MINIREVIEW / MINISYNTHÈSE

H2AX: tailoring histone H2A for chromatin-dependent genomic integrity[1]

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Abstract: During the last decade, chromatin research has been focusing on the role of histone variability as a modulator of chromatin structure and function. Histone variability can be the result of either post-translational modifications or intrinsic variation at the primary structure level: histone variants. In this review, we center our attention on one of the most extensively characterized of such histone variants in recent years, histone H2AX. The molecular phylogeny of this variant seems to have run in parallel with that of the major canonical somatic H2A1 in eukaryotes. Functionally, H2AX appears to be mainly associated with maintaining the genome integrity by participating in the repair of the double-stranded DNA breaks exogenously introduced by environmental damage (ionizing radiation, chemicals) or in the process of homologous recombination during meiosis. At the structural level, these processes involve the phosphorylation of serine at the SQE motif, which is present at the very end of the C-terminal domain of H2AX, and possibly other PTMs, some of which have recently started to be defined. We discuss a model to account for how these H2AX PTMs in conjunction with chromatin remodeling complexes (such as INO80 and SWRI) can modify chromatin structure (remodeling) to support the DNA unraveling ultimately required for DNA repair.

Key words: H2AX, DNA repair, double-stranded DNA breaks, phosphorylation.

Résumé : Au cours de la dernière décennie, la recherche sur la chromatine s'est concentré sur le(s) rôle(s) de la variabilité des histones en tant que modulateurs de la structure et de la fonction de la chromatine. La variabilité des histones peut résulter soit de modifications post-traductionnelles ou de variations intrinsèques au niveau de la structure primaire : les variants d'histones. Dans cette revue, nous porterons notre attention sur un des variants d'histone le plus exhaustivement caractérisé au cours des dernières années, l'histone H2AX. La phylogénie moléculaire de ce variant semble avoir évolué en parallèle avec celle de H2A1, une histone somatique de référence chez les eucaryotes. D'un point de vue fonctionnel, H2AX semble être principalement associé au maintien de l'intégrité du génome, au moyen de sa participation à la réparation les bris double-brin à l'ADN, introduits de façon exogène par un dommage environnemental (radiation ionisante, produits chimiques), ou dans le processus de recombinaison homologue durant la méiose. Au plan structurel, ces processus impliquent la phosphorylation d'une sérine associée au motif SQE présent à l'extrémité du domaine C-terminal

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Abbreviations: 53BP1, p53 binding protein 1; Arp4, actin-related protein 4; ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; Brca1, breast cancer susceptibility protein 1; CDT, cytolethal distending toxins; DNA-PK, DNA-dependent protein kinases; DNA-PKcs, DNA-PK catalytic subunit; DSB, double-stranded DNA breaks; dTip60, *Drosophila* Tip60; Esa1, essential Sas2-related acetyltransferase-1; HAT, histone acetyl transferase; HDAC, histone deacetylase; HR, homologous recombination; HTA, histone H2AX in yeast; HU, hydroxyurea; INO80, Inositol 80-containing complex [inositol-1-phosphate synthase (INO1)]; IR, ionizing radiation; IRIF, ionizing-radiation-induced foci; LCL, lymphoblastoid cell lines; Mb, megabase; Mec1p, meiosis entry checkpoint 1 protein; MEF, mouse embryo fibroblasts; MMS, methyl methanesulfonate; Mre11, meiotic recombination 11; MRN, Mre11/Rad50/Nbs1; Nbs1, Nijmegen breakage syndrome 1; NHEJ, nonhomologous end-joining; NuA4, nucleosome acetyltransferase of H4; PIKK, phosphatidylinositol-3 kinase-like family of kinases; PTM, post-translational modification; RAG, recombination activating gene; RPA, replication protein A; Spo, sporulation; SWI/SNF, switch/sucrose nonfermenting; SWRI, Swi2/Snf2-related adenosine triphosphatase complex; Tel1p, telomere length 1; Tip60, Tat interactive protein; V(D)J, variable, diversity, joining.

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de H2AX et impliquent possiblement d'autres modifications post-traductionnelles (MPT) dont certaines commencent seulement à être définies. Nous discutons d'un modèle conçu pour expliquer comment ces MPT d'H2AX, conjointement avec les complexes de remodelage de la chromatine (comme INO80 et SWRI), peuvent modifier la structure de la chromatine (remodelage) pour permettre le déroulement de l'ADN finalement requis pour la réparation de l'ADN.

Mots clés : H2AX, la réparation de l'ADN, les bris double-brin à l'ADN, la phosphorylation.

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Generalities about H2AX

The histone core is composed of 2 copies each of the 4 core histones, H2A, H2B, H3, and H4. The fundamental unit of chromatin contains 146–180 bp of DNA wrapped around this histone octamer (Ausió and Abbott 2004), and it is known as the nucleosome. Each of the 4 core histones has a central "histone fold" domain, which consists of 3 alpha helices connected by 2 loops (Arents and Moudrianakis 1995). The core histones interact via the "histone fold" domain, providing stability to the nucleosome (Luger et al. 1997). The histone fold is flanked by regions that have very little secondary or tertiary structure ("tails") and a less well-defined contribution to the stability of the nucleosome (Ausió and Abbott 2004). These N- or C- terminal tails interact extensively with the DNA. The variation (histone variants and PTMs) of these tail domains plays an important role in the folding of the chromatin fiber. Histone tails are one of the 3 major structural components (besides linker DNA length and linker histones) that can affect chromatin conformation (Ausió and Abbott 2004).

Histone H2AX is a rather unique heteromorphous core histone H2A variant (Ausió and Abbott 2002), which represents 2%–25% of the mammalian histone H2A pool, depending on the cell line or tissue examined (Rogakou et al. 1998). Its amino acid sequence was first determined in 1989 (Mannironi et al. 1989). This H2A variant is evenly distributed throughout the genome (Siino et al. 2002). H2AX is characterized by a unique and invariant SQE motif at the C-terminal tail, which is a consensus sequence for PIKK. In mammalian cells, 3 PIKKs are known to phosphorylate the serine residue in the SQE motif of H2AX. These PIKK members, which play an important role in DSB repair, are ATM, DNA-PK, and ATR (Stiff et al. 2004). Because a lot of DSB repair research has been carried out in yeast, *Saccharomyces cerevisiae*, it is important to note that the major histone H2A variant (which is encoded by genes *HTA1* and *HTA2*) in this organism contains the SQE motif (Redon et al. 2002), which can be phosphorylated by the mammalian homologs of ATR and ATM, Mec1p and Tel1p, respectively (Downs et al. 2000).[5] The *HTA1* and *HTA2* isoforms contribute 95% of the H2A complement in yeast (Pilch et al. 2003), the rest corresponding to HTZ1/HTA3 (the H2AZ equivalent in yeast) (Jackson and Gorovsky 2000). Immediately upstream of the SQE motif, mammalian H2AX variants also contain a second phosphorylatable motif: SQ in mouse and TQ in human (Rogakou et al. 2000). Therefore, human H2AX can be phosphorylated at Threonine 136 as well as Serine 139 (Rogakou et al. 2000).

Histone H2AX: a phylogenetic perspective

The evolution of the H2AX variant, within the context of other histone H2A variants, is shown in Fig. 1. With few exceptions, such as in the nematode *Caenorhabditis elegans*, which does not have H2AX, and the fruit fly *Drosophila*, which contains an H2AZ/H2AX chimeric H2A (H2AvD), histone H2AX is evenly distributed throughout the eukaryote kingdom. As already pointed out, in *S. cerevisiae* the major H2A component is an H2AX, and, similarly, an H2AX also replaces the canonical H2A in *Giardia* and fungi. Therefore, as can be seen in Fig. 1, the canonical H2A1 and H2AX histone H2A variants appear to have co-evolved in different eukaryotic lineages, having had multiple evolutionary origins (Malik and Henikoff 2003). This evolution is in high contrast to that of other histone variants, such as H2AZ, H2ABbd, and macroH2A, whose the diversification has been the result of single differentiation events along the phylogenetic tree. The single evolutionary origin of macroH2A has been the most recent (Fig. 1).

At the protein level, the evolutionary process involves the alteration and extension of a region of over 20 amino acids at the C-terminal domain of H2A (see Fig. 2A) with the appearance of a highly conserved SQE phosphorylatable motif (Fig. 2B). At the gene level, the unique gene encoding this variant in higher eukaryotes is transcribed as 2 different mRNAs. Although both contain the 3′ stem loop characteristic of the replication-dependent histone mRNAs, 1 of them consists of a 3′UTR extension with a polyA signal that is characteristic of replication-independent histone genes. The occurrence of these 2 different RNA populations is the result of alternative processing. This allows a fraction of H2AX to be expressed during S phase while another fraction is expressed during G1 (Alvelo-Ceron et al. 2000). This later fraction exchanges with the canonical H2A and probably with phosphorylated H2AX in the process of DSB DNA repair during the G phase of the cell cycle, as it will be discussed later.

Sequence comparison of the protein sequence extension of H2AX beyond the protein sequence of the canonical H2A for different representatives within the eukaryotes (Fig. 2B) provides some interesting information. First, as already described, an SQE phosphorylation motif appears extremely conserved. In addition it reveals the presence of a highly conserved GKK motif. The high conservation of this motif suggests that in addition to phosphorylation, the C-terminal extension of H2AX may be subject to other PTMs at these lysine residues, which may also participate in the DSB repair process (Moore and Krebs 2004; Wyatt et al. 2003) or

[5] To clarify confusions arising from the protein names used in the 2 most commonly studied systems in DSB repair, a comparison of the yeast and mammalian homologs involved in this process is presented in Table 1.

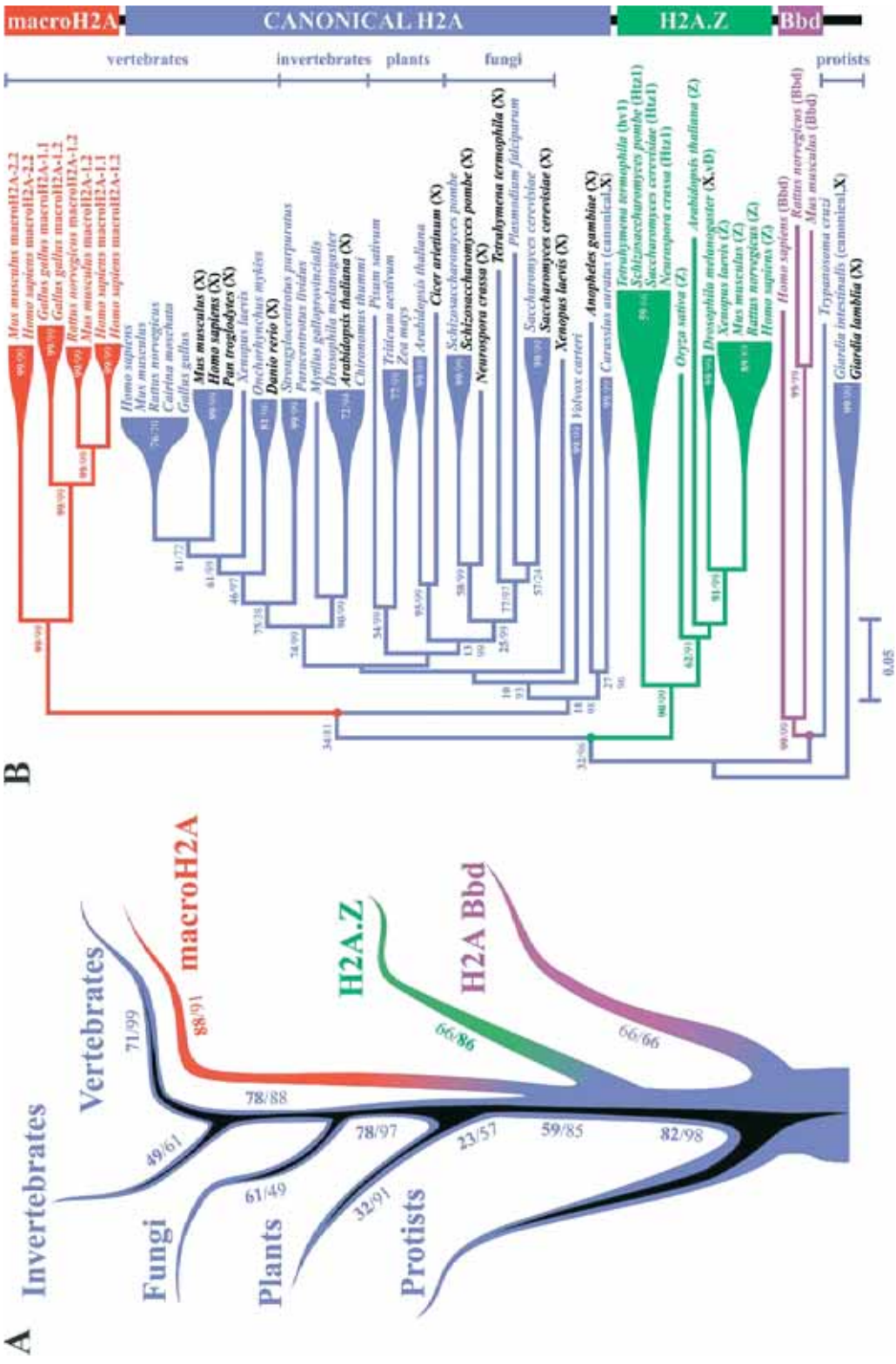
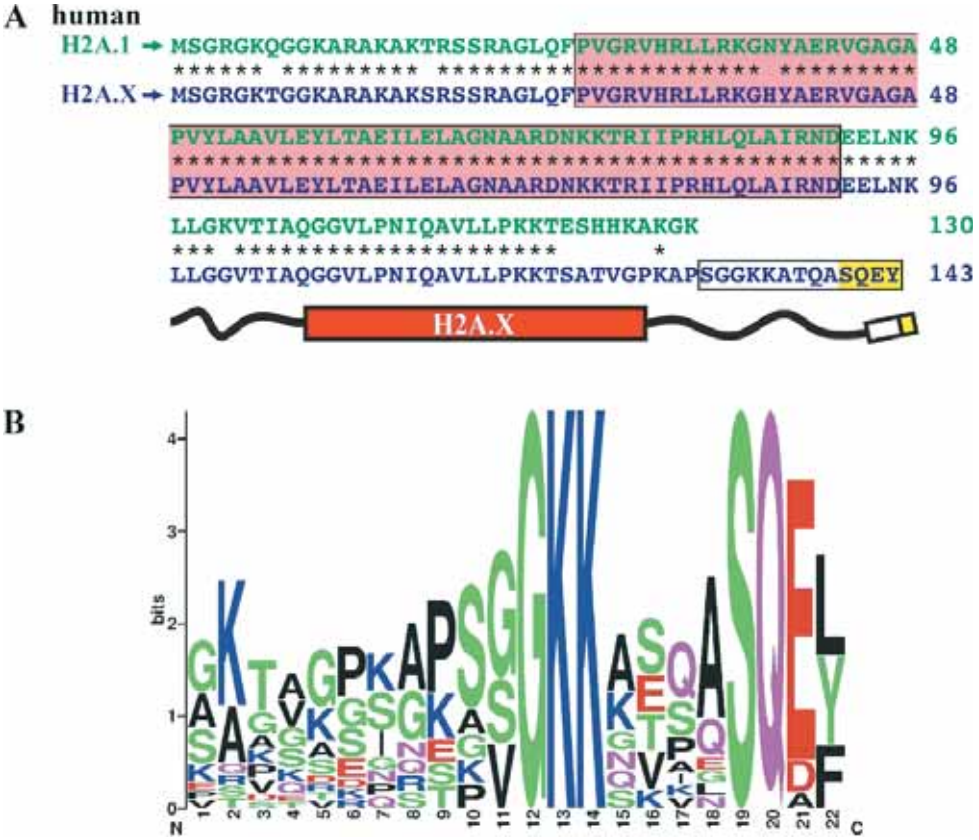


Fig. 1. (A) Schematic representation of the phylogenetic relations among different H2A histones in eukaryotes based on the neighbor-joining tree shown in (B). The canonical H2A lineage is represented in blue, and H2AX is in black. The Barr body-deficient H2A (H2A Bbd) (blue), the H2AZ (green), and the macroH2A (red) lineages stem from the main trunk through single differentiation events taking place during histone H2A evolution. The numbers for interior branches represent bootstrap (boldface) and interior-branch test (normal) values, both based on 1000 replications and only shown when greater than 50%. (B) Phylogenetic neighbor-joining tree reconstructed from uncorrected *p* distances showing the relations among H2A genes from representative organisms of the *Eukarya* superkingdom. The topology reveals the early differentiation as well as the monophyletic origin for the 3 aformentioned variant lineages. The H2AX variants (black and in boldface) are otherwise ubiquitous in the major eukaryotic groups, arising through multiple differentiation events from canonical H2A histones (blue), with the unique exception of *Drosophila melanogaster* H2AX variant.

Fig. 2. (A) Sequence alignment of human histone variants H2A1 and H2AX. The stars identify the identical amino acids, and the pink box corresponds to the histone fold. A schematic representation of H2AX is also shown. (B) The last C-terminal 22 amino acids of histone H2AX from different organisms (see Fig. 1B) were sequence aligned and displayed in a Logos format (Schneider and Stephens 1990). In this representation, the size of the letters is proportional to the frequency with which an amino acid appears at a given position in the sequence alignment. The overall height of all of the letters at any given position is proportional to the conservation of the site. The letters are color coded according to the physical and chemical structural characteristics of the amino acids they represent.



in other important nuclear metabolic processes yet to be described.

The protein extension at the C-terminal end that occurred during the evolutionary diversification of H2AX from the main canonical H2A components is a region that is very close to the binding site of histone H1 in the nucleosome and hence can, in itself or through its PTMs, such as phosphorylations, have structural effects of its own (Arents and Moudrianakis 1995; Ausió et al. 2001).

H2AX phosphorylation and DSB repair

H2AX is phosphorylated extensively in response to a specific type of DNA damage called DSB. DSB, as the name implies, takes place when both strands of DNA are broken, exposing free DNA ends. If improperly repaired, DSB can have deleterious consequences for the cell. These include chromosomal aberrations such as chromosomal breaks, translocations, and aneuploidy (Fernandez-Capetillo et al. 2004*b*). DSB repair can be achieved by 2 different pathways: NHEJ and HR. NHEJ involves the direct joining of the broken ends. HR takes advantage of the homologous DNA sequence from other parts of the genome to repair the broken ends. DSBs are mainly repaired by NHEJ in mammalian cells (Drouet et al. 2005). However, HR is found to be predominant in yeast in diploid cells and the S-phase of haploid cells (HR is used whenever a sister chromatid is available, not just a homologous chromosome) (Moore and Krebs 2004), and during meiosis.

In eukaryotic cells, DNA DSBs can have diverse origins. DSBs are induced accidentally by chemical agents, such as bleomycin, camptothecin, HU, and MMS, and ionizing radiation (Balajee and Geard 2004; Bird et al. 2002; Nazarov et al. 2003; Ward and Chen 2001). DSBs can be replication-fork-associated when a replication fork collides with an unrepaired DNA single-stranded break (Arnaudeau et al. 2001). Viral agents, such as retroviruses, also induce DSBs in host DNA by an integrase-mediated process (Daniel et al. 2004). CDT, a toxin produced in some Gram-negative bacteria, are able to induce DSBs (Thelestam and Frisan 2004). In addition to accidental damage, DSBs occur in the cell in a controlled environment, such as during V(D)J, apoptosis, and meiotic recombination (Chen et al. 2000; Mahadevaiah et al. 2001; Rogakou et al. 2000). These programmed DSBs are all essential processes for the survival of the cell or the organisms themselves. Mechanisms of DSB detection and repair in both accidental and physiological conditions are essential for genomic integrity. Phosphorylation of H2AX is a marker for both accidental and natural DNA DSB. Immediately after the occurrence of DSBs, numerous DNA repair fac-

tors are recruited and modified at the DSB site. Among them, H2AX is extensively phosphorylated over approximately 2 Mb immediately to the DSB (Rogakou et al. 1999). The phosphorylated form of H2AX is commonly denoted as γ-H2AX.

In what follows, we briefly review the occurrence of γ-H2AX triggered by DSB DNA damage and other physiologically relevant processes involving DSBs.

DSB damage induced by external agents

Time-dependent phosphorylation of H2AX in response to DSB damage induced by extracelluar agents has been studied extensively because of the availability of reagents and controllable experimental conditions. These studies have allowed the examination of the role of γ-H2AX in DSB repair. It has been shown that γ-H2AX is generated with 1 min and γ-H2AX foci appear within 3 min after irradiation (Rogakou et al. 1999). The phosphorylation of H2AX rapidly increases and peaks at around 30 min after irradiation (Rogakou et al. 1998). At this point, γ-H2AX starts being dephosphorylated, with a half-life of approximately 2 h (Redon et al. 2002).

The formation of IR-induced γ-H2AX foci was initially shown to be ATM-dependent in ATM knockout cell lines (Burma et al. 2001). However, later on it was observed that both ATM and DNA-PK appear able to phosphorylate H2AX redundantly after IR exposure (in actively growing and plateau phase human fibroblasts, growing MEFs and LCLs, and in chicken cells), although there are kinetic and growth conditions under which ATM predominates (Stiff et al. 2004). Thus, ATM may play the dominant role of phosphorylating H2AX. ATM is critical at least soon after irradiation (Stiff et al. 2004).

It is activated by DNA damage through a process that involves autophosphorylation and dimer dissociation, presumably as a result of the chromatin alteration caused by DNA DSB (Bakkenist and Kastan 2003). The ATM-mediated H2AX phosphorylation represents an important step in determining subsequent events in the signal transduction pathway that participates in the DNA repair process. However, DSB repair factors such as Nbs1, Mre11, 53BP1, and Brca1 (see Table 1) are also phosphorylated in response to IR by ATM (Balajee and Geard 2004; Taylor et al. 2004). Thus, besides H2AX phosphorylation, ATM and DNA-PK are also both required for the repair of IR-induced DSBs because cells deficient in either of these factors are hypersensitive to IR and exhibit DNA repair defects (Bassing et al. 2002). Indeed, deletion of the ATM and DNA-PK genes has more severe consequences to the cells than H2AX deletion (Kurimasa et al. 1999; Xu et al. 1996) because, besides H2AX phosphorylation, these PIKK members are also involved in other cellular mechanisms. For instance, ATR has been known to participate in the initiation of G_2 arrest signaling and phosphorylation of cellular proteins in other DNA damage response mechanisms (Zimmerman et al. 2004).

The dephosphorylation of H2AX is just as important as the phosphorylation process. Indeed, the H2AX homolog in *Drosophila melanogaster,* H2AvD, is also phosphorylated during DSB repair. The removal of the phosphorylated form of H2AvD involves the dTip60 chromatin-remodeling complex (Kusch et al. 2004), which mediates the acetylation of the phosphorylated H2AvD and further exchange of this acetylated and phosphorylated H2AvD with an unmodified H2AvD (Kusch et al. 2004). However, this finding does not preclude the involvement of other mechanisms. Protein phosphatase 1 has been shown to remove the phosphate group from γ-H2AX-containing chromatin in vitro and in situ (Nazarov et al. 2003).

The existence of a γ-H2AX replacement mechanism raises the question of whether there is a replication-independent H2AX variant. As described in the evolution section, the H2AX gene can be transcribed as mRNA with or without a polyadenylated tail (Alvelo-Ceron et al. 2000). It is possible that the polyA- form is involved in the replication-dependent deposition of H2AX every 5 nucleosomes during S phase of the cell cycle (Pilch et al. 2003). However, only 1 in 10 of the H2AX-containing nucleosomes are phosphorylated during DSB repair (Pilch et al. 2003). The fraction of the replication-independent H2AX (polyA+) may be responsible for the unmodified H2AX, which replaces this γ-H2AX after the DSB has been repaired.

Apoptosis

Apoptosis or programmed cell death is an essential process for multicellular organisms and involves an important alteration of chromatin. Phosphorylation of H2AX is also associated with apoptosis. The initiation of DNA fragmentation during apoptosis induces DSB and, thus, induces the formation of γ-H2AX (Rogakou et al. 2000). γ-H2AX was detectable as soon as the DNA DSB occurred during this process, and it was observed in all apoptotic systems examined (Rogakou et al. 2000). An indirect effect of apoptosis is the localization of γ-H2AX at telomeres. This is due to the apoptotic events resulting from the loss of telomere function. Mice lacking the RNA component of telomerase ($mTR^{-/-}$) are unable to express a functional telomerase that contains both RNA and catalytic protein subunits. Therefore, telomere sequence loss during replication is not compensated in these mutants, and the $mTR^{-/-}$ mice telomeres are gradually shortened, resulting in the loss of the telomere function. These mice have a decrease in fertility and an increase in T cell apoptosis (Hao et al. 2004). The loss of telomere function or the critically short telomeres are recognized as DSBs (Hao et al. 2004). Not surprisingly, γ-H2AX is localized to the shortened telomeres (Hao et al. 2004).

V(D)J recombination

V(D)J recombination induces programmed DSB formation in cells of the mammalian immune system (developing thymocytes) (Chen et al. 2000). This programmed DSB from V(D)J cleavage is mediated by RAG and repaired by NHEJ. Repair of DSBs by NHEJ in mammals requires at least DNA-PK and the DNA ligase IV/XRCC4 protein complexes (Drouet et al. 2005). DNA-PK is activated by nucleosomes, and the activated DNA-PK is capable of phosphorylating H2AX within the nucleosome (Park et al. 2003). Nbs1 and γ-H2AX are known to associate with the V(D)J recombination-induced DSB site. In developing thymocytes, Nbs1 and γ-H2AX are associated with the T cell receptor alpha locus in response to RAG protein-mediated V(D)J cleavage (Chen et al. 2000). Although the γ-H2AX foci formation is universal in T cell receptor recombination, it has been shown that γ-H2AX is dispensable for this process (Chen et al. 2000).

Table 1. The DSB repair factors in humans and their yeast homologs (Krogh and Symington 2004).

Human	Yeast	DSB repair pathway and function
Rad51	Rad51	HR, involved in strand exchange.
Rad52	Rad52	HR, indispensable DNA end/single-strand-binding protein, stimulates complementary single-strand adhesion.
Mre11/Rad50/Nbs1 (MRN complex)	Mre11/Rad50/Xrs2 (MRX complex)	Recruitment to DSB site, yeast MRX complex involved in both HR and NHEJ, but mammalian MRN complex only involved in HR
Ku70	Yku70/HDF1	NHEJ, a component of DNA-PK in human
Ku80	Yku80/HDF2	NHEJ, a component of DNA-PK in human
DNA-PK (Ku70/80 and DNA-PKcs)	—	NHEJ, kinase complex recruited to DSB site
Ligase IV	DNL4	NHEJ ligases
XRCC4	LIF1	NHEJ
ATM	Tel1	PIKK kinase
ATR	Mec1	PIKK kinase
Brca1	—	HR, associates with SWR
RPA	—	HR and NHEJ, single stranded DNA binding protein

Note: DSB, double-stranded DNA breaks; HR, homologous recombinations; MRN, meiotic recombination 11/Rad50/Nijmegen breakage sydrome 1; NHEJ, nonhomologous end-joining; DNA-PK, DNA-dependent protein kinases; ATM, ataxia telangiectasia mutated; PIKK, phosphatidylinositol-3 kinase-like family of kinases; ATR, ATM- and Rad3-related; RPA, replicated protein A.

Meiosis

Programmed DSB formation also occurs in germ cells during meiotic recombination (Hunter et al. 2001; Mahadevaiah et al. 2001), when DSBs are repaired by HR. Indeed, phosphorylated H2AX has been shown to be temporally and spatially linked to Spo11, a topoisomerase II-like protein responsible for DSB formation during meiosis (Mahadeviah et al. 2001). Moreover, more H2AX is phosphorylated in testes than in any other unirradiated mouse tissues, possibly because of the extensive DSB formation in the male germinal cells during meiosis (Mahadevaiah et al. 2001). The distribution of phosphorylated H2AX varies during spermatogenesis. γ-H2AX foci can be observed in intermediate and B spermatogonia, and in preleptotene to zygotene spermatocytes, whereas γ-H2AX exhibits a more homogeneous nuclear distribution in type A spermatogonia and round spermatids, and coalesces in the sex body in pachitene spermatocytes (Hamer et al. 2003). The overall relevance of H2AX to meiosis and in particular to spermatogenesis (Lewis et al. 2003) is underscored by the observation that disruption of H2AX expression in mice is required for fertility and maturation of male testes but is expendable in females (Celeste et al. 2002; Scherthan 2003).

The role of H2AX in the meiotic process goes beyond DSB repair. As stated previously, phosphorylated H2AX has been also shown to accumulate in the highly condensed chromosomal sex body during meiotic prophase. This event has been shown to happen in a way that is independent of meiotic recombination-associated DSB (Fernandez-Capetillo et al. 2003*b*). Furthermore, there is evidence to suggest that H2AX has a critical role in controlling the topological distribution and movement of telomeres during meiosis (Fernandez-Capetillo et al. 2003*a*).

Stalled replication forks

During replication, DSBs are also present at stalled replication forks (Ward and Chen 2001). This damage signal is initiated by ATR, and the activation of the pathway prevents entry into mitosis to allow for either DNA repair or apoptosis (Zimmerman et al. 2004). H2AX is phosphorylated by ATR in response to DNA replication stress, such as stalled replication forks (Ward and Chen 2001). The specificity of ATR in this process is underscored by the fact that it does not show a significant contribution to the phosphorylation of H2AX after DSB induced by IR (Stiff et al. 2004; Zimmerman et al. 2004).

The different sources of DSBs described result in the activation of different PIKKs. However, γ-H2AX is found at the sites of all DSBs regardless of their origin. Therefore, H2AX phosphorylation is considered to be a marker for the site of DNA DSBs. In addition to DSB repair, γ-H2AX also participates in checkpoint control of the cell after DNA damage (Fernandez-Capetillo et al. 2002; Stewart et al. 2004). Therefore, the cell cycle does not proceed until DNA damage is repaired. The sequential events and the different components involved during each of the DSB repair processes are yet to be elucidated.

H2AX foci: γ-H2AX and its partners, their role in DSB repair

Modified nucleosomal partners (PTM)

While H2AX is extensively phosphorylated during DSB repair, other histone modifications have also been shown to have an important role. For instance, methylated lysine 79 of histone H3 is involved in targeting 53BP1 to DSBs in mammalian cells (Huyen et al. 2004). 53BP1 is a DNA DSB response protein. Its tandem tudor domain binds to the methylated lysine 79 of histone H3. This domain is sufficient for targeting 53BP1 to DSBs initially, but it does not allow the retention of 53BP1 at DSB. A different domain of 53BP1 located N-terminal to the tandem tudor domain binds

to γ-H2AX (Ward et al. 2003). Since 53BP1 needs to interact with both γ-H2AX and methylated histone H3 to function properly, it is reasonable to hypothesize that the γ-H2AX and the methylated histone H3 are in close proximity, possibly in the same nucleosome. There might be a designated nucleosome that has a modified H2AX as well as a modified canonical H3 acting synergistically in the initiation of DSB repair as a part of a DSB histone code. A similarly related event has been described in *Schizosaccharomyces pombe*. In this instance, Crb2, a homolog of 53BP1 in mammalian cells, is recruited to DSB by methylated histone H4 at lysine 20 (Sanders et al. 2004).

Histone H2B was recently found to be phosphorylated at the N-terminal serine 14 at the DSB site (Fernandez-Capetillo et al. 2004*a*). Furthermore, histone H2B is not a direct target for PIKK (Fernandez-Capetillo et al. 2004*a*). Although the formation of phosphorylated H2B is γ-H2AX independent, the assembly of the irradiation-induced $H2B^{ser14P}$ foci is γ-H2AX-dependent (Fernandez-Capetillo et al. 2004*a*). This indicates that the formation of irradiation-induced $H2B^{ser14P}$ foci is downstream of the γ-H2AX signal.

Another post-translational modification of histones that has been shown to contribute to DSB repair is acetylation. H4 acetylation in yeast by Esa1, a HAT subunit of NuA4 chromatin remodeling complex, is critical for DSB repair (Bird et al. 2002). Also in mammalian cell lines, HDAC4 is associated with 53BP1 at the ionizing-radiation-induced foci, and HDAC 4 inhibition increases sensitivity to IR (Kao et al. 2003).

These modified nucleosomal partners are likely to act synergistically to transiently alter chromatin structure and function as well as to provide an initiation histone code that triggers the repair events.

Nonnucleosomal partners

The phosphorylation of H2AX represents an early response to DSBs (Stiff et al. 2004). In addition, H2AX foci colocalize with IRIF consisting of 53BP1, Brca1, MRN complex, and ATM in mammalian cells (Fernandez-Capetillo et al. 2002, 2004*b*; Paull et al. 2000). The generation of γ-H2AX foci triggering the recruitment of numerous proteins to the DSB site indicates that DSB repair is a highly amplified process in the cell. However, the initial recruitment of factors such as Nbs1, 53BP1, and Brca1 does not require the phosphorylation of histone H2AX, while the retention of these factors to form IRIF does require H2AX (Celeste et al. 2003). Furthermore, the recruitment of the IRIF components is a sequential event. γ-H2AX and 53BP1 foci form very rapidly (within minutes) in response to IR, followed by the appearance of Brca1 foci (within hours) (Celeste et al. 2003). HR repair factors, either Rad50 or Rad51, were found to colocalize with γ-H2AX foci after DNA damage following recruitment of the product of the tumor suppressor gene *BRCA1* and the formation of the Brca1 foci (Paull et al. 2000). The HR repair is mediated by the recruitment of HR repair proteins, Brca1, Rad50 and Rad51, through γ-H2AX (see Table 1).

After exposure to IR, the recruitment of NHEJ repair factors is also observed in human cells (Drouet et al. 2005), and this is a dose- and time-dependent process (Drouet et al. 2005). These proteins include DNA-PK (Ku70/80, DNA-PKcs), XRCC4, and DNA ligase IV proteins (see Table 1) (Drouet et al. 2005). As shown in the yeast system, repair factors from both HR and NHEJ repair pathways are recruited within the first 2 h of IR exposure. These proteins include Rad51, Rad52, Rad54, Rad55, and Yku80 (see Table 1) (Morrison et al. 2004). The difference in preference of the repair pathways between mammalian cells and yeast is still unclear. One might speculate that the histone code is different as a result of the different levels of H2AX and H2A phosphorylation in mammalian cells and in yeast, respectively. The relevance of phosphorylation in the overall repair process is underscored by the fact that wortmanin, a kinase inhibitor, when added before DSB induction eliminates focus formation by Rad51 and Brca1 (Paull et al. 2000). As well, it reduces the colocalization of RPA, a protein involved in both HR and NHEJ repair (see Table 1), which was recently shown to be associated with γ-H2AX in a time-dependent manner after DNA damage (Balajee and Geard 2004).

In addition to DNA repair factors, 3 chromatin remodeling complexes were found to associate with the phosphorylated Ser129 of histone H2A (γ-H2A) in yeast. They are NuA4 HAT, INO80, and SWR1 chromatin remodeling complexes (Downs et al. 2004; Morrison et al. 2004; van Attikum et al. 2004) (Fig. 3). The recruitment of these chromatin remodeling complexes to the DSB site through γ-H2A is a time-dependent process. The NuA4 HAT complex with acetyltransferase activity is recruited before the association of the ATP-dependent chromatin remodeling complexes INO80 and SWR1 (Downs et al. 2004). The NuA4 HAT complex is responsible for the acetylation of the N-terminal tail of histones H2A and H4 (Boudreault et al. 2003). All 3 chromatin remodeling complexes contain 1 common subunit, Arp4 (Downs et al. 2004). This subunit directly binds to γ-H2A. However, the interaction of INO80 and γ-H2A is mediated by the Nhp10 subunit of the INO80 complex (Morrison et al. 2004).

Recent evidence has shown that the highly conserved yeast chromatin remodeling complex, INO80, from the SWI/SNF super family, is involved in DSB repair in addition to the well-established function in transcription (Morrison et al. 2004). As mentioned earlier, this 12-subunit complex is recruited to the DSB site through the physical interaction between the Nhp10 protein subunit of the complex and γ-H2A (Morrison et al. 2004). In addition, the Rvb1/Rvb2 protein subunits of the complex are similar to the RuvB helicase in bacteria, which is involved in DNA recombination and repair. This implies the involvement of the INO80 complex in DSB repair (Morrison et al. 2004). The finding of the recruitment of this chromatin remodeling complex to the DSB site provides evidence for the unfolding of chromatin during DSB repair. Perhaps the remodeling–unfolding of the chromatin requires the assistance of chromatin remodeling machineries. In yeast, the INO80 complex is either directly involved in the Rad52 DNA repair pathway (HR) or might be part of the NHEJ pathway.

In addition to INO80, another remodeling complex, SWRI, which probably mediates the exchange of H2AX by nonphosphorylated H2AX (Cairns 2004) (Fig. 3D), can also participate in the chromatin remodeling process (Downs et al. 2004). In *Drosophila*, in which this exchange has been shown to take place (Kusch et al. 2004), and possibly in humans, this role is played by Tip60. Mammalian homologs of

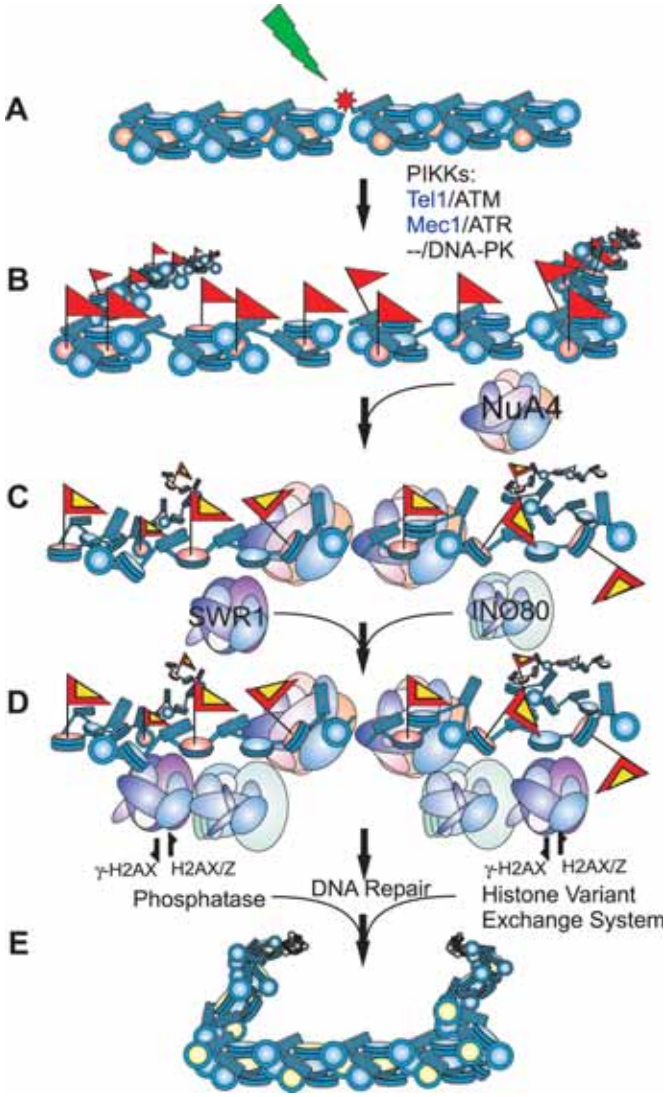


Fig. 3. A putative model for the role of H2AX phosphorylation in double-stranded DNA break (DSB) repair. (A) The induction of DSB in chromatin results in a rapid recruitment of homologous recombination (HR) or nonhomolgous end-joining (NHEJ) proteins followed by the activation and the recruitment of PIKK (phosphatidylinositol-3 kinase-like family of kinases) members (yeast and human homologs are shown in blue and black, respectively). In our chromatin representation, the circular discs in blue represent histone octamers, and the darker bands represent the DNA wrapping around the octamer. The orange discs represent H2AX-containing octamers. (B) PIKK kinases phosphorylate different targets, including H2AX. The phosphorylation of H2AX (red flags) by PIKK members flags the chromatin for recruitment of DNA DSB repair factors and remodeling complexes. The presence of γ-H2AX spanning a large region of the chromatin may alter the chromatin structure (Downs et al. 2000). (C) This, in combination with histone acetyl transferase (HAT) activities, such as those of the NuA4 complex that acetylate H2AX (yellow flags), may further alter chromatin structure and (or) provide the signaling for the recruitment of the ATP-dependent chromatin remodeling complexes INO80 (Inositol 80-containing complex [inositol-1-phosphate synthase (INO1)]) and SWR1 (SWRI, Swi2/Snf2-related adenosine triphosphatase complex) (Downs et al. 2004; Morrison et al. 2004; van Attikum et al. 2004) (D). (D) INO80 is a multi-subunit complex that binds directly to γ-H2AX through 1 of its constitutive subunits (see the text). The activity of INO80 is presumably responsible for the appearance of single-stranded DNA. The presence of RuvB-like protein subunits in INO80 suggests that this complex, in collaboration with SWRI, which mediates the exchange of γ-H2AX by nonphosphorylated H2AZ/H2AX, may play an important role in HR repair. However, as pointed out in the text the presence of phosphatases that hydrolyze the γ-phosphate cannot be ruled out. (E) Upon repair by either NHEJ or HR the chromatin fiber reverts to its folded organization.

some of the protein subunits from these chromatin remodeling complexes are well-documented. For example, almost all of the protein subunits from the yeast NuA4 HAT complex are conserved in humans (Doyon et al. 2004). The yeast NuA4 catalytic subunit Esa1 is very similar to the higher eukaryotic Tip60 (Kusch et al. 2004). The mammalian homolog of Arp4 is Baf53 (Downs et al. 2004). Therefore, the mechanism by which γ-H2AX mediates DSB repair in mammalian cells is expected to be similar to that of the yeast.

The hypothetical roles of γ-H2AX

Numerous pieces of evidence provide possible insights into the role of H2AX within the cell, in particular as it pertains to DSB DNA repair. Three hypotheses have been proposed in attempt to explain the role of γ-H2AX in this process.

First, the protein complexes formed at the DSB site may facilitate repair by holding the broken ends in position by themselves (Pilch et al. 2003). Studies in support of this hypothesis have shown that γ-H2AX is dispensable for the DNA strand joining in V(D)J recombination and retroviral DNA integration (Chen et al. 2000; Daniel et al. 2004). Cells respond to retroviral DNA integration in a way similar to DSB induction during V(D)J recombination by NHEJ (Daniel et al. 2004). Both V(D)J recombination and retroviral DNA integration involve enzymes, the RAG1/2 protein complex and the viral integrase complex, respectively, that hold the broken DNA ends together (Daniel et al. 2004). The presence of these protein complexes may be redundant with H2AX phosphorylation and, hence, H2AX could be dispensable in these 2 systems. Because no proteins have been found to hold the broken ends of DNA together in DSBs induced by IR and chemical agents, the presence of γ-H2AX is required for the proper function of DSB repair and genomic stability.

A second hypothesis proposes that γ-H2AX can act as a component in the signal transduction pathway or is part of the histone code that recruits DSB repair factors. In support of this notion, DNA repair factors such as Nbs1 and 53BP1, as well as Nhp10 (part of the INO80 complex in yeast), have been shown to interact physically with γ-H2AX (Celeste et al. 2003). Furthermore, the accumulation, but not the initial recruitment, of the repair proteins requires γ-H2AX (Celeste et al. 2003).

Finally, it has been proposed that γ-H2AX directly affects the chromatin structure in the region surrounding a DSB, making the region accessible to repair factors. In this regard,

histone H2AX has been implicated in the alteration of the chromatin structure in *Saccharomyces cerevisiae* (Downs et al. 2000). Because the C-terminal tail of H2AX is located near to the entry and exit sites of DNA in the nucleosomes, the phosphorylation of this tail could relax the chromatin fiber by altering the linker DNA trajectory (Ausió et al. 2001). This change in chromatin conformation may be the result of the presence of the 2 negative charges introduced by the phosphate at this location. Conversely, histone H1 (which binds to the same nucleosomal location) could have an inhibitory role in the process. Indeed, it has been experimentally shown that Hho1p linker histone suppresses HR (Downs et al. 2003; Harvey and Downs 2004). The opening of the chromatin fiber would allow the recruitment of the H2AX-dependent DSB repair proteins to the chromatin forming IRIF.

These hypotheses are not necessarily mutually exclusive. Indeed, it is possible that several of the proposed mechanisms may act synergistically to facilitate DNA repair. For example, repair factors could be directly recruited to DSB by the phosphorylation of the SQE motif at the C-terminal tail of H2AX, which could also be involved in the alteration of chromatin conformation.

A working model

The histone modifications, and the recruitment of the chromatin remodeling complexes and DNA repair factors are an essential part of repairing DSBs. The phosphorylation of H2AX precedes most of the repair mechanisms. It is possible to envisage a model in which the role of γ-H2AX in DSB repair is that of a signal mediator and DSB repair facilitator. γ-H2AX could act as part of the histone code that flags the region of DNA damage. This would allow the activation and recruitment of the critical DNA repair factors in both HR and NHEJ pathways as well as the recruitment of chromatin remodeling complexes. γ-H2AX could also directly, or in combination with histone acetylation by NuA4 complex, alter the conformation of chromatin facilitating the recruitment. The subsequent recruitment of chromatin remodeling complexes could additionally alter the conformation of the chromatin fiber for further facilitation of the DSB repair process (see Fig. 3).

Although H2AX sequence and function have been highly conserved throughout evolution (see Figs. 1 and 2), the mode of action of this histone H2A variant might be slightly different in different species. The role of H2AX in the yeast and mammalian systems has been studied extensively. However, there are major differences between the 2 systems. First, yeast has a much smaller genome than mammals. Also, because the yeast orthologs (HTA1, HTA2) of H2AX make up 95% of the H2A population, the involvement of chromatin modification through phosphorylation at the C-terminus might be more significant than that observed in mammalian systems. Therefore, this may affect the frequency of γ-H2AX-containing nucleosomes in the yeast chromosome. Another major difference is that yeast employs HR as the predominant pathway to repair DSB whereas the mammalian systems, including human, mainly use NHEJ. After IR exposure, yeast contains a huge amount of phosphorylated H2A-containing nucleosomes because of the large occurrence of these H2AX orthologs in the yeast genome. This most likely results in a major alteration of the chromatin folding (Downs et al. 2000), which probably facilitates the HR repair pathway. In mammals, the more subtle changes in the chromatin folding may simply reflect the preferential use of the NHEJ DSB repair pathway by these organisms.

Concluding remarks

At present, the detailed molecular mechanisms of DSB DNA repair as they pertain to NHEJ or HR in yeast and in metazoans are not completely understood. Also, whether the structural implications of γ-H2AX for chromatin transcend the signaling role or not still awaits further elucidation. Despite the many remaining unanswered questions, γ-H2AX exhibits a ubiquitous occurrence in DSB repair throughout the eukaryote kingdom regardless of the DSB origin and the different pathways used for its repair. There is no doubt that the coevolution of H2AX with the canonical H2A counterpart has played an important role in the maintenance of the eukaryotic genome integrity.

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