

Phosphorylation of Histone H2B at DNA Double-Strand Breaks

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Abstract

Posttranslational modifications of histone tails regulate numerous biological processes including transcription, DNA repair, and apoptosis. Although recent studies suggest that structural alterations in chromatin are critical for triggering the DNA damage response, very little is known about the nature of DNA damage-induced chromatin perturbations. Here we show that the serine 14 residue in the NH₂-terminal tail of histone H2B is rapidly phosphorylated at sites of DNA double-strand breaks. At late time points after irradiation, the phosphorylated form of H2B, H2B-Ser^{14P}, accumulates into irradiation-induced foci. H2B-Ser^{14P} foci formation is not associated with the apoptotic phosphorylation of H2B but is strictly dependent on the phosphorylated isoform of H2AX. Our results broaden the spectrum of histone modifications that constitute the DNA damage “histone code” and suggest a model for the underlying chromatin structure within damage-induced foci.

Key words: DNA damage • epigenetics • histone H2B • chromatin • histone code

Introduction

It is well established that DNA double-strand breaks (DSBs) induce chromosomal aberrations that may result in mutations and cancer. To preserve chromosomal integrity, mammalian cells have evolved systems to faithfully repair DSBs, as well as mechanisms to rapidly eliminate cells harboring irreparable damage. The ataxia-telangiectasia mutated (ATM) kinase, which is mutated in the cancer predisposition syndrome ataxia telangiectasia, is involved in sensing the presence of DSBs and transmitting signals to the cell cycle checkpoint and apoptotic machineries. A recent study demonstrated that ATM is activated upon genotoxic stress via intermolecular autophosphorylation on serine 1981 (1). The finding that few DSBs can induce widespread ATM phosphorylation suggested that direct binding to DSBs is not essential, but rather that alterations in chromatin structure triggered by the break could activate ATM at a distance (1). Indeed, several chromatin-modifying treatments that relax the topological constraints on DNA induced global ATM phosphorylation (1). This new paradigm suggests that epigenetic changes in chromatin may play a critical role in triggering the DNA damage response.

The basic building block of chromatin is the nucleosome, which consists of DNA wrapped around an octamer containing two copies of each H2A, H2B, H3, and H4 core histone. It has been proposed that specific patterns of posttranslational modifications of histone NH₂- or COOH-terminal regions form a “histone code” (2). According to this hypothesis, combinations of histone modifications (including acetylation, phosphorylation, methylation, ubiquitination, and sumoylation) induce chromatin reorganization either directly by changing contacts with underlying DNA or through modification-induced recruitment of chromatin-associated effector proteins. Distinct patterns of chromatin modifications and associated changes in chromatin folding regulate downstream events including transcription, DNA repair, replication, and apoptosis.

To date, the only identified posttranslational histone modification in mammalian cells that is associated with DNA DSBs is the phosphorylation of H2AX (γ -H2AX) (3). γ -H2AX is induced in response to external damage and forms at sites of physiological DSBs during antigen receptor rearrangements in lymphocytes and meiotic recombination in germ cells (4–6). γ -H2AX spreads over a region

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Abbreviations used in this paper: ATM, ataxia-telangiectasia mutated; DAPI, 4',6'-diamidino-2-phe-nylindole; DSB, double-strand break; IR, ionizing radiation; IRIF, ionizing radiation-induced foci; MEF, mouse embryonic fibroblast; PIKK, phosphatidylinositol 3-OH-kinase-related kinase.

spanning millions of bases surrounding the lesion, becoming microscopically detectable as so-called nuclear foci (7). Chromatin remodeling associated with H2AX phosphorylation is critical for protecting the genome from spontaneous-, irradiation-, V(D)J-, and class-switch recombination-induced DSBs (8–11). According to the histone code hypothesis, there may be additional histone modifications at DSBs that could synergize with γ -H2AX. A recent study documented that the Ser-14 residue in H2B is phosphorylated in response to several apoptotic stimuli, some of which are potent inducers of DNA damage (12). Here we show that H2B-Ser14P is associated with sites of DSBs and forms irradiation-induced foci (IRIF) that are dependent on γ -H2AX. We suggest that H2B-Ser14P and γ -H2AX work in concert to establish a heterochromatin-like state within which both damaged DNA and repair factors are concentrated.

Materials and Methods

Cell Lines and Mice. Generation of H2AX^{+/+} and H2AX^{-/-} mouse embryonic fibroblasts (MEFs) as well as the reconstituted H2AX^{-/-} MEF lines expressing the S136/139A mutant isoform of H2AX have been described (9, 13). Thymocytes were isolated from 4–6-wk-old H2AX^{+/+} and H2AX^{-/-} mice. H2B-GFP-expressing CHO cells were a gift from Tom Misteli (National Cancer Institute). The H2B-GFP construct has been described (14).

Immunostaining and Western Blotting. Cells were seeded onto 18-mm² coverslips and irradiated with 10 Gy or mock treated. At various times after irradiation, cells were fixed using either methanol or paraformaldehyde and processed for immunocytochemistry as described (15). The primary antibodies used for immunofluorescence were mouse anti- γ H2AX (1:1,000) (Upstate Biotechnology) and rabbit anti-H2B-Ser14P (1:600) described by Cheung et al. (12). Alexa 568- and Alexa 488-conjugated secondary antibodies (Molecular Probes) were used at 1:250. DNA was counterstained with 4',6'-diamidino-2-phe-nylindole (DAPI). Phosphorylated H2B Ser14 was detected in acid-extracted protein lysates by Western blot analysis.

Generation of DSBs with Laser Microdissecting Microscope. MEFs were grown on 8-well chamber slides. The DNA intercalating dye Hoechst 33258 was added at 10 μ g/ml and incubated for 20 min at 37°C. The slide was mounted on the microscope stage of an LSM 3100 (Leica), and cells were irradiated with a 337.1-nm laser along a user-defined path to generate localized DSBs.

Online Supplemental Material. Online supplemental material showing the presence of H2B-S14P at DSBs and on the male meiotic sex body is available at <http://www.jem.org/cgi/content/full/jem.20032247/DC1>. In Fig. S1, ATM-1981P staining was combined with TUNEL labeling of laser-induced DSBs. The use of ATM-1981P allows the visualization of breaks in H2AX-deficient cells. Fig. S2 demonstrates that DSB-associated phosphorylation of H2B is independent of both Chk2 and Chk1 signaling pathways. Fig. S3 shows the presence of H2B-S14P in the male meiotic sex body, which is also enriched in γ -H2AX.

Results

H2B-S14P Forms IRIF at Sites of DSBs. To investigate the distribution of H2B-Ser14P in response to DSBs, immortalized MEFs were exposed to different doses of ionizing ra-

diation (IR) and subjected to indirect immunofluorescence analysis. In untreated samples, H2B-Ser14P stained diffusely throughout the nucleoplasm, although small discrete nuclear foci were detectable in some cells (Fig. 1 A). In contrast, large irradiation-induced H2B-Ser14P foci (IRIF) accumulated in MEFs over a period of several hours after treatment. For example, at the 4-h time point \sim 30% of the cells contained H2B-Ser14P IRIF, after which the percentage of cells with foci gradually decreased (Fig. 1 B). H2B-Ser14P IRIF were also detected in other mouse and human lines including NIH3T3, U2OS, IMR90, HeLa, and CHO cells (unpublished data). The number of H2B-Ser14P IRIF per cell correlated with the severity of the damage, suggesting a direct relationship with the number of induced DSBs (Fig. 1 C). Importantly, H2B-Ser14P IRIF showed colocalization with γ -H2AX foci, therefore confirming their presence at DSBs (Fig. 1 D). Unlike γ -H2AX and H2B-Ser14P, similar analyses did not show any significant concentration of phosphorylated H3 and H4 histones at break sites (H3 Ser-10, H4 Ser-1, and H3 Thr-3; unpublished data). In contrast to γ -H2AX, the overall intensity of the H2B-Ser14P signal did not change significantly after irradiation. To determine if H2B-Ser14P IRIF formation is thus a consequence of the redistribution of histone H2B at sites of DSBs, we performed live cell imaging before and after exposure to IR in cells that had been transfected with an H2B-GFP construct. No reorganization of H2B-GFP was noted at time points up to 5 h, nor at 16 h after exposure to IR (Fig. 1 E). Thus, H2B-Ser14P IRIF formation represents a specific posttranslational modification rather than a redistribution of the total H2B pool in response to irradiation. The finding that the kinetics of H2B-Ser14P IRIF formation is significantly delayed with respect to γ -H2AX (Fig. 1 B) may indicate that H2B-Ser14P foci are a late event in response to DSBs or that H2B-Ser14P mark a subset of DSBs (e.g., unrepairable DSBs or abnormal chromosomal rearrangements induced by excessive damage).

Many factors involved in the DNA damage response assemble into IRIF only at late time points after IR (16, 17). However, the time at which foci become cytologically visible does not necessarily reflect the initial appearance of factors at DSBs, since microscopic techniques are not sensitive enough to detect small numbers of molecules. To test whether H2B-Ser14P also forms rapidly at sites of DNA damage, we generated a large amount of DSBs into a defined nuclear volume with the “laser scissors” technique, which allows for the early cytological detection of factor migration to DSBs (13, 18). We found that H2B was phosphorylated together with H2AX as early as 1 min after the laser treatment (Fig. 1 F). In contrast, there was no significant change in the distribution of H2B-GFP upon laser treatment even after a 5-h observation period (Fig. 1 G). Thus, although the assembly of H2B-Ser14P into IRIF is not visible at early time points, phosphorylation of Ser-14 in H2B occurs rapidly at sites of DSBs.

H2B-Ser14P Formation at DSBs Is Not Associated with Apoptosis but Is Dependent on Phosphatidylinositol-3-OH Kinase-related Kinase Activity and γ -H2AX. To determine if the H2B-Ser14P formation at DSBs shares the same signal trans-

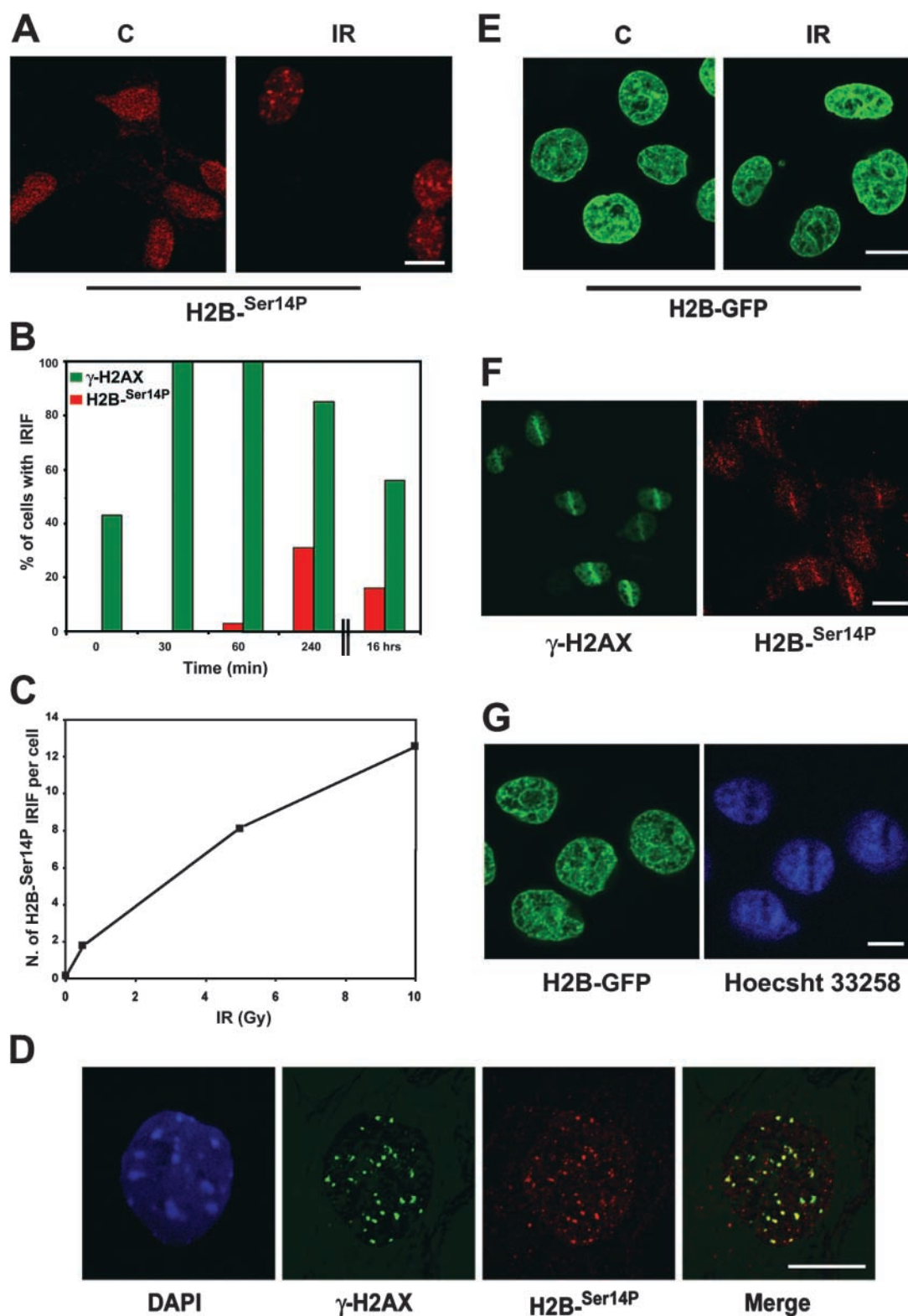


Figure 1. H2B Ser 14 is phosphorylated at DSBs. (A) Immunostaining of H2B-Ser14P (red) in MEFs 4 h after treatment with 10 Gy of IR together with the corresponding nonirradiated control (C). Bar, 10 μ m. (B) Percentage of cells with H2B-Ser14P or γ -H2AX foci at various times after a single exposure to 10 Gy of IR. (C) Average number of H2B-Ser14P IRIF per cell after exposure to IR (0, 0.5, 5, and 10 Gy). (D) Immunostaining of H2B-Ser14P (red) and γ -H2AX (green) in MEFs 4 h after treatment with 10 Gy. DNA was counterstained with DAPI. Bar, 5 μ m. (E) H2B-GFP-expressing CHO cells 5 h after treatment with 10 Gy of IR (IR) and nonirradiated control [C]. No foci-like H2B-GFP distribution was observable in irradiated cells. Bar, 10 μ m. (F) Immunostaining of H2B-Ser14P (red) and γ -H2AX (green) in MEFs that had been exposed to laser damage. Bar, 20 μ m. (G) H2B-GFP expressing CHO cells 5 h after damage by laser scissors. No significant alteration in the distribution of H2B was noted by live cell imaging of these cells during the entire observation period. Note that the path of the laser can be deduced from the bleached nuclear region of the Hoechst 33258 image. Bar, 10 μ m.

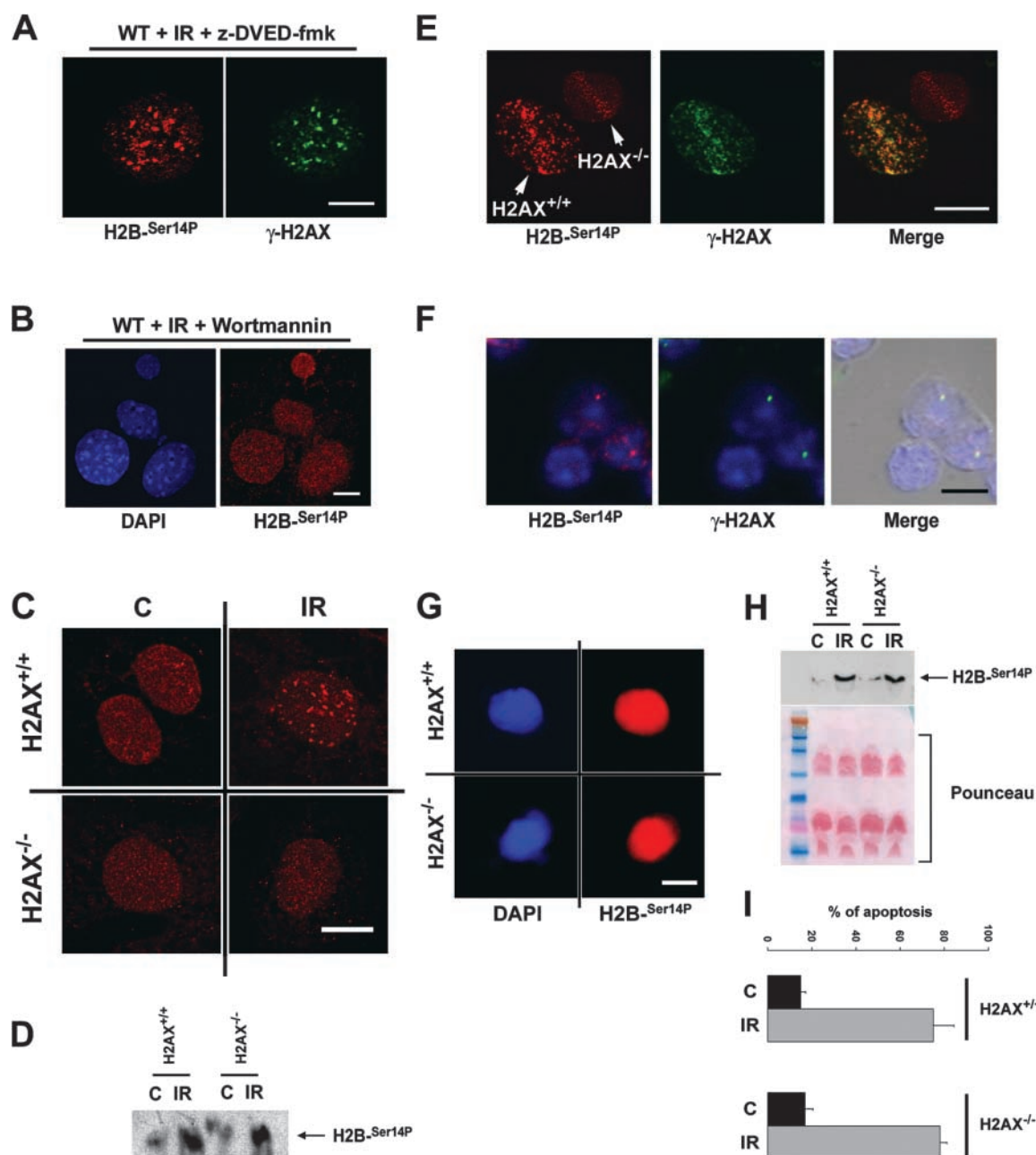


Figure 2. H2B-Ser14P IRIF formation is not associated with the apoptotic phosphorylation of H2B but is dependent on γ -H2AX. (A) Immunostaining of H2B-Ser14P in wild-type MEFs 4 h after treatment with 10 Gy of IR. The caspase inhibitor z-DEVD-fmk (200 μ M) was added to the cells 1 h before the exposure to IR and was maintained in the medium throughout the length of the experiment. Bar, 10 μ m. (B) Immunostaining of H2B-Ser14P in wild-type MEF 4 h after treatment with 10 Gy of IR. The PI3K inhibitor wortmannin (200 μ M) was added to the cells 15 min before irradiation and was maintained in the medium throughout the length of the experiment. Bar, 10 μ m. (C) Immunostaining of H2B-Ser14P (red) in H2AX^{+/+} and H2AX^{-/-} MEFs 4 h after mock treatment (C) or exposure to 10 Gy of IR. Bar, 5 μ m. (D) Western blot analysis of H2B-Ser14P levels in H2AX^{+/+} and H2AX^{-/-} MEF 4 h after exposure to 100 Gy. (E) Immunostaining of H2B-Ser14P (red) and γ -H2AX (green) in a mixed population of H2AX^{+/+} and H2AX^{-/-} MEFs that had been exposed to laser damage. Bar, 5 μ m. (F) Immunostaining of H2B-Ser14P (red) and γ -H2AX (green) in freshly isolated thymocytes. Bar, 5 μ m. (G) Example of the massive H2B-Ser14P (red) staining in H2AX^{+/+} and H2AX^{-/-} apoptotic thymocytes (see H and I). Bar, 5 μ m. (H) Western blot detection of H2B-Ser14P in histone extracts that were prepared by acid extraction from H2AX^{+/+} and H2AX^{-/-} thymocytes 8 h after exposure to 5 Gy of IR. The Ponceau staining of the nitrocellulose membrane is shown as a loading control. (I) Apoptosis in irradiated thymocytes (IR) measured by flow cytometric analysis of the percentage of cells with a sub-G₁ DNA content. Nonirradiated cells (C) that were kept in culture media during the 8-h period were used as a control. When shown (A, B, E, and F), DNA was counterstained with DAPI (blue).

duction pathway as the apoptotic phosphorylation of H2B, we analyzed the formation of H2B-Ser14P IRIF in the presence of apoptosis inhibitors. During apoptosis, the phos-

phorylation of H2B at S-14 is dependent on caspase-3-mediated cleavage of mammalian sterile twenty kinase (Mst1) (12) and is therefore abolished in the presence of the caspase

inhibitor z-DEVD-fmk. However, in MEFs pretreated with z-DEVD-fmk the formation of H2B-Ser14P IRIF remained unaltered (Fig. 2 A). Although irradiation induces foci formation and cell cycle arrest, it does not lead to apoptosis in MEFs (19 and unpublished data), which again unlinks the H2B-Ser14P IRIF from programmed cell death.

The S-14 residue in H2B is not a consensus phosphorylation site for phosphatidylinositol-3-OH kinase related kinases (PIKKs), which target SQ motifs (20). However, PIKK-induced phosphorylation is essential for the accumulation of various factors, including Mre11 complex, Brca1, and γ -H2AX into IRIF (18). To determine whether H2B-Ser14P IRIF was also dependent on PIKKs, we pretreated cells with the drug wortmannin at a concentration known to inhibit these kinases (200 μ M). Treatment with wortmannin completely abolished the formation of H2B-Ser14P IRIF (Fig. 2 B). Thus, H2B-Ser14P IRIF formation is dependent on PIKKs even though these kinases are unlikely to directly phosphorylate Ser-14 in H2B.

H2AX is a direct target of PIKKs (7) and is essential for the formation of IRIF of several DNA damage response factors (9, 18). To determine whether the formation of H2B-Ser14P is dependent on H2AX, we analyzed the formation of foci in H2AX^{+/+} and H2AX^{-/-} MEFs. Although H2AX deficiency did not affect the overall nuclear staining pattern or levels of H2B-Ser14P, IRIF failed to form in the mutant cells (Fig. 2 C). Moreover, H2B-Ser14P IRIF were undetectable in H2AX-deficient cells that had been reconstituted with a phosphorylation mutant isoform of H2AX (13) (unpublished data), demonstrating that the formation of these foci is directly dependent on the formation of γ -H2AX. In contrast, the overall phosphorylation levels of H2B Ser 14 in response to IR were not dependent on H2AX, as determined by Western blotting (Fig. 2 D). Moreover, a normal H2B-Ser14 phosphorylation pattern was observed at early time points when H2AX-deficient cells were exposed to laser treatment (Fig. 2 E and Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20032247/DC1>). Finally, the use of the laser scissors showed that H2B phosphorylation at DNA breaks is independent of both ATM-Chk2 and ATR-Chk1 signaling pathways (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20032247/DC1>). We conclude that although the phosphorylation of H2B at DSBs is H2AX independent, formation of H2B-Ser14P IRIF depends on the phosphorylation of H2AX, which is downstream of phosphatidylinositol 3-kinase-related kinase activity.

H2AX is phosphorylated in chromatin regions surrounding the TCR- α locus in thymocytes actively undergoing V(D)J recombination (4). Moreover, γ -H2AX formation in thymocytes is dependent on RAG-mediated cleavage (4). Consistent with the staining pattern observed in irradiated MEFs (Fig. 1 D), we found that H2B-Ser14P foci colocalized precisely with γ -H2AX in freshly isolated thymocytes (Fig. 2 F), indicating that H2B-Ser14 phosphorylation also occurs during physiologically programmed rearrangements. Unlike MEFs, thymocytes are exquisitely sensitive to irradiation-induced apoptosis. 8 h after treatment with 500 cGy, >70%

of thymocytes were clearly apoptotic as determined by flow cytometry (Fig. 2 I). As previously reported (12), H2B-Ser14P exhibited a global nuclear staining pattern in a fraction of the apoptotic thymocytes (Fig. 2 G). In contrast to IRIF formation, this massive apoptotic phosphorylation of H2B-Ser14P was independent of H2AX, as determined by both Western blotting and immunofluorescence analysis (Fig. 2, G and H). Moreover, the percentage of apoptotic nuclei was identical in H2AX^{+/+} and H2AX^{-/-} thymocytes (Fig. 2 I), indicating that although H2AX is also massively phosphorylated during DNA fragmentation (21), H2AX itself does not play an active role in apoptosis. Together, these results unlink the DSB-associated H2B-Ser14P formation from the apoptotic phosphorylation of H2B.

Discussion

There is increasing evidence that epigenetic regulation of nucleosomes plays a key role in the response to DSBs. Alterations in the configuration of chromatin appear to be necessary for the initial recognition of DSBs (1). Beyond the detection of the lesion, the ability to efficiently repair DSBs may also require chromatin-remodeling activities. For example, BRAF-35, a complex that interacts with the DNA repair factor Brca2 has the capacity to deacetylate histones (22). A newly discovered Brca2-associated protein, EMSY, forms IRIF and interacts with the HP1 β chromoshadow domain, which recognizes methylated lysine residues within histones (23). Moreover, a histone deacetylase HDAC4 also assembles in nuclear foci and is critical for irradiation-induced survival (24). HDAC4, like γ -H2AX, is required for the formation of 53BP1 IRIF (24), suggesting the possibility that chromatin configuration may be intimately linked to the cytological observation of foci. However, although HDACs normally act on acetylated lysines to induce chromatin compaction, it remains unclear which, if any, specific histone residues in the vicinity of DSBs are targeted by Brca2- or HDAC4-containing deacetylases. At present, the only known histone modification that is enriched at DSBs in mammalian cells is the phosphorylation of H2AX. Here we have uncovered an additional site of histone phosphorylation at DSBs that supports the involvement of histone tail modifications in the signaling/repair of DSBs.

The finding that H2B Ser-14 phosphorylation at DSBs is H2AX independent, whereas H2B-Ser14P foci formation is γ -H2AX dependent is paradoxical. One interpretation for the absence of repair foci is that factors are not recruited sites of DNA damage. However, we have found that both events are independent and that despite a normal recruitment of factors to DSBs at early time points, factors may still not form IRIF (13). There are at least two possible explanations for why H2B-Ser14P foci formation is dependent on the phosphorylation marks on H2AX. First, γ -H2AX may be required to retain the H2B S-14 kinase after its initial recruitment to DSBs. This could be mediated via weak interactions between the kinase and the SQ motif in the H2AX tail, thousands of which are modified by phosphorylation.

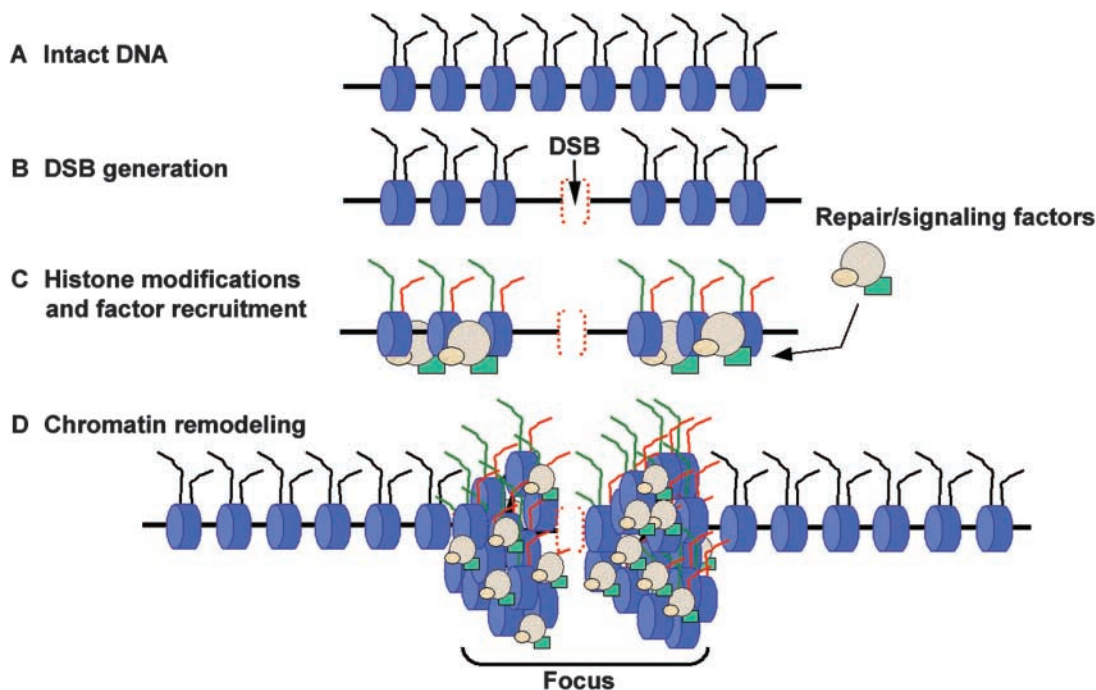


Figure 3. The chromatin compaction model. (A) Intact DNA molecule showing the nucleosomes (blue) with protruding tail motifs (black). (B) Generation of a DSB. (C) Histone tails become specifically modified in the chromatin surrounding the lesion (i.e., γ -H2AX [green], H2B-Ser^{14P} [red]). At the same time, DNA repair/signaling factors accumulate in the region. (D) The DSB-induced pattern of histone modifications promotes the compaction of the chromatin and associated factors cytologically detectable as foci.

However, such direct interactions appear to be unlikely given that H2AX is required for IRIF formation of almost all factors, some of which undoubtedly assemble at DSBs by independent pathways. To account for the universal role of H2AX in IRIF formation, we favor an alternative model that posits that H2AX phosphorylation has a direct effect on the chromatin structure surrounding a DSB (Fig. 3). In this scenario, the time-dependent increase of the intensity of IRIF may not be due to an increase in accumulation of factors at a DSB but rather to the condensed state of the DNA. As long as the break is not repaired, a higher amount of DNA will become condensed around the lesion, thereby leading to a higher concentration of factors that are associated with the damage (Fig. 3). These two possible functions of H2AX phosphorylation, the tethering of factors and chromatin compaction, are not mutually exclusive since the γ -H2AX mark may provide both a docking site and promote a change in chromatin folding.

The known properties of γ -H2AX are consistent with a role in chromatin compaction. For example, H2AX is required for the chromatin condensation and transcription silencing of the sex chromosomes during spermatogenesis (25). Moreover, H2AX regulates the long-distance synapsis of DNA ends during immunoglobulin class-switch recombination (26). Interestingly, we have found that H2B-Ser^{14P} shows a similar staining pattern to that of γ -H2AX in mouse spermatocytes, being particularly enriched in the highly compacted XY chromosomes, also known as the sex body (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20032247/DC1>). H2B-Ser^{14P} has already been re-

ported to be associated with chromatin condensation both *in vivo* and *in vitro* (12, 27). The H2B peptide tail has the unique property of self-aggregating when phosphorylated at S-14, and therefore this modification could play a direct role in regulating chromatin condensation (12). Thus, it is possible that H2B-Ser^{14P} can act in concert with H2AX to promote chromatin condensation at sites of DSBs (Fig. 3).

The chromatin compaction model predicts that H2AX would even be required for the foci formation of its own kinase. Indeed, we have found that the formation of ATM^{Ser1981P} foci is abrogated in the absence of H2AX, whereas the recruitment of the ATM^{Ser1981P} to DSBs is H2AX independent (unpublished data). A previous study indicated that DSB-induced chromatin relaxation might directly trigger ATM activation (1). Such a chromatin alteration may also facilitate access of DNA repair/signaling machinery to DSBs and target enzymes that covalently modify histone tails. If the lesion persists, modifications on histone tails including phosphorylation of H2AX-Ser-136/139 and H2B-Ser14P (and possibly histone deacetylation) may cooperate to establish a heterochromatin-like state, which may prevent the premature separation of DNA ends. The presence of fragmented chromosomes associated with defective foci formation in H2AX^{-/-} cells (9, 10) supports this model. Understanding how histone modification patterns dictate dynamic changes in chromatin topology at DNA damage sites is a major challenge for future research.

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