Histone marks: repairing DNA breaks within the context of chromatin

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Abstract
Inherited or acquired defects in detecting, signalling or repairing DNA damage are associated with various human pathologies, including immunodeficiencies, neurodegenerative diseases and various forms of cancer. Nuclear DNA is packaged into chromatin and therefore the true in vivo substrate of damaged DNA occurs within the context of chromatin. Our work aims to decipher the mechanisms by which cells detect DNA damage and signal its presence to the DNA-repair and cell-cycle machineries. In particular, much of our work has focused on DNA DSBs (double-strand breaks) that are generated by ionizing radiation and radiomimetic chemicals, and which can also arise when the DNA replication apparatus encounters other DNA lesions. In the present review, we describe some of our recent work, as well as the work of other laboratories, that has identified new chromatin proteins that mediate DSB responses, control DSB processing or modulate chromatin structure at DNA-damage sites. We also aim to survey several recent advances in the field that have contributed to our understanding of how particular histone modifications and involved in DNA repair. It is our hope that by understanding the role of chromatin and its modifications in promoting DNA repair and genome stability, this knowledge will provide opportunities for developing novel classes of drugs to treat human diseases, including cancer.

Nuclear DNA in eukaryotes is bound by proteins and associated RNA to form a complex collectively termed as chromatin. The primary function of chromatin is to compress the large, linear genomes of eukaryotes into the volume of the nucleus. As DNA is wrapped around histone proteins to form the basic unit of chromatin, called the nucleosome, all DNA-based processes have evolved mechanisms to function within the context of chromatin. For example, DNA sequence-specific binding proteins, such as transcription factors, must be able to access their target sequences and can therefore be inhibited by chromatin states. Additionally, duplex DNA is unwound into single-stranded DNA while being transcribed or replicated. Chromatin is therefore highly dynamic and regulated during transcription and replication to allow these processes to occur and to ensure both epigenetic and genome integrity.

The integrity of nuclear DNA, as well as the chromatin that binds it, are constantly under threat due to the high incidence of DNA damage that occurs within cells [1,2]. For example, reactive by-products from metabolic reactions, base mismatches occurring during replication, misregulated DNA cutting enzymes or exposure to exogenous DNA damaging agents can all create DNA lesions that must be accurately repaired to ensure genome fidelity. Notably, chromatin may function to at least partially inhibit exposure of DNA to the nuclear environment containing these DNA damaging agents. Nevertheless, once DNA is damaged, cells respond by mounting a set of events termed the DDR (DNA damage response). The DDR represents an ever-expanding network of cellular pathways that function to detect the DNA lesions, signal their presence by mediating various responses, and ultimately lead to the repair of the damaged DNA [1,2].

One type of lesion is the DNA DSB (double-strand break) that occurs when both strands of the DNA double-helix break in close proximity to each other. This type of break yields a discontinuous DNA molecule, creating a situation that is particularly deleterious to cells, as free DNA ends are susceptible to degradation and religation, resulting in translocations and chromosome loss. Because of this, DSBs constitute the most cytotoxic of all DNA lesions. Under normal circumstances, DSBs are repaired by either HR (homologous recombination) or NHEJ (non-homologous end-joining) [3]. HR requires DNA end processing and a homologous DNA molecule for the accurate copying and repair of a DSB, while NHEJ utilizes little to no DNA end processing with ligation occurring to repair the break. In this review, we highlight recent findings detailing how DSB repair occurs within chromatin and, in particular, the role of histone modifications and histone modifying enzymes in DSB repair.
Histone modifications

The principal protein components of chromatin are histones. Histones are highly abundant and basic proteins that interact with DNA to form the basic unit of chromatin, the nucleosome [4]. The nucleosome consists of an octamer containing two copies each of the four core histones, H2A, H2B, H3 and H4, along with ~146 bp of DNA packaged around the histone protein core. Mammalian genomes also encode a number of histone variants that resemble core histones but have evolved to carry out specialized functions [5]. For example, the histone variant H2AX is nearly identical with H2A except for a divergent and extended C-terminus that plays crucial roles during the DDR [6–8]. Histones are also highly modified on specific amino acid residues with a complex assortment of chemical PTMs (post-translational modifications) including phosphorylation, acetylation, methylation, citrullination and ADP ribosylation [9–11]. In addition, histones are posttranslationally modified on lysine residues by the covalent attachment of small polypeptides such as ubiquitin and SUMO (small ubiquitin-like modifier) [12]. Importantly, histone marks are reversible because cells contain chromatin modifying enzymes that catalyse the addition of these modifications as well as enzymes that remove them [9]. Acting together, these enzymes dynamically regulate the PTM landscape of histones to allow the chromatin to perform a diverse array of functions, including gene regulation, DNA replication, chromosome segregation and DNA repair.

How do histone PTMs regulate chromatin structure and function? PTMs of histones can influence and modulate chromatin in several ways. The protein–DNA interactions in chromatin can be mediated through the interaction of the negatively charged DNA backbone with positively charged lysine and arginine residues. Acetylation or citrullination of lysine or arginine residues respectively can change the charge of the amino acid residue and thereby weaken its interaction with either DNA or other charged protein interfaces [9,13]. Similarly, reversal of these marks can increase electrostatic interactions within chromatin. Thus histone modifications have the potential to regulate DNA-protein and protein–protein interactions by changing the charge of the modified amino acid.

It is also well established that certain PTMs can act as high-affinity binding sites for proteins containing PTM-binding domains [14]. For example, bromodomains bind specifically to acetylated lysine residues, whereas chromodomains can exhibit a binding preference for methylated lysine residues [9,13,15]. Other important histone-mark-binding domains operating within DDR proteins include twin BRCT (breast cancer 1, early-onset) [BRCA1 C-terminal] domains that bind phosphorylated epitopes on target proteins as well as UBD and SIM regions, that bind ubiquitin and SUMO respectively [16,17]. Indeed, eukaryotic cells have evolved diverse families of proteins containing these, and other, PTM-binding domains. A well-established example of how histone modifications regulate chromatin functions comes from studies of lysine methylation of the N-terminal tail of histone H3 [4]. Trimethylation of Lys4 of histone H3 creates a binding site for transcriptional complexes, leading to gene activation and euchromatin formation. Conversely, methylation of Lys9 of histone H3 provides an interaction motif for the chromodomains of HP1 proteins, which facilitate the formation of heterochromatin and ensuing gene repression [18,19]. Thus, depending on the context of the amino acid residue where methylation occurs, different chromatin states and functions are established.

Histone modifications can also function by influencing other histone marks through cross-regulatory mechanisms. For instance, this can occur either on the same protein or in trans on other histones or nucleosomes [20]. Given that over one in five amino acids of the core histones encode either a lysine or an arginine residue, all potentially modifiable with various PTMs, the potential for ‘cross-talk’ and complexity of histone marks is easily recognized. Furthermore, because these and other histone amino acid residues have the potential to be modified by several distinct PTMs, this raises the possibility that some histone marks function to inhibit the occurrence of other marks on the same residue. This type of regulation could be particularly relevant for lysine residues, which can be modified by acetylation, methylation, ubiquitylation or SUMOylation. Thus researchers have the formidable challenge of deciphering the mechanisms that govern these modifications on histones, as well as to understand their functions within the framework of chromatin for all DNA-based processes, including the DDR and in particular, DNA DSB repair.

Marking H2AX at DSBs

The clearest demonstration of how chromatin is modified at DSBs came from the discovery that the histone variant H2AX is phosphorylated on its C-terminal tail following DNA damage to form γH2AX. This phosphorylation is mediated primarily by the DDR kinases ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3-related) and DNA-PK. Strikingly, γH2AX occurs within large, IRIF (ionizing radiation-induced nuclear foci) that spread over 1 Mb of chromatin surrounding a DSB as detected by specific antibodies against γH2AX in fluorescence microscopy or ChIP-Seq (chromatin immunoprecipitation-sequencing) analyses [6,21,22]. While the exact functions of IRIF are unclear, these structures are vital for an effective DDR since H2AX−/− mice exhibit genome instability, are hypersensitive to DNA damage and are cancer-prone, potentially due to the inappropriate channelling of DSBs into alternative error-prone DNA repair pathways [23–25]. It seems most likely that γH2AX-dependent IRIF act to amplify DSB signalling through activating many kinases and other signalling components, as well as enhancing DSB repair by concentrating repair factors in the vicinity of the lesions and perhaps by also facilitating the holding of the DNA ends in juxtaposition.

γH2AX is a key regulator of IRIF formation because, in its absence, many DDR proteins fail to form foci.
effectively at DSB sites [23,24,26]. A key discovery in terms of understanding IRIF formation was the finding that γH2AX is directly bound by the MDC1 (mediator of DNA-damage checkpoint 1) protein, which then recruits other DDR factors to unrepaired DSBs [7]. This binding is mediated by the twin BRCT domains in MDC1. For example, γH2AX-dependent retention of MDC1 is now known to mediate the DSB localization of four ubiquitin E3 ligase proteins, RNF (RING finger) 8, RNF168, HERC2 and BRCA1 [27–30] whose substrates include H2AX as well as other histones and, most probably, various DDR proteins. Ubiquitylated histones, including H2AX and H2A, in turn appear to mediate the chromatin association of the BRCA1-interacting protein RAP80 (receptor-associated protein 80) though its UIM (ubiquitin-interacting motif) [31]. Ubiquitylated H2AX might also participate in IRIF formation by promoting the formation of γH2AX through a cross-regulatory mechanism [32] although another study demonstrated that γH2AX production was independent of ubiquitylation [33,34].

Additional modifications of H2AX that participate in the DDR also occur. For instance, H2AX is acetylated on Lys5 by the HAT (histone acetyltransferase) TIP60 (60 kDa Tat-interactive protein) [35], which regulates H2AX ubiquitylation through a ‘cross-talk’ mechanism to affect H2AX dynamics after DNA damage. H2AX is also phosphorylated on its C-terminal tyrosine residue by the non-canonical tyrosine kinase WSTF [36]. Upon DNA damage, this tyrosine residue is dephosphorylated by the phosphatase EYA [37]. Down-regulation of either WSTF or EYA impairs the DDR, highlighting the relevance of this tyrosine modification on H2AX function [36,37]. Significantly, the Scully group has recently identified additional IR-induced H2AX modifications by using a reconstituted system in H2AX−/− mouse ES cells. In that study, acetylation of Lys36 and phosphorylation of Thr101 on H2AX were detected by MS following IR and, importantly, rendering these sites unmodifiable resulted in defective IR resistance [34,38] that was independent from γH2AX-dependent HR [34]. Thus the histone variant H2AX is decorated by several different histone marks on multiple residues (Figure 1), which all contribute to chromatin responses required for an effective DDR.

Figure 1 | DNA damage-responsive histone modifications
A graphical list of all the histone modifications involved in the DDR that are discussed in the review. See the text for details.

Modification of core histones in response to DNA damage
H2AX is not the only histone modified by PTMs in response to DNA damage, as numerous DDR-induced modifications of core histones have been described (Figure 1). Two key PTMs occurring on histone H4 that function in the DDR are histone H4K16 (H4 Lys16°) acetylation and H4K20 methylation. Acetylation of H4K16 occurs upon DNA damage and is mediated by the HATs TIP60 and MOF [39–41]. MOF appears to regulate global levels of H4K16Ac, while TIP60 affects the appearance of this mark at site-specific DSBs. Importantly, depletion of either HAT results in defective HR and NHEJ. The roles of these HATs in the DDR could depend on the nature of the DNA lesion and/or the location of the DNA break since TIP60 can be activated by trimethylation [H3K9me3 (H3 Lys9 trimethylation)], a mark associated with heterochromatin [42]. How these two HATs regulate H4K16Ac upon DNA damage is unclear, especially given that MOF does not appear to localize to DSBs [41]. Since TIP60 and MOF are required for efficient DSB repair by HR and NHEJ, these functions are likely to be exerted through additional non-histone targets.

The precise mechanism of how acetylation of H4K16 promotes DSB repair also remains elusive. A potential insight into this question has been provided by the recent demonstration that H2B is ubiquitylated upon DNA damage by the E3 ubiquitin ligases RNF20/40 [43,44]. H2B ubiquitylation occurs at DSB sites, and depletion of these ubiquitin E3 ligases renders cells defective in DSB repair, particularly HR. Notably, in a defined in vitro system, nucleosome arrays containing ubiquitylated H2B were decompacted compared with unmodified H2B, and H4K16Ac acts similarly in inhibiting higher-order chromatin compaction in vitro [45,46]. Furthermore, combining H2B-Ub and H4K16Ac into the same nucleosomal templates resulted in a synergistic decompaction of these nucleosome arrays [46]. Thus these two histone marks could function together at DSB sites by opening up chromatin for subsequent DSB processing by resection, which is required for effective repair by HR.

Methylation of histone H4K20 is another well-established histone mark associated with the DDR, which creates a
binding site for the Tudor-domain region of the DDR protein 53BP1 [47]. Two recent studies have suggested that the WHSC1/MMSET (Wolf–Hirschhorn syndrome associated methyltransferase protein 1) targets H4K20 in a DNA damage-dependent manner such that it promotes the subsequent targeting of 53BP1 to DNA damage sites [48,49]. This work highlights how further studies are warranted to decipher the impact of defective histone methylation and DNA repair on this and potentially related diseases. In addition, methylation of histone H3 on Lys⁵⁶ by Metnase/SETMAR and demethylation by JHDM1a was observed [50]. H3K36me2 (H3 Lys⁶ dimethylation) was also shown to be induced by DNA damage and Metnase was concomitantly localized to a site-specific break by ChIP (chromatin immunoprecipitation) analysis. Furthermore, impairment of this specific histone methylation resulted in defective loading of the NHEJ factor Ku (Ku70/Ku80), as well as the DSB repair and signalling protein NBS1 (Nijmegen breakage syndrome 1) to DSB regions. The localization of Metnase and the methylation of H3K36me2 were confined to regions of chromatin adjacent to DSBs, suggesting that this histone mark acts locally to promote NHEJ through an as-yet-unidentified mechanism.

Induction of DSBs can result in global changes to certain histone marks, as well as the appearance of histone marks associated with repressed chromatin. Indeed, we performed an extensive screen of histone PTMs to determine which histone marks are globally affected by various DNA-damaging agents [51]. Our analyses revealed that most histone marks are not detectably perturbed by DNA damage. Contrary to some previous reports, we determined that mitotic histone H3 phosphorylations, such as H3S10, were reduced upon DNA damage due to checkpoint activation resulting in a decrease in mitotic cells rather than DNA damage per se. By contrast, histone H3 acetylations, particularly Lys⁹ and Lys⁶⁶, were deacetylated upon DNA damage in a cell-cycle independent manner, indicating that they directly respond to DNA damage induction [51,52]. This was in agreement with additional studies [53,54], although others have reported converse data [55,56] and the reasons for these discrepancies are unclear. Interestingly, we found that H4K16Ac was dynamically regulated at DSB sites, with deacetylation occurring rapidly followed by hyperacetylation.

Our above work established that deacetylation of particular histone marks takes place at DSBs. Our subsequent work then provided a rationale for these observations, as we found that the HDACs (histone deacetylases; HDAC1 and HDAC2) are rapidly recruited to DSBs and, under normal conditions, co-regulate H3K56Ac and H4K16Ac levels [52]. Importantly, we found that such regulation appears to be important for DSB repair because depletion of HDAC1 and HDAC2 impaired DNA repair, especially NHEJ, and rendered cells hypersensitive to DSB-inducing agents [52]. Additional histone marks associated with transcriptional repression have been detected at DSBs. Indeed, several studies have shown the recruitment of Polycomb proteins, including the histone methyltransferase EZH2 and the E3 ubiquitin ligase complex containing BMI1 and RING1b, to DSB regions [57–62]. Consistent with these proteins functioning at sites of DNA damage, DNA damage-induced H3K27me3 [61] and H2A ubiquitylation occur at DSBs. Collectively, these studies indicate that Polycomb repressive complexes promote transcriptional repression at sites of DNA damage and also serve to facilitate DSB repair by HR and NHEJ. Of note, Hong et al. found that the recruitment of the Polycomb protein PHF1 to sites of DNA damage required the NHEJ factor Ku, suggesting that DNA repair proteins might directly regulate the dynamics of Polycomb complexes at DSBs [62]. Considering these findings, it seems clear that repressive histone marks are key features of chromatin containing unrepaired DSBs.

**Getting specific about chromatin at DSBs**

An in-depth understanding of chromatin dynamics involved in gene expression has now been achieved, in part due to researchers’ ability to analyse specific genomic loci by ChIP followed by other techniques, including deep sequencing [63]. A similar analysis at DSBs has been hampered because most DNA damaging agents act non-selectively throughout the genome, precluding the utility of ChIP-based techniques. Several groups have challenged this limitation, however, by creating DSB-inducing systems that act at defined genomic loci. The earliest of these systems employed ectopic expression of a rare-cutting restriction enzyme, such as I-SceI or I-PpoI, to analyse chromatin changes at defined DSB sites [40,64]. Although informative, these systems had limitations due to the analysis only being performed on a few DSBs. More recently, the Greenberg group created a unique DSB system where transcription of the locus can be monitored. This led this group to suggest that, upon DSB induction, local chromatin compaction occurs with a concomitant repression of transcription [65]. H2A ubiquitylation by RNF8 was also observed at the DSB locus in this study and was shown to be required for transcriptional repression. This system has the advantage of a DSB-inducing enzyme that can be ‘turned-off’ to allow repair to resume, although it also examines only one artificial DSB loci. By contrast, Legue and co-workers developed a robust inducible restriction enzyme system whereby hundreds of DSBs are generated across the genome (Figure 2) [22]. Utilizing this system, ChIP-on-chip (ChIP followed by DNA array hybridization analysis) and ChIP-Seq analyses were performed to give high-resolution maps of γH2AX at sites of many DSBs simultaneously (Figure 2) [22,66]. This analysis has the advantage of analysing multiple DSBs across the genome to study whether or not all DSBs are treated identically by the DDR, regardless of genomic location and the histone modification landscape in which the DSB occurred. Chromatin changes at replication-induced DSBs can also now be monitored by iPOND, a powerful technique for analysing proteins, as well as PTMs, at sites of newly synthesized DNA [54]. Dysfunctional telomeres also represent site-specific DSBs and have been used to analyse both global and local chromatin changes in aged
Legube and co-workers have developed an inducible restriction enzyme (AsiSI) system that is compatible with ChIP-Seq analysis to create high-resolution maps of histone marks across the genomic landscape of multiple DSBs [22,66]. For this system, AsiSI is fused to a modified ER (oestrogen receptor) domain to keep the restriction enzyme cytoplasmic (upper panels). Upon 4-OHT (4-hydroxytamoxifen) treatment, AsiSI-ER translocates to the nucleus where it recognizes and cleaves an 8 bp sequence to create a DSB in a sequence and site-specific manner. These DSBs induce γH2AX that can be visualized in cells by immunofluorescence with an antibody against γH2AX [bottom panels, nuclear DNA is detected with the DNA-binding dye DAPI (4′,6-diamidino-2-phenylindole)]. These DSBs can then be analysed by ChIP-Seq to create genomic maps of γH2AX, and potentially other histone marks and proteins, surrounding multiple DSBs (middle panels, a γH2AX ChIP-Seq profile at one AsiSI-induced DSB is shown as an example).

Conclusions
Determining how DSBs within chromatin are repaired by HR and NHEJ is a fundamental challenge for researchers and for furthering our understanding of how genetic and epigenetic information is maintained. Although many studies have contributed to our knowledge of this subject, relatively few mechanistic insights have been gained to allow us to create clear models for how these processes occur. This is perhaps due to the fact that chromatin is extremely plastic and dynamic, allowing genomes the necessary flexibility required for adapting to various processes and events. As chromatin exists in many states, its responses to DSBs are likely to vary depending on the stage of the cell cycle, the type of cell responding, the genomic location where the damage occurs as well other conditions that influence chromatin. These factors could make comparisons between studies difficult and we caution that chromatin work often relies heavily on the specificity of commercially available antibodies, which can give spurious results [51,69]. Regardless of these issues, with the emerging technologies of genome-wide DNA sequencing and quantitative MS, as well as the development of powerful DSB-inducing systems (as exemplified in Figure 2) and ever-more sophisticated microscopy and imaging methods [68], we anticipate that the next few years will witness dramatic improvements in our understanding of DSB repair processes within chromatin. In addition, it is a particularly exciting and important time to be studying chromatin and DNA repair as it is clear that these processes are involved in human diseases, including cancer. Indeed, because many cancer treatments function through DSB induction and cancers can exhibit hypersensitivity to DNA damaging agents and/or DDR inhibition, targeting of chromatin-based pathways could provide new opportunities for cancer treatments [2].
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