DNA repair: **Spot(light)s on chromatin**

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Chromatin modifications regulate many nuclear processes. Recent studies on the phosphorylation of a histone 2A variant have revealed that this chromatin modification is a general and evolutionarily conserved cellular response to DNA double-strand breaks.

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Cell survival and maintenance of genome integrity are critically dependent on efficient repair of DNA double-strand breaks. Double-strand breaks are an inescapable form of DNA damage for proliferating cells. They are generated when DNA replication forks collapse because of obstructions in the template strands [1]; by the action of exogenous DNA damaging agents [2]; and during programmed DNA rearrangements [3]. Eukaryotic cells have evolved multiple systems for repairing double-strand breaks, which use either homologous recombination or non-homologous end joining at their mechanistic core [2]. To operate adequately, these repair systems are intimately coupled to DNA damage surveillance and signaling functions [4]. A remarkable finding by the Bonner lab [5,6] has shed new light on a very early step in the cellular response to double-strand breaks.

Bonner and colleagues [5,6] have discovered that, within seconds after induction of genomic double-strand breaks by ionizing radiation, mammalian cells respond by a specific and extensive chromatin modification around the damaged sites, in the form of phosphorylation of a histone 2A variant. In doing so, they have developed the tools to detect double-strand breaks essentially at their onset. Recently, it has become clear that this modification is not limited to mitotic double-strand break repair in mammalian cells. Instead, it appears to be a general cellular response in many, if not all, processes that involve double-strand break intermediates, including mitotic double-strand break repair in yeast [7], V(D)J recombination — the process by which variable (V), diversity (D) and joining (J) elements are joined to generate mature immunoglobulin and T-cell receptor genes — in lymphoid cells [8] and meiotic recombination in mice [9].

Chromatin modification during double-strand break repair

The eukaryotic genome is organized into a nucleoprotein structure called chromatin. The basic unit of eukaryotic DNA organization is the nucleosome, which consists of a stretch of 146 base pairs of DNA, wrapped around two copies each of the histone proteins H2A, H2B, H3 and H4, in addition to linker DNA bound by histone H1 [10]. Repeats of this unit are packaged into higher-order chromatin structures. Three classes of H2A are present in mammalian cells, and among these histone H2AX makes up 2–25% of the H2A pool [5].

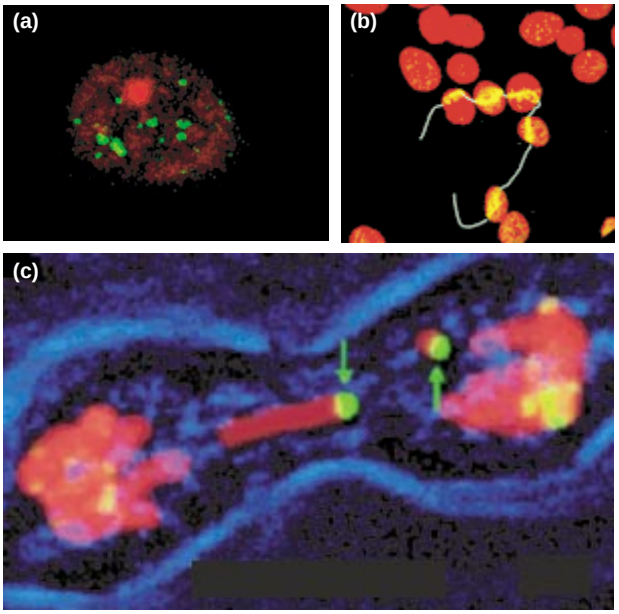
Rogakou *et al.* [5] discovered that, following exposure to various agents that cause double-strand breaks, mammalian cells responded by phosphorylating serine 139 in the unique, and evolutionary conserved, carboxy-terminal extremity of H2AX. This response was directly proportional to the amount of double-strand breaks that were introduced, and reached a maximum after 10–30 minutes. Hundreds to thousands of phosphorylated H2AX (γ-H2AX) molecules were formed per double-strand break, implying that a single double-strand break leads to the modification of a chromatin domain containing megabase pairs of DNA [5]. Furthermore, using an antibody specific for γ-H2AX and by inducing double-strand breaks in partial nuclear volumes, Rogakou *et al.* [6] visualized the spectacular modification of chromatin domains around the damaged sites (Figure 1). To date, detection of γ-H2AX is probably the most specific and efficient technique for spotting double-strand breaks in cells.

γ-H2AX formation during V(D)J recombination

Whether γ-H2AX formation is limited to the repair of DNA damage-induced double-strand breaks in somatic mammalian cells, or whether it is a more general cellular response to double-strand breaks was addressed by the Nussenzweig, Reid and Bonner labs [8]. Double-strand breaks are also transient intermediates during a number of programmed genomic rearrangements. For instance, during the process of V(D)J recombination in lymphoid cells, immunoglobulin and T-cell receptor genes are reorganized by site-specific recombination [3]. The process is initiated by double-strand breaks introduced by the RAG1 and RAG2 proteins. The general double-strand break repair machinery is then recruited to reassemble the broken ends into functional genes.

To address whether γ-H2AX is formed during V(D)J recombination, γ-H2AX labeling by immunofluorescence was combined with detection of DNA by fluorescence *in situ* hybridization to pinpoint the T-cell receptor α locus during V(D)J recombination. In 20% of freshly isolated immature murine thymocytes, which are active in V(D)J recombination, Chen *et al.* [8] detected one to two γ-H2AX

Figure 1



Visualization of the γ-H2AX response following DNA double-strand breaks. (a) Nucleus of an irradiated human MCF7 cell after exposure to 0.6 Gy and 30 minutes recovery. Nucleic acid staining is red and γ-H2AX foci are green. (b) Laser-directed DNA double-strand breaks in MCF7 cells. The path of the laser is indicated with a white line. (c) Broken chromosome arms in a *Muntiacus muntjak* cell (2n = 6) undergoing a defective mitosis. Green arrows point to ends of isolated chromosome arms with γ-H2AX foci. (Images provided by E. Rogakou, reproduced with permission from [6].)

foci (Figure 2a). The γ-H2AX foci were dependent on the presence of the Rag proteins that mediate V(D)J recombination, and most of the γ-H2AX localized to the T-cell receptor α locus.

Interestingly, Chen *et al.* [8] found that γ-H2AX nearly always coincided with the Nijmegen breakage syndrome protein (Nbs1), which is part of a complex, also containing Rad50 and Mre11, that has been implicated in the cellular response to double-strand breaks. This colocalization suggests a role for the Rad50–Mre11–Nbs1 complex in V(D)J recombination. It could help explain the high rates of chromosomal translocations involving immunoglobulin and T-cell receptor loci, and the increased susceptibility to lymphoid malignancies observed in patients with a defect in Nbs1 or Mre11 [11].

γ-H2AX formation during meiosis

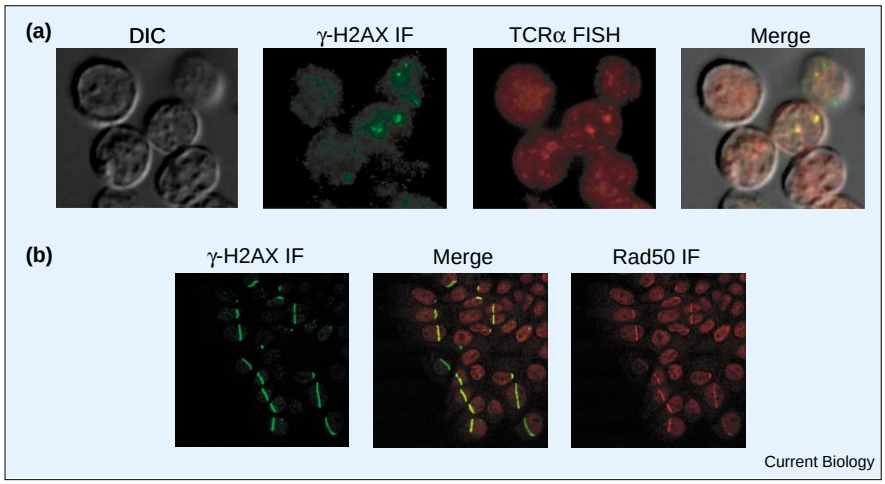
Double-strand breaks are also intermediates in the generation of genetic diversity during meiotic recombination in *Saccharomyces cerevisiae* [12]. They appear prior to synapsis, the process that ultimately ensures proper disjunction of homologous chromosome pairs. In mice, meiotic double-strand breaks have not been detected physically, but recent results [13,14] have indicated that recombination between homologous chromosomes is initiated by double-strand breaks that appear before the initiation of synapsis. With this in mind, Burgoyne and colleagues [9] reasoned that the detection of γ-H2AX could be used to mark double-strand breaks during meiosis. They indeed detected γ-H2AX during the early stages of meiotic prophase I, where chromosomes are not yet synapsed. At later stages, the number of the γ-H2AX signal dropped dramatically, disappearing from the chromatin of fully synapsed homologous chromosomes. Furthermore, this pattern of γ-H2AX formation was not detected during meiosis of an asynaptic mutant. These results provide compelling evidence that, just as in yeast, meiotic recombination in mice is initiated by double-strand breaks whose processing is linked to homologous chromosome synapsis.

Functional significance of γ-H2AX formation

The Jackson lab [7] addressed whether γ-H2AX formation is functionally significant using yeast genetics. *S. cerevisiae* has only a single H2A variant, but it does have the carboxy-terminal extension specific for the mammalian H2AX subfamily. Downs *et al.* [7] showed by genetic ablation that this extension is not essential for cell viability. However, the truncation mutation, as well as a point mutation changing the critical serine residue into alanine, did affect the survival of cells following the induction of double-strand breaks. Further analysis of strains lacking the H2A carboxy-terminal extension showed that γ-H2A is not critical for cell-cycle checkpoint functions after damage induction, or for transcriptional induction of DNA damage-responsive genes. The efficiency of repairing double-strand breaks, however, was seen to be affected when assessed by a plasmid reporter assay for non-homologous end joining. In contrast, an indirect measurement of double-strand break repair through homologous recombination indicated that this repair system was only marginally affected. Thus, in yeast, histone H2A may have a role in DNA repair that is perhaps more critical during non-homologous end joining. But it cannot yet be excluded that the effect of γ-H2AX formation on DNA repair is indirect, through a local difference in chromatin conformation resulting from phosphorylation [7].

In a collaborative effort, the Gellert and Bonner labs investigated the relationship between double-strand break-induced γ-H2AX foci and those formed in human cells by double-strand break repair factors, specifically Rad50, Rad51 and Brca1 [15]. Paull *et al.* [16] found that all three repair factors can localize with γ-H2AX and genomic sites of DNA damage (Figure 2b). Time-course experiments revealed that γ-H2AX foci appear first. They are generally followed by colocalization of Brca1 in the foci, and then by either Rad50 or Rad51 in different subsets of cells.

Figure 2



γ-H2AX localizes to sites of DNA double-strand breaks. **(a)** In freshly isolated murine thymocytes, γ-H2AX localizes to the T-cell receptor α (TCRα) locus detected by fluorescence *in situ* hybridization (FISH). The cells are visualized by differential interference contrast (DIC) microscopy. **(b)** Rad50 detected by immunofluorescence (IF) localizes to γ-H2AX and laser-induced DNA double-strand breaks in human MCF7 cells. The laser path is clearly apparent. (Images for panels (a,b) provided by M. Difilippantonio and A. Nussenzweig, and T. Paull, respectively.)

Given that Rad51 is very likely involved in homologous recombination only, while Rad50 is probably required for both homologous recombination and non-homologous end joining, it is conceivable that the γ-H2AX response of somatic human cells is important for both double-strand break repair systems.

Kinases involved in γ-H2AX formation

The kinases involved in the responses of mammalian cells to double-strand breaks include the DNA-dependent protein kinase (DNA-PK), the ataxia telangiectasia mutated protein (ATM) and the AT-related protein (ATR) [4]. These serine/threonine kinases belong to the family of phosphatidylinositol-3-OH kinase-related kinases (PIKKs), which have the amino acid motif SQE as a target site for phosphorylation. Two members of the PIKK family, Mec1 and Tel1, are conserved in *S. cerevisiae* [4]. The SQE motif is present in the conserved carboxy-terminus of human H2AX and in the yeast equivalent. Experiments with yeast *mec1* and *tel1* mutant strains showed that γ-H2A formation upon double-strand break induction was largely dependent on Mec1 [7]. In the *mec1* strain, γ-H2A formation was not entirely abolished, but the residual phosphorylation was no longer detectable in a *mec1 tel1* double mutant strain. As Mec1 is able to phosphorylate H2A *in vitro*, these results suggest that Mec1 is the primary kinase involved in γ-H2A formation.

In mammalian cells the role of the kinases was addressed with the use of wortmannin, a potent inhibitor of the entire PIKK family [4]. Paull *et al.* [16] showed that pre-incubation of cells with wortmannin before double-strand break induction abolished γ-H2AX focus formation, as well as the later appearance of Rad51 and Brca1 foci. Interestingly, when cells were incubated with the drug 5 minutes after damage induction, no effect on focus formation by any of these proteins was detected. These results implicate PIKK proteins in the phosphorylation of H2AX upon double-strand break induction, and γ-H2AX as the seed for focus formation by other repair proteins such as Rad51 and Brca1. Examination of the γ-H2AX response in DNA-PK and/or ATM-deficient mammalian cells led to the conclusion that, in addition to DNA-PK, at least one other kinase, possibly ATM and/or ATR, can phosphorylate H2AX after double-strand break formation [16]. This functional redundancy could reflect the critical importance of this very early event in the cellular response to double-strand breaks.

Future directions

Given that the γ-H2AX response has now been detected during mitotic double-strand break repair, V(D)J recombination and meiosis, it is likely a truly general cellular response to double-strand breaks. One might therefore expect that the formation of γ-H2AX foci will shortly be demonstrated in other processes in which double-strand breaks have been implicated, such as class switch recombination [17], somatic hypermutation of immunoglobulin genes [18,19] and transposition [3]. Numerous other intriguing questions remain to be answered about the involvement of γ-H2AX in the cellular response to double-strand breaks. For example, does this histone modification in mammalian cells assist DNA repair or DNA damage signaling, or both. γ-H2AX might promote these processes by recruiting factors to damaged sites. Another possibility is that γ-H2AX could trigger changes in higher-order chromatin structure around damaged sites in a way that would facilitate DNA repair or trigger the signaling response.

The organization of higher-order chromatin structures in interphase cells is still poorly understood. Some recent studies [20] propose that interphase chromatin fibers might be organized into domains of several megabase

pairs, which in turn, might contain smaller DNA loops. Remarkably, Bonner and colleagues [5,6] have estimated that a single genomic double-strand break induces the modification of a chromatin domain containing megabase pairs of DNA. It is therefore tempting to speculate on the nature and dynamics of γ-H2AX foci. Does γ-H2AX formation initiate at or near broken ends, and then spread out along the chromatin fibers? Does γ-H2AX spread from a loop containing a double-strand break to other smaller loops within one chromatin domain? Are changes in superhelix density in the broken loops sensed by the kinases responsible for phoshorylating H2AX, or do they first recognize broken ends? Is γ-H2AX modification of chromatin fibers used by the various DNA repair systems to synapse broken ends or bring them in proximity of a homologous template? These are only a few of the many intriguing questions that remain to be answered.

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