H2AX Y142F mutant, which cannot be phosphorylated, is able to restore both IR resistance and HR as efficiently as wtH2AX, suggesting that Y142 phosphorylation is not required for these DNA damage response functions of H2AX. Indeed, we failed to detect H2AX peptides containing phosphorylated Y142 in the experiments described here. Conceivably, only a small fraction of H2AX is normally phosphorylated on Y142 at a given instant in cycling cells and is dephosphorylated in response to IR. Alternatively, other technical factors might have made the Y142 phosphorylated peptide difficult to detect by mass spectrometry.

A striking, robust and apparently evolutionarily conserved function of H2AX is its contribution to HR between sister

chromatids.^{18,19,39–41} In vertebrates, this function is mediated by interaction of γ -H2AX with MDC1; however, we noted previously that H2AX/MDC1-mediated HR and BRCA1-mediated HR are genetically separable functions.^{18,19} Further, MDC1 mutants lacking the ability to recruit RNF8, BRCA1, the MRN complex or 53BP1 to chromatin are nonetheless able to restore HR to *MDC1*^{-/-} cells. Therefore, the mechanisms by which MDC1 mediates HR remain to be determined. The genetic analysis of H2AX described here underscores the significance of H2AX-mediated HR for the cellular resistance to IR. However, additional DSB repair functions of H2AX are implied by its contribution to

class switch recombination (CSR) at *IgH* loci, a function that is completed by NHEJ.^{35,42} This suggests that H2AX can mediate both HR and NHEJ, under the appropriate circumstances. However, our attempts to elicit evidence of an NHEJ function of H2AX have thus far yielded no direct evidence in support of this idea.³⁷ In this regard, it may be that H2AX is not required for the rapid religation step of NHEJ—a function that might dominate in the rejoining of two adjacent, easily repaired tandem I-SceI-mediated chromosomal DSBs. In contrast, CSR lesions, which are initiated by interaction of the cytosine deaminase enzyme, AID, with target sites within the *IgH* locus, may be more complex than those generated by I-SceI.^{43,44} A further constraint on repair of CSR-associated DSBs is imposed by the extensive (~100 kb) distance between the DSB ends. Possibly, as the complexity of or distance between the two ends of the break increases, H2AX and its associated proteins might assume a greater functional significance in NHEJ. Indeed, perhaps H2AX mediates a synapsis function that supports HR/SCR under some circumstances and NHEJ under others.

Materials and Methods

Plasmids and antibodies. The hygromycin resistant expression vectors for HA-tagged human H2AX, S139A and Y142 mutants were described previously.^{18,19} The other H2AX mutants were generated from the HA-wtH2AX expression plasmid using the QuickChange II site-directed mutagenesis kit (Stratagene). Commercial rabbit polyclonal antibodies used in this study were anti- γ -H2AX (Cat# DR1017) from AbCam, anti-HA-tag (Cat# sc-805) from Santa Cruz Biotechnology, anti-histone H4 (Cat# 06-598) from Upstate and anti-53BP1 (Cat# NB 100-304) from Novus Biologicals. Mouse anti-mouse MDC1 antibody was a gift from Junjie Chen.⁵

Cell lines, cell culture and transfection. Isogenic *H2AX*^{+/+} and *H2AX*^{-/-} HR and NHEJ reporter ES clones were generated as described previously.^{18,19} Transfection of mouse ES cells was performed using Lipofectamine 2000 (Invitrogen) as described previously.¹⁹ Mouse *H2AX*^{-/-} ES cells stably expressing H2AX were generated as described previously.¹⁹

Western blotting and immunofluorescence staining. Histones were acid-extracted and analyzed by western blotting

as described previously.¹⁹ For immunofluorescence staining, mouse *H2AX*^{-/-} ES cells stably expressing H2AX and its variants were grown on glass coverslips overnight, irradiated, recovered for 30 minutes, fixed in ice-cold methanol or methanol/acetone, stained with antibodies and imaged by using a Zeiss microscope.

Immunoprecipitation. Histones were acid-extracted from ~10⁸ irradiated mouse *H2AX*^{-/-} ES cells stably expressing HA-tagged wtH2AX as described previously,¹⁹ precipitated in acetone at -20°C overnight, air-dried, dissolved in Lysis Buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.1% NP40, 5 mM NaF, 5 mM Na-Orthovanadate, 5 mM Na-Butyrate, 1 mM PMSF and 1.5 ug/mL protease inhibitor cocktail tablets [Roche]), immunoprecipitated using anti-HA Affinity Matrix (Cat# 1815016, Roche) and resolved by SDS-PAGE electrophoresis. After Coomassie Blue staining, protein bands corresponding to HA-tagged wtH2AX protein were cut from the SDS-PAGE gel and prepared and sent for sequencing by mass spectrometry (Taplin Mass Spectrometry Facility, Harvard Medical School).

I-SceI-induced repair assays. Analysis of I-SceI-induced GFP⁺ frequencies in HR or NHEJ reporter mouse ES cells was performed as described previously.^{18,19,37,45} In co-transfection, each expression plasmid and the I-SceI plasmid were used in equal amounts. Transfection efficiency was measured by parallel transfection of wtGFP expression vector, at an amount one tenth that of the I-SceI expression vector. Statistical analysis was performed with Student's two-tailed paired t-test.

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/XieCC9-17-sup.pdf

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