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ORIGINAL PAPER

Histone H2AX in DNA repair

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Abstract The paper reviews the recent reports on the role of the phosphorylated histone H2AX (γ-H2AX). The modification of this histone is an important part of the cellular response to the induction of DNA double strand breaks (DSB) by ionising radiation and other DSB-generating factors. In irradiated cell the modification is carried out mainly by ATM (*ataxia--telangiectasia* mutated) kinase, the enzyme that starts the alarm signalling upon induction of DSB. γ-H2AX molecules are formed within 1–3 min after irradiation and form foci at the sites of DSB. This seems to be necessary for the recruitment of repair factors that are later present in foci of damaged nuclei. Modification of a constant percentage of H2AX molecules per DSB takes place, corresponding to chromatin domains of megabase pairs of DNA.

Key words DNA double strand breaks • DNA repair • histone γ-H2AX • ionising radiation

Introduction

In eucaryotic cells, DNA is bound to various proteins and forms a complex nucleoprotein structure called chromatin. Indispensable and ubiquitous chromatin components are histones, small proteins of highly conserved primary structure. Two molecules of each H2A, H2B, H3 and H4 type, form the nucleosomal core around which a 146 base pair (bp) stretch of DNA is wrapped. Histone H1 is bound to about 40 bp linker DNA. A sketch of the nucleosomal core structure is shown in Fig. 1. The repeats of this unit structure together with the linker region form the so-called beads on a string, that can be seen on photographs of relaxed chromatin in an electron microscope.

Histones *in vivo* are subject of various modifications, such as phosphorylation, acetylation, ubiquitination. Phosphate and acetate moieties usually are transferred by nuclear enzymes on specific serine and lysine residues, respectively, usually located near the amino or carboxyl termini of the histone molecules. Thus, their chemical properties are altered and this brings about changes in their interaction with DNA and with other proteins, and, in consequence, in the chromatin structure. Such changes are a necessary requirement for various functions of DNA performed in cells. For example, relaxation of a condensed higher-order structure is necessary to provide access of transcription factors and specific acetylations are part of that mechanism [3, 12].

It has long been known that histones are heterogenous, but not all aspects of this heterogeneity are understood. For example, the family of H2A histones consists of three subfamilies present in varying proportions in various cell types: H2A1-H2A2, H2AZ, and H2AX. In mammals, histone H2AX represents 2–25% of the H2A family [15].

Recently, a specific repair function has been ascribed to histone H2AX and this shows that DNA repair still has many

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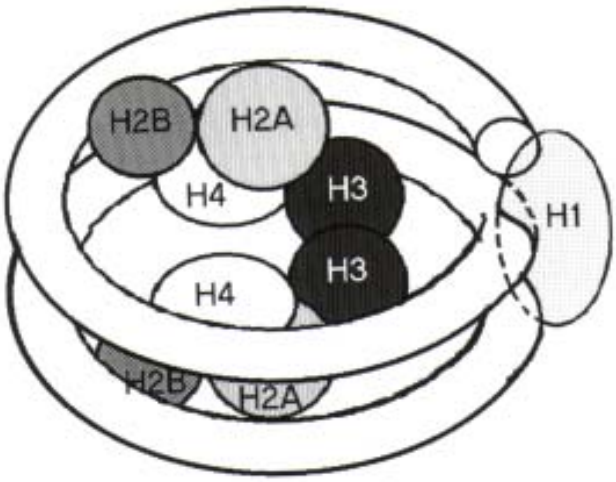

Fig. 1. Histone positions in a nucleosome. The nucleosome contains about 145 bp of DNA and eight histone proteins, two from each of four histone families, H4, H3, H2B, and H2A. Histone H1 binds the two DNA turns.

secrets to unravel. This paper describes the current state of knowledge on the biological role of histone γ-H2AX.

Histone H2AX is phosphorylated in response to induction of DNA double strand breaks

One effect of ionising radiation (or other agents that introduce double-stranded breaks into DNA) *in vivo* or *in vitro* is induction of post-translational modifications of proteins which can be detected by electrophoresis on two-dimensional gels on the basis of altered electrophoretic mobility and molecular mass. Labelling with ^{32}P can be used to indicate freshly phosphorylated molecules. Among changes found in nuclear proteins after exposure to ionising radiation, the most recently described is phosphorylation of histone H2AX at serine 139 (at the C-terminus of the molecule) and named γ-H2AX [18].

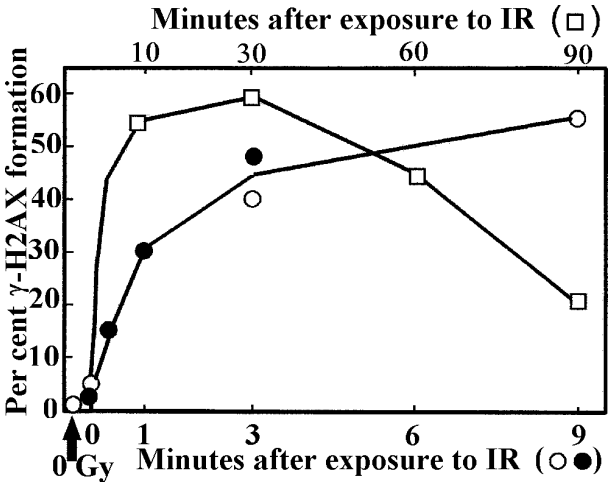

Fig. 2. Relative amount of γ-H2AX at various times after exposure to ionising radiation. The H2AX components were quantitated with ImageQuant software version 3.3 (Molecular Dynamics) without any contrast or brightness enhancement. The open circle indicated by the arrow shows the percentage of γ-H2AX measured in unirradiated cells. The open and filled symbols (circles and squares) denote separate experiments [18].
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The same modification of H2AX takes place in cells undergoing DNA fragmentation due to apoptosis [17]. DSB are transient intermediates in genomic rearrangements within immunoglobulin and T-cell receptor genes [6, 14] and these processes also are accompanied by generation of γ-H2AX.

Histone H2AX phosphorylation is immediate and evolutionarily conserved

γ-H2AX molecules appear very soon after exposure of cells to ionising radiation; half-maximal amounts are reached after 1 min and maximal amounts after 10 min. Fig. 2 shows an example of γ-H2AX formation in Chinese hamster ovary (CHO) cells irradiated with 200 Gy of γ-rays on ice and then returned to an incubator for recovery at 37°C. As soon as 20 s after return to the incubator, γ-H2AX was detected, and the maximal level of γ-H2AX was reached after 10 min (circles,

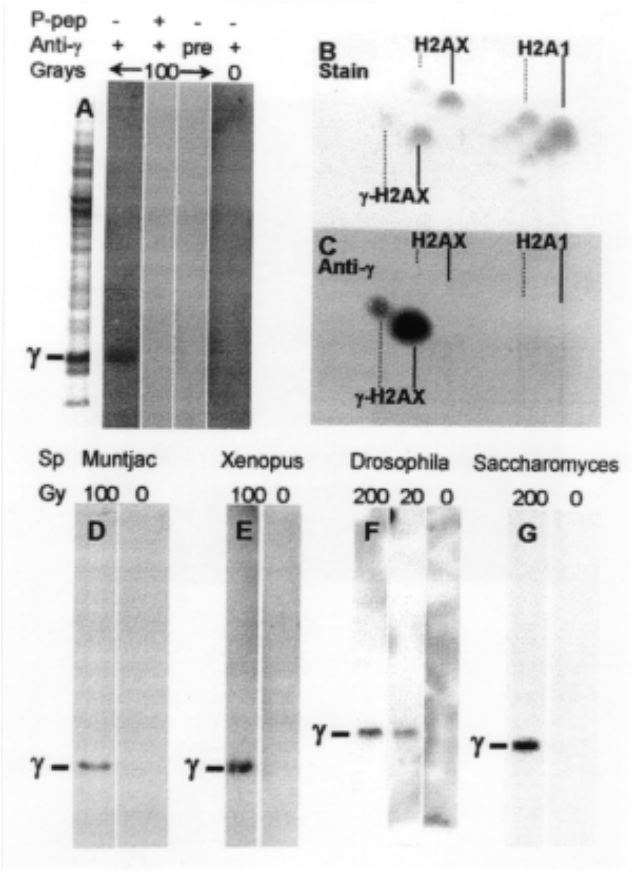

Fig. 3. Immunoblots of γ-H2AX histone**.** After exposure to the indicated doses of ionising radiation and 30-min recovery, cells were harvested. Extracts were prepared and analysed by gel electrophoresis and immunoblotting on polyvinylidene difluoride (PVDF) membranes as described in [16]. A – Human MCF7 breast cancer cells. Blots of fractionated total sodium dodecyl sulphate (SDS) extracts were probed as indicated with anti-γ preimmune serum (pre) or with anti-γ containing 1 μM immunising peptide (P-pep). The left-most lane shows the protein staining pattern on SDS gels. B and C – Human SF268 astrocytoma cells. Cells were irradiated with 100 Gy and analysed on high-resolution two-dimensional electrophoresis on polyacryl-amide gels (first dimension – acetic acid-urea-Triton X100; second dimension – acetic acid-urea-cetyltrimethyl ammonium bromide. For experimental details, see [18]. D–G – Migration of the respective H2AX homologues, H2AX in *Muntiacus muntjak* (D) and *Xenopus laevis* (E), H2AvD in *Drosophila melanogaster* (F), and H2A in *Saccharomyces cerevisiae* (G) are indicated by γ. For experimental details, see [16].
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Fig. 2) and then it gradually declined during the following 90 min (squares, Fig. 2).

Fig. 3 shows the effect of a massive radiation doses, 100 or 200 Gy, on the formation of γ-H2AX in cells of various types. Since the detection is carried out after electrophoresis with a γ-H2AX – specific antibody or a protein-specific stain, the amount generated must be big enough to allow for the detection sensitivity and this is why high radiation doses were used. As mentioned below, lower doses can be applied when anti-γ-H2AX antibody with a fluorescent tag is applied to stain cells on microscopic slides. Fig. 3 also shows that γ-H2AX is evolutionarily conserved from yeast to man, indicating that its formation is part of a universal response to DSB. Judging from the kinetics of -γ-H2AX formation, this is an early step of the response which is further supported by other experiments, as described below.

The ATM kinase is the main enzyme that phosphorylates histone H2AX

The presence of DSB in nuclear DNA activates three related protein kinases, all belonging to the family of phosphoinositide-3 kinases (PI-3K): DNA-PK (DNA-dependent protein kinase), ATM (*ataxia-telangiectasia* mutated; its function is impaired in the heritable hypersensitivity to ionising radiation), and ATR (*ataxia-telangiectasia* related). It was postulated that these kinases form part of a monitoring system that detects DNA damage, generates alarm signalling cascades and recruits repair enzymes to the damaged sites (for review, see [19]). The substrate specificities of these kinases *in vitro* are similar, but *in vivo* they have distinct substrates according to their functions. For example, ATM phosphorylates proteins that function in the cellular "check--point mechanisms" leading to cell cycle arrest. DNA-PK is indispensable in a DSB repair system called non-homologous end-joining.

All three kinases may carry out phosphorylation of H2AX *in vitro*, but H2AX phosphorylation is practically absent in Atm-/- cells (devoid of both alleles of the Atm gene), whereas it is normal in DNA-PKcs-/- cells (lacking the catalytic subunit of the DNA-PK kinase). This is shown in Fig. 4, which compares the amounts of the normal histone H2A component with that of γ-H2AX in irradiated murine fibroblasts [4]. Probably, DNA-PK is responsible for the minimal levels of H2AX phosphorylation in Atm-/- cells, since low doses of wortmannin, an inhibitor of PI-3K, hence, also of DNA-PK, eliminate this trace amount of γ-H2AX. This observation is consistent with the finding, that a fraction of nuclear ATM co-localises with γ-H2AX at sites of DSB and therefore, in response to DNA damage [1]. So, the likely scenario after irradiation is that ATM is activated at the site of DSB, it immediately phosphorylates histone H2AX at the site of the break and by that, a signal is produced to indicate that a DSB is present and must be repaired. This may then initiate the recruitment of DNA repair or damage-signalling factors [4].

Although most experimental results consistently show the role of ATM in phosphorylation of H2AX, one human cell line (M059J, a mutant line lacking DNA-PK protein [10]) is deficient in γ-H2AX formation. Similarly to other mammalian cell lines, the wild type M059K converts over 60% of its H2AX to the γ-form 15 min after irradiation with 200 Gy of

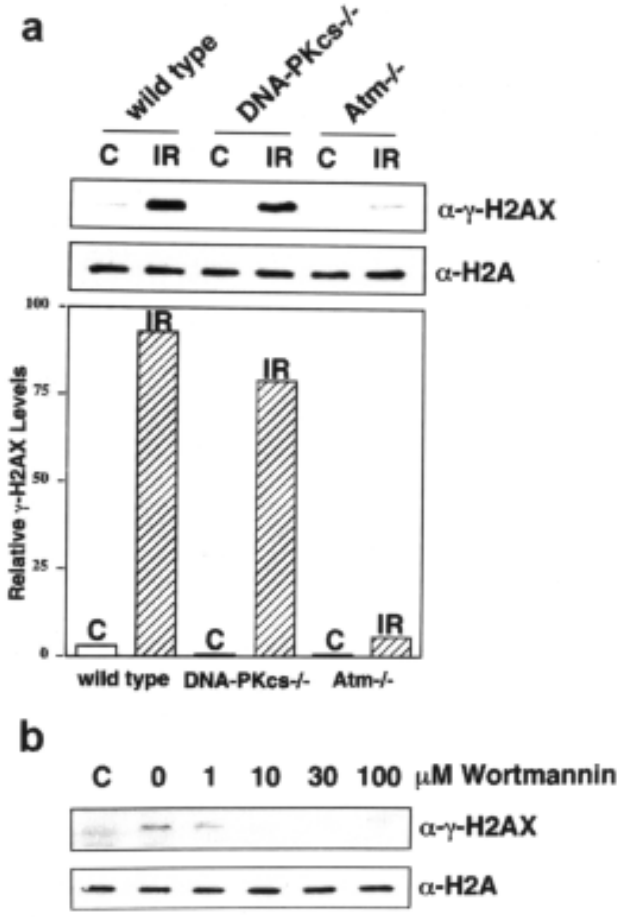

Fig. 4. Lack of H2AX phosphorylation in Atm-/- cells. **a –** Wild type, DNA--PKcs-/-, or Atm-/- fibroblasts were mock-irradiated (C) or irradiated (IR), harvested after 30 min, and analysed by Western blotting. The autoradiograph in the figure was scanned, the bands were quantified using NIH Image software, and the relative levels of γ-H2AX were plotted on the *y* axis. After IR the level of γ-H2AX in Atm-/- cells is about 5% of that in wild type cells. A similar pattern of H2AX phosphorylation was observed in three independent experiments. **b** – Atm-/- fibroblasts were incubated with increasing concentrations of wortmannin for 30 min and then irradiated. Cells were harvested 30 min after irradiation, and SDS extracts were analysed by Western blotting [4].

γ-rays, under the same conditions the mutant M059J line contained no more than 25% γ-H2AX [13]. The DNA-PK activity is about 10 times higher in human as compared to rodent cells. Hence, there may be differences in γ-H2AX generation between human and rodent cells. However, another, more plausible reason for the reduced H2AX phosphorylation in M059J cells could be the low level of ATM in these cells [9, 10] rather than the absence of DNA-PK. Such an explanation would be more consistent with other experimental results.

γ-H2AX is present in the repair foci

When DSB are induced in DNA, a complex cellular response is triggered. One component of this response is accumulation of proteins involved in DNA repair or damage signalling. They can be seen as foci in the fluorescent microscope when specific antibodies with fluorescent tags are applied, e.g. [11].

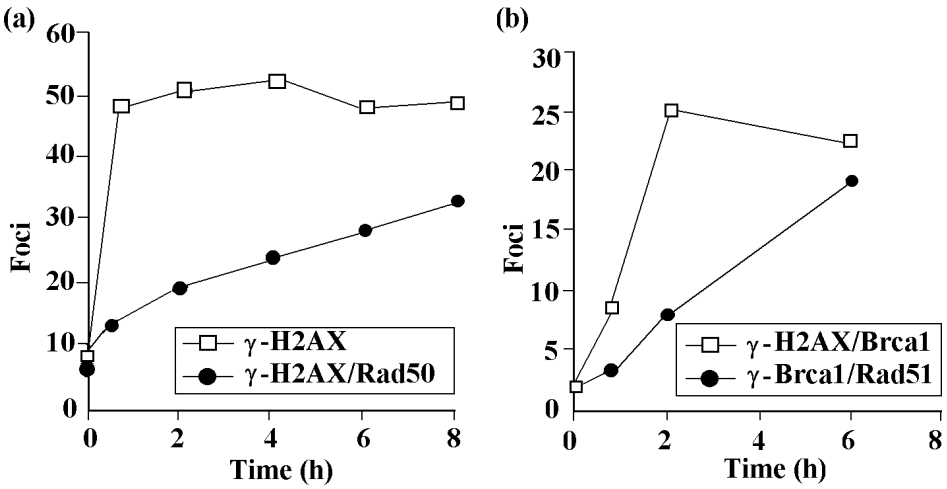

Fig. 5. Quantification of radiation-induced foci [13]. (a) IMR90 cells, either unirradiated or irradiated with 12 Gy, were stained with γ-H2AX and Rad50 antibodies. At least 30 images were collected from each time point and the number of overlapping foci was determined using NIH Image software. Foci were counted in this way for each time point: unirradiated (0) or irradiated (0.5, 2, 4, 6 and 8 h recovery). The sets of data from each time point are Poisson distributed, and the average value for each data set is plotted on the graph. The Y axis represents the number of foci per average cell nucleus area. The number of γ-H2AX–Rad50 overlapping foci (filled diamonds) is represented in comparison with the total number of γ-H2AX foci (open squares). (b) An analysis of γ-H2AX–Brca1 overlapping foci (open squares) is shown in comparison to Brca1–Rad51 overlapping foci (closed squares) at four different time points: 0 (unirradiated cells), 45 min, 2 h, and 6 h, using methods described in (a) [13].
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Also an anti-γ-H2AX antibody with a fluorescent tag was applied and a pattern of γ-H2AX foci was found within minutes after γ-irradiation with doses considerably lower than that used for biochemical studies, namely 0.6–2 Gy [16]. When DSB were introduced with a pulsed microbeam UVA 390 nm laser in a partial nuclear volume, foci formed at the path of the microbeam [16], confirming that γ-H2AX foci are formed at sites of DSB.

Observation of the time course of localisation of various proteins in the foci indicated that γ-H2AX foci appeared first [13]. At a later time they seemed to be co-localised with Brca1 foci (visualised via an antibody with a different fluorescent tag) followed by accumulation of Rad 50 or Rad 51 in different subsets of cells. Rad 50 is a part of the MRN complex (Mre11 – Rad 50 – Nbs1), which is active in both recombinational repair and non-homologous end-joining [7] and has been shown to co-localise with γ-H2AX [5].

Interestingly, Rad 50 and Rad 51 foci are rarely present in the same cell after damage induction [11], indicating that there are 2 repair pathways mutually excluding one another. In both cases the assembly of repair factors is dependent on the previous generation of the γ-H2AX foci. When irradiated cells are post-treated with wortmannin, there is no effect on the formation of foci. Pre-treatment completely prevents the accumulation of all components of the foci. These data show that H2AX phosphorylation takes place immediately after damage infliction and is indispensable for the sequential assembly of the studied repair proteins [13].

Are the foci really critical for DSB repair? The kinetics of focus formation (see Fig. 5) and of the fast component of DSB repair (half-time 15–30 min) are not consistent. On the other hand, the slow component of DSB repair (1–4 h or more) would be consistent with the accumulation of repair factors in foci. In this context, it is important that slowly repaired residual damage is better correlated with cell survival than the rate of fast repair [2]. Also, there is a decreased efficiency of DSB repair by the non-homologous end-joining in a yeast mutant with a truncated H2AX C-terminus; due to the truncation, the histone molecules lack the consensus phosphorylation site for PI-3K type kinases [8].

Table 1. Percentages of H2AX molecules are γ-modified per Gy of γ-rays (according to [18], modified).

Cell type	Origin of cells	H2AX/total H2A (%)	Total numbers of γ-H2AX per DSB	Per cent γ-H2AX per DSB	Modified bp of DNA per DSB
VA13	Transformed human fibroblast	2.6	530	0.033	2.0×10^6
HeLa	Human ovary carcinoma	2.4	490	0.035	2.1×10^6
IMR90	Normal human fibroblast	9.8	2100	0.035	2.1×10^6
CHO	Chinese hamster ovary	9.4	2240	0.040	2.4×10^6
SF268	Human astrocytoma	25.0	8800	0.059	3.5×10^6

The stained H2A2, H2A1, and H2AX species on two-dimensional gels were recorded as TIFF images and quantitated with ImageQuant software version 3.3, as described in [18]. The γ-H2AX/H2AX ratio was determined 30 min after exposure of cells to 25 Gy. The following conversion factors and assumptions were used. 1) The mammalian G1 genome contains 6×10^9 bp of DNA, 30×10^6 nucleosomes (200 bp/nucleosome) and 60×10^6 H2A molecules (2 molecules/nucleosome). 2) 25 Gy induces about 875 DSB per G1 genome. 3) H2AX is randomly distributed in the chromatin [18].

Megabase chromatin domains are involved in DSB repair *in vivo*

As discussed by Paull *et al*. [13], modification of the H2AX molecules may be important to alter the local chromatin structure in the surrounding of a DSB and thus, increasing the accessibility of DNA to various repair factors. Rogakou *et al*. [18] calculated that approximately 1% of the H2AX becomes γ-phosphorylated per 1 Gy of ionising radiation. This radiation dose induces about 35 DSB per mammalian G1 genome which in 6×10^9 bp, generates γ-H2AX distributed over 1% of the chromatin, i.e., about 0.03% of chromatin appears to be modified per DSB, corresponding to about 2×10^6 bp of DNA per DSB. Table 1 shows data from reference [18] used for this calculation. It can be seen that several cell lines were examined which differed in the amount of the H2AX histone; nevertheless, the percentage of modified H2AX per DSB was approximately the same in all cells studied (Table 1).

In conclusion, experiments on generation and distribution of γ-H2AX indicate that per DSB a chromatin domain corresponding to about 2×10^6 bp of DNA is modified. This important conclusion opens new and intriguing perspectives on DNA repair. It should be added that, while this paper was being reviewed, an extensive review appeared in *Science* by A. Celeste and twenty co-authors (*Science* 2002, vol. 296, pp. 922–927); it includes the new results obtained for B-cells and fibroblasts of H2AX-/-mice. These new data confirm and extend the early experimental results, reviewed above.

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