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REVIEW ARTICLE

Modifying chromatin architecture during the response to DNA breakage

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Abstract
The human genome is compacted in a dynamic macromolecular complex, chromatin, whose structure presents a considerable barrier to the cellular machinery which responds to DNA double-strand breaks. This review discusses current understanding of the processes that modify chromatin architecture to enable, first, the sensing of DNA breakage, next, the assembly of the protein complexes that resolve the lesion, and finally, the restoration of epigenetic marks after its repair. The importance of these fundamental biological processes is underscored by the growing appreciation that they are aberrant in human diseases, and that their modulation could provide new approaches to disease therapy.

Keywords: *DNA repair; histone; protein phosphorylation; protein ubiquitylation; γH2AX; double-strand DNA break; homologous recombination; end joining*

Chromatin packages the genome and regulates its function

The human genome is duplicated, read and repaired in cells not as a naked DNA strand, but as a complex and dynamic macromolecular structure, chromatin, in which the DNA is bound intimately to proteins. Chromatin serves two main functions; to compact DNA, and to regulate its function. The physical compaction of genomic DNA in chromatin helps to overcome the problem of packing its ~2 m length in the ~200 μm^3 average nuclear volume in a human cell. About 146 bp of double-stranded (ds) DNA wraps around an octameric complex of the core histones H2A, H2B, H3 and H4 to form nucleosomes, which resemble beads spaced ~200 bp apart on the ds DNA "string". Further compaction involves the linker histone H1, which has divalent DNA-binding sites that bridge inter-nucleosomal DNA, folding and organizing nucleosomes into the so-called 30 nm chromatin fiber. Higher-order chromatin structures are formed through the binding of non-histone proteins, and include regions of euchromatin or heterochromatin, distinguished by their apparent degree of compaction and the density of associated proteins like the heterochromatin protein 1 (HP1) family.

The second role of chromatin is to control genome duplication and function, through dynamic changes in its structure that achieve distinct states permissive for different transactions (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Polo and Almouzni, 2006; Downs *et al.*, 2007). Broadly speaking, chromatin structure can be altered through three different routes in human or other complex eukaryal cells. *Histone chaperones* promote the incorporation of specific histone variants which alter structure to fulfil distinct functions. *Chromatin remodelers* disrupt histone–DNA interactions in an ATP-dependent manner, and can mobilize nucleosomes across DNA, or facilitate the removal of histones from the nucleosome. Finally, a *histone code* specifies different chromatin states through covalent post-translational modifications (PTMs) to the histones. The code is, in part, translated via non-histone proteins – termed *effectors* – which contain domains that bind different histone-tail modifications, and are recruited through them to specific regions of chromatin (Taverna *et al.*, 2007).

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Histone PTMs involve lysine (K), arginine (R), serine (S), threonine (T), tyrosine (Y), histidine (H) and glutamic acid (E), located either in the N- or C-terminal "tails" of the histones, or less frequently, in their globular core domains. Modifications to these residues include the addition of small chemical groups such as methyl (me), acetyl (ac), or phosphate (p) moieties, as well as the conjugation of ubiquitin (Ub) or related peptides. There is some evidence that histone PTMs can alter chromatin compaction by affecting the recruitment or interactions of nucleosomal proteins. For example, H4K16 acetylation, a hallmark of transcriptionally active chromatin, restrains the folding of the 30 nm fiber into higher-order structures (Robinson *et al.*, 2008; Shogren-Knaak *et al.*, 2006). More specifically, however, histone PTMs recruit specific effector proteins, which contain recognition modules that have evolved to recognize different PTMs at discrete positions in the histone sequence. These include the bromodomain, which recognizes Ac modifications to K residues (Kac) at several positions in the histone sequences, the Royal family domains (e.g. chromodomain, Tudor domain) recognizing Me modified K (or R) residues (Kme, Rme), or the BRCT domain, which binds to histone H2AX modified by P addition to a specific S residue in its tail (Sp).

Well over 50 different sites for histone PTMs, and at least eight types of covalent modification, have so far been reported, comprising the individual elements of histone code. There is increasing evidence that the overall pattern of the individual elements in given genomic regions, rather than any single PTM per se, determines regional chromatin structure and function. A multiplicity of effector proteins can be recruited to particular types of PTMs in a manner dependent on their position in the histone sequence. When taken together, this combination of individual elements and larger patterns allows chromatin to assume a vast repertoire of different structural states in different parts of the genome.

Changes in chromatin structure may also be achieved via a fourth route. PTMs that occur not on histone tails, but the effector proteins that bind them, have recently been proposed to modulate chromatin structure through a so-called sub-code. For example, HP1γ phosphorylation can regulate its transcriptional silencing activity in experimental conditions (Lomberk *et al.*, 2006). However, whether such modifications play a physiologically relevant role is not yet well established.

Modifying chromatin architecture during the response to DNA breakage

The ability to resolve DNA double-strand breaks (DSBs) is central to the maintenance of genome integrity (Hartlerode and Scully, 2009). DSBs can arise endogenously (for example, in the wake of stalled DNA replication forks in proliferating cells), or exogenously, through exposure to ionizing radiation or other genotoxic agents, and their presence evokes a complex sequence of events that culminates in resolution. Broadly speaking, these events proceed in three overlapping phases, during which discrete cellular machineries *sense* the presence of broken DNA, *assemble* a complex of proteins around it to repair the lesion, and finally, *restore* the repaired region to its original configuration. At least two distinct pathways are used in vertebrate cells for DSB repair. In non-homologous end-joining (NHEJ), the broken ends are religated after processing, which may add or remove nucleotides, resulting in error-prone repair. During homologous recombination (HR), DSBs undergo error-free repair by a mechanism which retrieves intact genetic information from a sister chromatid or rarely, a homologous or non-homologous chromosome.

Overcoming the constraints imposed by chromatin architecture: an overview

The successful execution of the events involved in the response to DNA breakage mandates that some of the physical constraints (Luijsterburg *et al.*, 2008) imposed by the architecture of eukaryotic chromatin can be overcome. One major constraint arises from genome compaction, the net result of at least two forces. The crowding of macromolecules at very high concentrations in the cell nucleus favors the entropy-driven, spontaneous aggregation and thus, compaction, of genomic DNA. Compaction is reinforced and extended through protein–DNA interactions in chromatin, which, as discussed earlier, organize the genome into poly-nucleosomal arrays, 30 nm chromatin fibers and higher-order structures. Where DNA repair by excision has been reconstituted *in vitro*, even the nucleosomal incorporation of DNA can inhibit the repair process (e.g. Wang *et al.*, 1991; Hara *et al.*, 2000). Thus, the relaxation or loosening of chromatin structure is probably essential for DSB repair *in vivo*. However, remarkably little is known about the mechanisms by which this is achieved, an overview of which follows.

Bonner and colleagues first showed that phosphorylation of the H2A variant H2AX, which constitutes some 5–25% of the H2A pool in different mammalian cell types, is induced within minutes after the exposure of cells to IR. This modification occurs on S139 in the C terminal tail of H2AX, a motif conserved in evolution (Rogakou *et al.*, 1998; 1999). The abundance and spatial distribution of the S139-phosphorylated form of H2AX (γH2AX) in irradiated cells was found to correspond to a large region (~2 Mb) of DNA surrounding each DSB, forming visible nuclear foci in chromatin that reliably mark sites of breakage (Rogakou *et al.*, 1998; 1999).

Members of the phosphatidylinositol-3 kinase-like family of kinases (PIKK) have been implicated in γH2AX formation, including ataxia telangiectasia mutated (ATM), ATM-and Rad3-related (ATR) ATM related kinase (ATX), and DNA dependent protein kinase (DNA-PK). Each may be the principal mediator for H2AX modification triggered by DSBs formed in different settings (Burma *et al.*, 2001; Ward and Chen, 2001). Thus, ATM seems to be the principal player in the IR response, whereas ATR predominates during replication stress. However, it also clear that there is functional redundancy between different PIKK members, because H2AX phosphorylation persists in cells lacking individual kinases (Fernandez-Capetillo *et al.*, 2002; Brown and Baltimore, 2003).

It is conceivable, but not demonstrated, that the addition of negatively charged P groups to thousands of H2AX molecules in the nucleosomes surrounding each DSB could affect the recruitment or interactions of nucleosomal proteins. Moreover, γH2AX formation is followed by an increase in the acetylation of distinct K residues in the core histones H3 and H4 (Tamburini and Tyler, 2005), which interferes with the interaction of the core octamer with DNA in nucleosomes. In human cells, the histone acetyl transferase (HAT) complex TIP60, which is essential for DSB repair, has been shown to acetylate H2A, H3 and H4 (Yamamoto and Horikoshi, 1997; Kimura and Horikoshi, 1998). Interestingly, TIP60 binds to ATM, and may activate ATM by acetylation (Sun *et al.*, 2005), exemplifying that enzymes recruited to DSBs to alter the histone code may also create PTMs on non-histone proteins involved in this response.

Besides their possible effects on chromatin structure, H2AX phosphorylation and core histone acetylation also assist in the recruitment of chromatin remodeling complexes to DSB sites. These include INO80 and SWR1, conserved members of the SWI2/SNF2 superfamily of ATP-dependent chromatin remodeling complexes, each of which includes a component that mediates recruitment to γH2AX. These complexes remodel chromatin at DSB sites in different ways, INO80 by displacing nucleosomes (Shen *et al.*, 2000), or SWR1, by incorporating variant histones into them (Krogan *et al.*, 2003; 2004); their net effect is to relieve nucleosomal packaging, allowing access to DNA.

Changes in the dynamics of chromatin-bound proteins at the sites of DSBs may also assist in opening the structure of chromatin. The formation of visible γH2AX foci at DSBs is followed by the accumulation in them of many proteins known to participate in their sensing or repair (Paull *et al.*, 2000). These not only include PIKK members, MDC1 or 53BP1, which play a pivotal role in signaling DSBs, but also molecules implicated in their resolution, such as the MRE11-RAD50-Nbs1 (MRN) complex, or enzymes like the RAD51 recombinase. Fluorescence studies demonstrate that the accumulation of several of these proteins in foci is accompanied by changes in their dynamics that prolong their retention in these structures (Essers *et al.*, 2002; Yu *et al.*, 2003), which could in turn affect the local structure of chromatin.

Whilst many of these conjectures concerning how histone modifications, protein binding or other changes in the vicinity of DSBs may affect chromatin structure seem plausible, in reality however, very little is known about their precise effects at the structural or physical level. Our lack of knowledge is all the more striking when the higher-order organization of chromatin beyond the poly-nucleosomal array is considered. Chromatin is organized into chromosomes in somatic cells that appear to occupy discrete territories (~2 μm in diameter) within the cell nucleus (Cremer *et al.*, 2000). Within these territories, chromatin is organized in complex loops that are intimately attached to a proteinaceous, nuclear scaffold (Cremer *et al.*, 2001). Multiple loops that fall within or between chromosome territories may come together in shared regions or "factories" where major transactions like transcription may take place (Fraser and Bickmore, 2007; de Laat *et al.*, 2008). How this level of chromatin organization may relate to the processes involved in the DSB response remains wholly unclear. What is more, the topographic features of individual chromosomes examined by electron microscopy are suggestive of even higher levels of organization. Thus, chromosomes contain multiple, electron-dense chromatin domains of ~0.3 mm, surrounded by intervening spaces apparently devoid of chromatin (Fakan and van Driel, 2007). It has been proposed that chromatin may be accessible to the macromolecular complexes that carry out transactions like transcription and DNA repair primarily at the interface between the chromatin domains and the surrounding space (e.g. Cmarko *et al.*, 2003; Solimando *et al.*, 2009). If or how chromatin may be translocated to these accessible regions from within chromatin domains is unknown.

These general considerations underscore the lack of physical and structural understanding of how chromatin architecture can be modified during biological processes. However, a large number of observational studies over the past decade catalog the changes that may occur in damaged chromatin during the DSB response. Some of the key alterations, and their potential significance, are discussed in relation to the processes that lead to the sensing of broken DNA, the assembly of DSB-response complexes around it, and the restoration of chromatin following DSB repair.

Sensing the presence of broken DNA

How DNA breakage is sensed, and how this leads to H2AX phosphorylation, is poorly understood**.** The

earliest steps in this process are likely to involve alterations in chromatin structure. Thus, the exposure of cells to hypotonic stress, which is expected to trigger changes in chromatin structure, activates the ATM kinase (Bakkenist and Kastan, 2003). Inactivation of the hMOF or TIP60 HAT complexes, which modify K residues in the tails of histones H3 and H4, suppresses ATM activation (Gupta *et al.*, 2005; Sun *et al.*, 2005). The prolonged tethering to undamaged chromatin of damage-responsive proteins such as MRE11, NBS1 or MDC1 is enough to initiate γH2AX formation (Soutoglou and Misteli, 2008). These observations argue that DSB-induced chromatin alterations may help to trigger the cellular response to these lesions, but provide little insight into how they may do so.

Chromatin decompaction

More direct evidence comes from studies using a photoactivatable version of the GFP-tagged core histone H2B to examine chromatin structure at the site of laser-induced DSBs (Kruhlak *et al.*, 2006). Damaged chromatin undergoes an energy-dependent local expansion within seconds after DNA damage, corresponding to a decrease of about 35% in the density of 30 nm chromatin fibers in this region. These events are dependent on ATP turnover, but require neither H2AX nor ATM, suggesting a very proximal role in DSB sensing. The rapid and localized decompaction in chromatin structure triggered by DSBs may facilitate the recruitment of molecules that sense, signal or repair the lesions (Figure 1). In turn, the recruitment of DNA-binding proteins to the vicinity of DSB sites could help to maintain the structural "loosening" of chromatin in this region until repair has been completed.

Other changes in chromatin structure may also precede ATM activation by DSBs (Kim *et al.*, 2009). HMGN1, a non-sequence-specific nucleosome-binding protein, is necessary for ATM recruitment to chromatin both to undamaged chromatin as well as to DNA breaks. Its depletion or inactivation reduces ATM activation after DNA damage, in a manner that requires HMGN1-dependent acetylation of histone H3K14; this requirement can be bypassed by chemical inhibition of histone de-acetylation. These findings suggest that HMGN1 can regulate ATM activation and histone modification at sites of DNA breakage by modulating chromatin structure (Figure 1).

HP1β mobilization and recruitment

Another proximal event implicated in DSB sensing (Figure 1) may be the rapid and transient mobilization of the chromatin factor HP1β from damage sites (Ayoub *et al.*, 2008). Laser-induced DNA breaks swiftly mobilize GFP-HP1β, marked both by its visible dispersal from heterochromatic foci where it is present in high concentrations, and by an increase in its dynamics measured by FRAP locally within the damaged chromatin region. Local changes in histone-tail modifications are, however, not apparent. Instead, phosphorylation of HP1β on amino acid Thr51 accompanies mobilization, releasing HP1β from chromatin by disrupting hydrogen bonds that fold its chromodomain around H3K9me. Inhibition of casein kinase 2 (CK2), an enzyme implicated in DNA damage sensing and repair, suppresses Thr51 phosphorylation

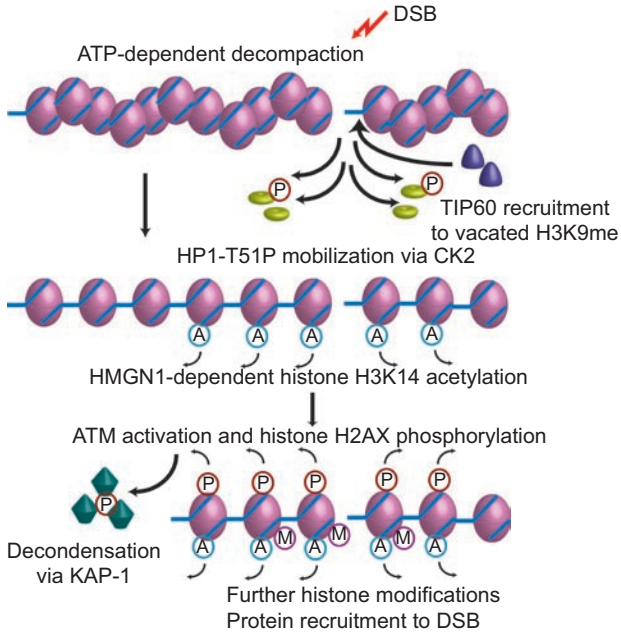

Figure 1. Modifying chromatin architecture at DNA breaks. Processes that modify chromatin architecture at DSBs are shown. Neither their hierarchy nor their interdependencies have yet been elucidated. ATP-dependent chromatin decompaction occurs rapidly after DNA breakage, relaxing 30 nm chromatin fibers by ~30% of their pre-damage state as visualized by transmission electron microscopy. HP1β is mobilized within seconds from chromatin surrounding DNA breaks, marked both by dispersal that is most evident in heterochromatic regions which contain a high concentration of the protein, as well as by an increase in its dynamics within the damaged region measured by fluorescence techniques. Mobilization depends on the casein kinase 2 (CK2)-dependent phosphorylation of HP1β Thr51 (circledP). It enables the recruitment to vacated histone H3K9me sites of the histone acetyltransferase TIP60, which in turn activates ATM. Acetylation (circled A) of histone H3K14 depends on the sequence-independent DNA-bending protein HMGN1; this process is necessary for efficient activation of the ATM kinase. ATM activation works directly to trigger phosphorylation of the variant histone H2AX on S139, ranging to ~2 Mb either side of the DSB. It also phosphorylates mediators like KAP-1, which induces chromatin decondensation throughout the nucleus of damaged cells. Further histone modifications (e.g. methylation, circled M), as well as the recruitment and binding of DSB-response proteins, may help to maintain the relaxed structure of chromatin in the vicinity of DSBs initiated by earlier processes.

and HP1β mobilization in living cells. CK2 inhibition, or a constitutively chromatin-bound HP1β mutant, diminishes H2AX phosphorylation. These findings suggest that a CK2-mediated signaling cascade helps to initiate the DNA damage response, altering chromatin by modifying a histone-code mediator protein, HP1, but not the code itself.

The visible dispersal of HP1β from damaged heterochromatic foci is transient; the protein visibly accumulates again there ~60 min afterwards (Ayoub *et al.*, 2008; 2009a). Interestingly, a recent paper (Luijsterburg *et al.*, 2009) describes a more rapid phenomenon of HP1β recruitment, with a T1/2 of ~75 s, after laser-irradiation of large strips of the nucleus, rather than single heterochromatic foci. This phenomenon of HP1β recruitment to DSB sites is neither dependent on Thr51, nor suppressed by the chemical inhibition of CK2, but instead requires the chromoshadow domain of HP1β. Recent studies suggest that HP1β mobilization and recruitment are mechanistically distinct, but spatially and temporally overlapping, behaviors of the protein in the vicinity of damaged chromatin (Ayoub *et al.*, 2009b). In a model to explain the bimodal response of HP1β to DNA breakage (Figure 2), the balance between these behaviors may differ according to nature of the damaged chromatin region, and the local concentration of HP1β found there before damage. Visible dispersal, most evident after damage to heterochromatic regions containing relatively high concentrations of HP1β, is accompanied by changes in the dynamics of HP1β that reflect mobilization from chromatin. Visible HP1β dispersal is transient, and over time, the balance between dispersal/recruitment tilts towards visible HP1β accumulation at and around damage sites. Whereas HP1β dispersal and mobilization depend on the phosphorylation of Thr51 by a CK2-dependent mechanism, HP1β recruitment is independent of Thr51, but instead, requires the chromoshadow domain.

How changes in HP1β dynamic behavior after DNA breakage can modulate H2AX modification is becoming clear. The CK2-dependent phosphorylation of Thr51 in HP1β, and the consequent mobilization of HP1β from chromatin, may provide a trigger for ATM activation. Thus, HP1 mobilization reveals in the vicinity of DNA breaks the histone H3 tails methylated on K9 to which HP1 would otherwise be stably bound. The TIP60 HAT complex is recruited to these exposed H3 K9me sites, activating the enzyme to modify ATM and enhance PIKK activity (Sun et al., 2009). However, chemical inhibition of the PIKK kinases can moderately diminish Thr51 modification (Ayoub *et al.*, 2008), suggesting that there is cross-talk between the pathways, rather than a strictly linear hierarchy of events. A non-exclusive possibility is that HP1β mobilization may in some way enhance the activity of PIKKs in the vicinity of DNA breaks, by altering the structure of chromatin to facilitate PIKK recruitment to damage sites, or improve their access to substrates. Enhanced PIKK activity could, in turn, promote HP1β modification, triggering a local, looping cascade of events to signal DNA breakage. The effect of PIKK enzymes on HP1β phosphorylation seems likely to be indirect, since HP1β neither contains the consensus phosphosite motif, nor is it represented in proteomic screens for PIKK substrates (Matsuoka *et al.*, 2007).

The spreading of H2AX phosphorylation around a DSB skips over heterochromatic regions marked by HP1 binding (Cowell *et al.*, 2007; Kim *et al.*, 2007), implying that features of the heterochromatin structure dependent on the HP1 proteins may prevent the activation of H2AX phosphorylation after DNA damage. In this light, HP1β mobilization from damaged chromatin could trigger structural alterations that enhance permissiveness for γH2AX formation, and for subsequent events in the DSB response. For example, as noted earlier, it has recently been shown that HP1 mobilization from DSBs unveils H3 K9me sites in the chromatin surrounding the lesion. HP1 displacement from H3 K9 me sites enables the recruitment and activation of the TIP60 HAT complex, which in turn modifies and activates ATM (Sun et al., 2009). However, the transience of HP1β mobilization from damage sites via CK2-mediated Thr51 modification, as well as the bimodal response of temporally overlapping mobilization and recruitment soon after DNA breakage, may reflect a requirement for other HP1 functions. For example, the suppression of transcriptional activities at or around damage sites positive for H2AX phosphorylation (Solovjeva *et al.*, 2007) may help to inhibit illegitimate transcriptional activity from a damaged DNA template undergoing repair.

Assembling DSB-response proteins at DNA breaks

Whilst the precise mechanisms that initiate H2AX phosphorylation at DSBs remain unclear, the events that follow are better understood. Multiple proteins required for the response to DSBs – including ATM, the MRN complex, MDC1 and 53BP1 – quickly accumulate around the site of breakage, co-localizing with γH2AX foci (Paull *et al.*, 2000). There is substantial variation in the kinetics of their accumulation. Whilst NBS1 and MDC1 accumulate within seconds after DSB induction, 53BP1 is recruited somewhat more slowly, and all these precede the much later recruitment of DNA repair enzymes like RAD51 some 30-60 min afterwards (Paull *et al.*, 2000; Lukas *et al.*, 2004; Bekker-Jensen *et al.*, 2005).

γH2AX formation and ATM activation

γH2AX formation appears to precede and promote the accumulation of DSB response proteins. Thus, several of the recruited proteins, such as NBS1, 53BP1 or MDC1, contain recognition modules such as the forkhead-associated (FHA) or BRCT domains that bind to γH2AX (e.g. Kobayashi *et al.*, 2002; Stewart et al., 2003; Ward *et al.*, 2003). Moreover, their accumulation in damage-induced foci is diminished in H2AX-deficient cells (Bassing *et al.*, 2002; Celeste *et al.*, 2002). Nevertheless, there is evidence that many proteins involved in the DSB response can access sites of breakage even in the absence of H2AX (Celeste *et al.*, 2003); this suggests that γH2AX formation may greatly

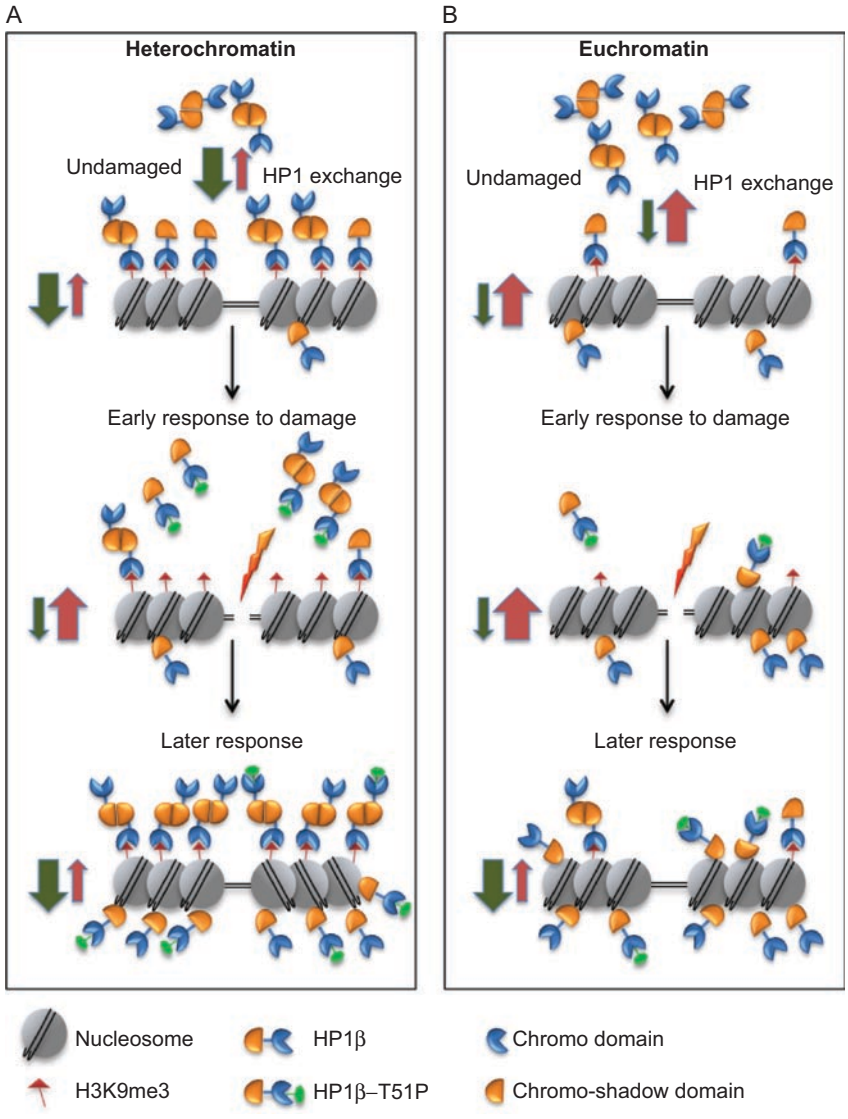

Figure 2. Model for the bimodal behavior of HP1β at sites of DNA breakage. A hypothetical model summarizing changes in HP1β dynamic behaviour after DNA damage in heterochromatin (A) or euchromatin (B) is summarized. In the undamaged state, HP1 exchange (green and red arrows) is more dynamic on euchromatin than heterochromatin, where H3K9me (red triangles) is more abundant. During the early response to DNA breakage, HP1 is mobilized from both euchromatin and heterochromatin via CK2-mediated phosphorylation of Thr51 (green ovals), releasing the chromodomain (blue) from H3K9me binding. However, visible dispersal of Thr51-phosphorylated HP1 (HP1Thr51P) is more evident in heterochromatic regions, where the protein is less dynamic and more highly concentrated in the undamaged state. Recruitment of HP1 to damaged chromatin via its chromoshadow domain (yellow) independent of Thr51 follows and may overlap temporally with mobilization. This may be more easily visible in euchromatin, due to its lower concentration there in the undamaged state. Late after DNA breakage, recruitment of HP1 via the chromoshadow domain predominates over mobilization in both euchromatin and heterochromatin. Recruitment includes HP1Thr51P; hence, even the recruited HP1 population may remain highly dynamic, due to its inability to bind H3K9me. The net effect of HP1 displacement from both euchromatin and heterochromatin is to promote TIP60 recruitment, in turn activating ATM (Sun et al., 2009). (Reproduced with permission from Ayoub *et al.*, 2009b).

enhance the accumulation of these factors at DSB sites without being utterly essential for their initial recruitment to them.

How γH2AX formation assists in the accumulation of DSB response proteins at damage sites has not yet been elucidated. γH2AX formation spreads for ~2 Mb around DSB sites, and thus, the foci contain thousands of γH2AX molecules. The large number of binding sites may make the probability of encounter with recognition modules in DSB-response proteins more likely, even if the interactions themselves are relatively weak. Moreover, direct effects of γH2AX formation on chromatin structure may also occur, assisting both in maintaining a relatively open structure permissive for the assembly of DSB-response foci, or possibly, in maintaining the apposition of the broken DSB ends.

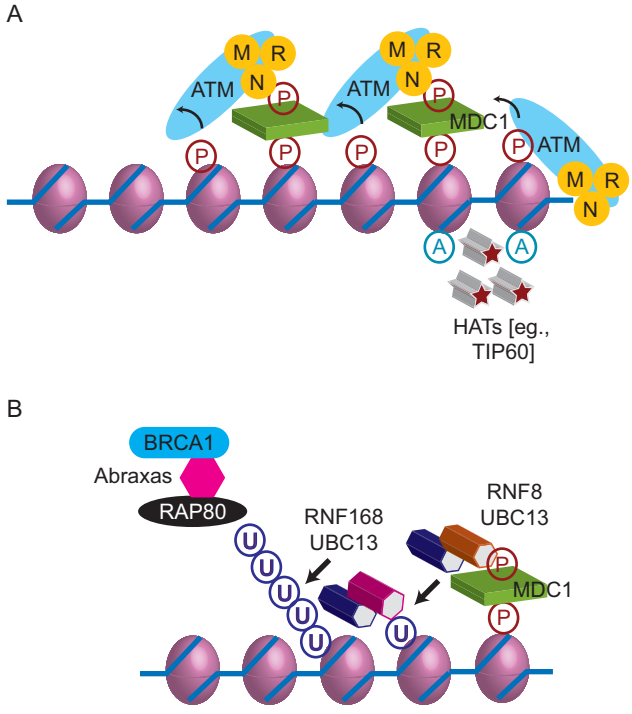

Figure 3. γH2AX phosphorylation and histone ubiquitylation in the DSB response. Panels A and B respectively summarize features of γH2AX formation and histone ubiquitylation at DSBs. The interdependencies between these processes are described in the main text; not all are depicted here. In A, the recruitment of the MRE11-RAD50-NBS1 complex (MRN) may facilitate ATM activation at free DNA ends, as well as DNA end-processing. γH2AX formation is induced over a large region of chromatin around the DSB at a variable and fluctuating density; the curved arrows are *not* meant to indicate that γH2AX formation spreads from the break. The adaptor protein MDC1, which binds to γH2AX, plays a key role with ATM in efficient H2AX phosphorylation. The WSTF protein constitutively phosphorylates H2AX on Y142, which is decreased after γH2AX formation, and may help to set a threshold for the longevity of γH2AX foci. MDC1 also plays a key role in the recruitment (B) of the RNF8 E3 ubiquitin ligase, with the E2 enzyme UBC13. RNF8-UBC13 activity may initiate histone ubiquitylation (circled U) surrounding a DSB, but another E3 ligase, RNF168, is necessary for efficient conjugation. Histone ubiquitylation forms a landing pad for the ubiquitin-binding modules of the RAP80 protein, which recruits BRCA1 via Abraxas.

MDC1, a factor which binds directly to γH2AX via its BRCT domain (Stucki *et al.*, 2005), and is an early migrant to DSB-response foci, appears to play a proximal and essential role in the accumulation of other proteins in the foci (Figure 3A). Loss of the MDC1–γH2AX interaction inhibits the accumulation of NBS1, 53BP1 or the active, phosphorylated form of ATM (Stucki *et al.*, 2005; Lou *et al.*, 2006). Moreover, MDC1 binds to the MRN complex, which in turn can bind and activate ATM, thus bridging between chromatin-bound γH2AX and a kinase responsible for its formation (You *et al.*, 2005). In particular, the NBS1 component of MRN contains a C-terminal ATM-binding site, as well as an N-terminal FHA domain that binds to the CK2-phosphorylated Ser-Asp-Thr (SDT) repeats in MDC1 (CK2) (Chapman and Jackson, 2008; Melander *et al.*, 2008; Spycher *et al.*, 2008). The sequential recruitment of active ATM to chromatin-bound γH2AX via MDC1-MRN could establish a feedback loop that extends H2AX phosphorylation to nucleosomes flanking the site of damage. Supporting the pivotal role of the MRN complex, tethering of its components to undamaged DNA elicits the H2AX-independent recruitment of DSB-response factors (Soutoglou and Misteli, 2008). Moreover, the propagation of H2AX phosphorylation by ATM may also require nucleosome mobilization, since depletion of the mammalian SWI/SNF chromatin remodeler complex reduces γH2AX formation whereas ATM autophosphorylation and its recruitment to chromatin after DNA damage remain intact (Park *et al.*, 2006).

Recent studies suggest that γH2AX formation may be induced over a wide but fixed region flanking a DSB, rather than spreading away from the break per se (Savic *et al.*, 2009). Within this region, the density of γH2AX may fluctuate with time and distance. ATM appears to be essential both to maintain a high density of γH2AX in this region, and elicit its maximal extension from the DSB site; other PIKK kinases can, however, promote γH2AX formation. By contrast, MDC1 is necessary for maximal γH2AX densities near the DSB, but not distal from it.

Beyond initiating and maintaining H2AX formation, the recruitment and activation of ATM at DSB sites affects chromatin structure by phosphorylating the KRAB-associated protein (KAP-1) (Ziv *et al.*, 2006). KAP-1 phosphorylated at DSB sites spreads rapidly throughout chromatin, triggering a relaxation in chromatin structure evident as decondensation (Figure 1). This response may be particularly relevant for the repair of DSBs in heterochromatin. Thus, DSBs in heterochromatic regions are repaired more slowly than in euchromatin (Goodarzi

et al., 2008), and require ATM signaling for their repair. Depletion of KAP-1, or the heterochromatin factor HP1, alleviates this requirement, suggesting that ATM signaling may work through these effectors.

Histone acetylation and histone methylation

As noted earlier, acetylation of lysine residues on core histones reduces their affinity for negatively-charged DNA, a plausible route to the relaxation of chromatin structure (Figure 1). Indeed, γH2AX formation is followed by an increase in the acetylation of H3 at tail residues K9, K14, K18, K23 and K27 and of H4, at K5, K8, K12 and K16 (e.g. Tamburini and Tyler, 2005). Acetylation of H3K56 (Das *et al.*, 2009), a residue in the globular domain of histone H3 located near the point at which ds DNA enters or exits the nucleosome, raises the possibility that its modification could affect nucleosome stability (Das *et al.*, 2009); however, this modification has also been implicated in the restoration of chromatin structure after repair, rather than in earlier events (Chen *et al.*, 2008).

Histone methylation has been implicated in the recruitment of proteins containing specific recognition modules, rather than in direct alterations to chromatin structure. For example, the methylation of histone H4 on K20 may help to guide 53BP1, one of the earliest migrants, to DSB-response foci (Botuyan *et al.*, 2006). 53BP1 contains tandem Tudor domains that specifically accommodate a di- but not tri-methylated lysine, explaining its specific interaction with H4K20me2. This binding site may work in conjunction with the BRCT domain of 53BP1, which mediates its interaction with γH2AX.

Histone ubiquitylation and DSB-response protein recruitment

Recent studies implicate histone ubiquitylation, besides other modifications, as an important route for the recruitment of additional DSB-response proteins to DNA breaks (Figure 3B). The RING-finger-containing nuclear factor, RNF8, accumulates at sites of DNA damage with γH2AX in a manner dependent on MDC1 (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). RNF8 contains an FHA domain at its N-terminus and at its C-terminus, a RING finger domain that marks it as an E3 ubiquitin ligase. It is recruited to DSBs via its FHA domain, which engages ATM-phosphorylated SpQ/TpQ motifs in MDC1. Working with another RING-containing E3 ligase, RNF168 (Doil *et al.*, 2009; Stewart *et al.*, 2009), RNF8 induces and maintains K6- and K63-type ubiquitin modifications on the core histones H2A and H2B, as well as the H2AX variant, at sites of DNA damage.

These covalent modifications, which conjugate the charged Ub peptide to the histones, could alter chromatin structure. However, it has become clear that they provide a landing pad for the recruitment of additional DSB-response factors to DSBs (Figure 3B). Thus, the breast and ovarian cancer suppressor protein, BRCA1, uses its phospho-Ser-binding BRCT domains to engage the adaptor protein Abraxas, forming a complex with the receptor-associated protein 80 (RAP80), which accumulates in foci at DSBs (Sobhian *et al.*, 2007; Wang *et al.*, 2007). RAP80 contains two ubiquitin-interacting motifs (UIM), which preferentially engage K6- and K63-linked polyubiquitin chains, enabling accumulation of the BRCA1-Abraxas-RAP80 complex at sites of DNA breakage.

The pathway specified by these interactions enables H2AX phosphorylation and MDC1 recruitment to sequentially recruit further DSB-response factors via histone ubiquitylation. The hierarchies within this pathway are being clarified (Huen *et al.*, 2007; Kolas *et al.*, 2007; Zhao *et al.*, 2007; Doil *et al.*, 2009; Stewart *et al.*, 2009). For example, although H2AX is ubiquitylated by RNF8, it is the formation of Ub-H2A conjugates that is essential for DSB-response factor recruitment. However, abrogation of γH2AX formation prevents Ub-H2A conjugation, exemplifying the hierarchical yet interdependent relationship between these two forms of histone modification.

Chromatin disassembly and remodeling during the DSB response

Histone chaperones such as the H3/H4 chaperone CAF-1 are recruited to sites of DNA damage, and the depletion or inactivation of chaperones can compromise repair, but there is little information concerning their precise role in the DSB response (reviewed in Corpet and Almouzni, 2009; Linger and Tyler, 2007). Chaperones mediate both chromatin disassembly and its re-assembly, and are essential in cells for basic cellular processes like DNA replication. This has made it hard to pick apart potential roles in the DSB response without *in vitro* biochemical systems that couple their function to these processes. Indeed, recent experiments in a yeast model system suggest that the absence of CAF-1 or Asf1 does not affect γH2AX formation or DSB repair, but instead, may together become essential after repair is complete to restore chromatin structure (Kim and Haber, 2009).

Histone chaperones generally work in co-operation with ATP-dependent chromatin remodeling complexes. Indeed, several remodeling complexes including the INO80 and SWR1 are known to be recruited to DSBs, and are essential for their resolution, in model organisms like yeast (Tsukuda *et al.*, 2005; van Attikum *et al.*, 2007; Kalocsay *et al.*, 2009). Available evidence from yeast models suggest that SWR1 may work to transiently deposit the variant histone H2A.Z

into chromatin surrounding a DSB, and to assist in nucleosome disassembly flanking the break, a function also performed by INO80. Nucleosome removal around a DSB may be particularly important in exposing DNA ends for resection, which is important in maintaining a checkpoint signal, as well as for DNA repair. Whether similar functions are performed by the human homologs of these complexes, whose subunit composition may vary from that in lower model organisms, is unclear.

Restoring chromatin architecture after DSB repair

The mechanisms that restore chromatin architecture to its normal state after the repair of DSBs remain largely undocumented. One prerequisite is likely to be the disassembly of DSB-response complexes, documented *in situ* at the sites of DSB repair by HR in a yeast model system (Miyazaki *et al.*, 2004). Several discrete mechanisms are likely to contribute to this process. Proteasomal degradation may assist in the turnover of MDC1 from DSB-response foci (Shi *et al.*, 2008), although the mechanism that triggers its removal from chromatin is not yet understood. Protein–protein interactions may regulate the extent and speed of this process. Thus, recent studies (Ayoub *et al.*, 2009c) on the disassembly of chromatin-bound foci containing the RAD51 protein, an essential factor for DSB repair by HR, implicate its interaction with a co-factor, the BRCA2 breast cancer susceptibility protein, regulated via CDK-dependent BRCA2 phosphorylation. Intriguingly, cells may monitor the disassembly of DSB-response foci before initiating DNA transactions that could conflict with their persistence. Yeast Rad51 is removed from the vicinity of a break at the same time that new DNA synthesis is initiated at the site (Miyazaki *et al.*, 2004). Vertebrate cells in which the disassembly of RAD5 foci is perturbed by targeted disruption of the BRCA2-RAD51 interaction alter the timing of their entry into mitosis via a mechanism that may be independent of the G2 checkpoint mechanism for DNA damage (Ayoub *et al.*, 2009c).

The removal of chromatin marks in the vicinity of DSBs after they have been repaired could involve the enzymatic removal of covalent modifications, or the replacement of modified histones with unmodified ones. These possibilities are not mutually exclusive; the efficient removal of γH2AX foci not only requires the phosphatases PP2A or PP4 in mammalian cells, but also their eviction from chromatin (Chowdhury *et al.*, 2005; Keogh *et al.*, 2006; Chowdhury *et al.*, 2008). Interestingly, the Williams-Beuren syndrome transcription factor (WSTF), a non-canonical tyrosine kinase that modifies H2AX, regulates γH2AX foci disassembly but not formation (Xiao *et al.*, 2009). WSTF constitutively phosphorylates Y142 in H2AX, but the abundance of this modification decreases after DNA damage concomitant with S139 phosphorylation, raising the possibility that the eviction of γH2AX from chromatin and/or its accessibility to de-phosphorylation in some way depend on this modification.

Finally, chromatin assembly factors including the histone chaperones CAF-1 and Asf1 play essential roles in restoring chromatin structure and cell cycle progression after DNA repair. Asf1 is necessary in this process to promote acetylation of free histone H3 on K56 via the histone acetyl transferase Rtt109; mimicking acetylation of K56 can bypass the requirement for Asf1, suggesting that this modification may signal the end of DSB repair (Chen *et al.*, 2008). Moreover, CAF-1 and Asf1 are together necessary in yeast for efficient recovery from damage-sensitive checkpoint arrest (Kim and Haber, 2009), which may require the restoration of chromatin structure in the vicinity of a repaired break.

Concluding remarks

The study of chromatin changes during DNA repair is a fast-moving field which has been reviewed both frequently and well by many expert authors. I have therefore attempted in this article to assemble the multitude of available observational studies into a coherent discussion of how chromatin architecture could be modified to facilitate each of three key steps necessary for the cellular response to DNA breakage: the sensing of broken DNA, the assembly of DSB-response proteins around the lesion, and finally, the restoration of chromatin architecture after repair. It reveals many key lacunae in our understanding of how DNA breaks are sensed and repaired in the context of chromatin in living cells, but also a profound lack of physical and structural information about how chromatin architecture is maintained and modified. It also seems clear that addressing these gaps in our knowledge must move beyond the cataloging of molecular species involved in these processes, towards an understanding of their mechanism. This may require the development of new genetic, biochemical or biophysical models or tools, since dynamic changes in chromatin architecture are essential to so many basic cellular processes other than DSB repair, raising many technical challenges to experimental analyses of cause and effect. Nevertheless, both the importance and urgency of such an exercise is underscored by the growing appreciation that aberrations in the molecules that modify chromatin architecture during the response to DNA breaks may underlie the pathogenesis of human diseases, and that their modulation by small molecules could provide new approaches to therapy.

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