

## Review

# Buried territories: heterochromatic response to DNA double-strand breaks

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## Abstract

Cellular response to DNA double-strand breaks (DSBs), the most deleterious type of DNA damage, is highly influenced by higher-order chromatin structure in eukaryotic cells. Compared with euchromatin, the compacted structure of heterochromatin not only protects heterochromatic DNA from damage, but also adds an extra layer of control over the response to DSBs occurring in heterochromatin. One key step in this response is the decondensation of heterochromatin structure. This decondensation process facilitates the DNA damage signaling and promotes proper heterochromatic DSB repair, thus helping to prevent instability of heterochromatic regions of genomes. This review will focus on the functions of the ataxia telangiectasia mutated (ATM) signaling cascade involving ATM, heterochromatin protein 1 (HP1), Krüppel-associated box (KRAB)-associated protein-1 (KAP-1), tat-interacting protein 60 (Tip60), and many other protein factors in DSB-induced decondensation of heterochromatin and subsequent repair of heterochromatic DSBs. As some subsets of DSBs may be repaired in heterochromatin independently of the ATM signaling, a possible repair model is also proposed for ATM-independent repair of these heterochromatic DSBs.

**Key words:** DNA double-strand breaks, DNA damage response, DSB repair, heterochromatin, decondensation

## Introduction

The human genome encounters hundreds of thousands of DNA lesions per cell per day, posing a great threat to the integrity of genetic material. These DNA-based injuries arise exogenously from environment agents such as UV light, ionizing radiation, and genotoxic chemicals, or endogenously from metabolism-associated reactive oxygen species and replication stresses, and from programmed cellular events, such as V(D)J recombination and classical switch recombination, two essential processes during immune development [1–4]. Eukaryotic cells have evolved multiple, but functionally distinct responses and repair pathways to combat various types of DNA damage [1,3–5]. Among these types of damage, the most deleterious lesions that challenge genomic stability are DNA

double-strand breaks (DSBs) [1,6]. Although DSBs do not occur as frequently as other types of damage, failure to properly detect, signal, and repair will lead to either unrepaired DSBs that will kill cells or genomic instability, a hallmark of cancer and a cause of many other human diseases [1,7].

Cellular response to DSBs is conserved from yeast to human and activated upon DNA breakage to ensure proper repair of DNA breaks [1,5,7]. This process is well coordinated with other cellular activities such as replication, transcription, translation, and metabolism [1]. As eukaryotic DNA is packed into a higher-order chromatin structure, the compacted structure of chromatin adds a layer of control on the processes of the DNA damage response [8–10]. These processes include break induction, break detection, damage

signaling, cell-cycle regulation (checkpoint), senescence and apoptosis, repair and post-repair restoration of pre-damage chromatin structure [8,11–13]. With a different level of compaction, euchromatin and heterochromatin respond with different modes of action to attack by DNA damage-inducing agents [8,10,14]. Here we focus mostly on heterochromatic response to DSBs in mammalian cells.

## Heterochromatin versus Euchromatin

In eukaryotic cells, DNA is packed with histones into chromatin structure where DNA-based activities such as replication, transcription, and DNA damage/repair occur [15–17]. Chromatin compaction first starts when 147 bp of DNA wraps around a histone octamer to form the basic repeat unit of chromatin called nucleosome [18–20]. The histone octamer consists of four core histone proteins (H2A, H2B, H3, and H4), two each. Nucleosomes are interconnected by 20–60 bp of linker DNA, forming an 11-nm ‘beads-on-a-string’ fiber. With histone H1 binding to the entry/exit sites of the linker DNA into the nucleosomes, the 11-nm fiber can coil into a 30-nm helical structure, known as the 30-nm fiber [18,20,21]. It has become clear that chromatin compaction is affected by many factors including DNA sequence, length of linker DNA, the use of histone variants, and epigenetic modifications of chromatin components [18,20,21]. Heterochromatin is also maintained by many heterochromatin-associated proteins such as heterochromatin protein 1 (HP1), methyltransferase suppressor of variegation 3-9 (SUV39), and histone deacetyltransferases (HDACs) [22,23].

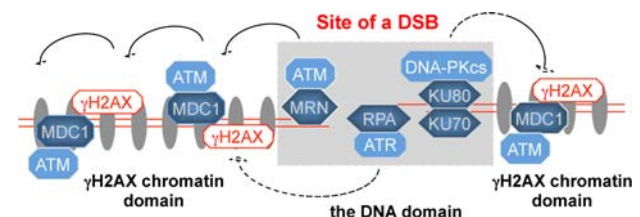
Euchromatin and heterochromatin differ in chromatin compaction, and represent two functionally and structurally distinct chromatin regions, which, respectively, comprise ~90% and ~10% of the human genome [24]. Euchromatin is lightly packed at the level of the 11-nm fiber, and enriched in genes that are transcriptionally active [18,20,21]. In contrast, heterochromatin is condensed to the level of the 30-nm fiber or higher, and predominantly transcriptionally inactive [18,20,21]. Euchromatin is often decorated by hyperacetylation of histone tails and/or trimethylation on lysine 4 of histone H3 (H3K4me3), whereas heterochromatin is characterized by histone hypoacetylation, trimethylation on lysine 27 of histone H3 (H3K27me3), and di- and tri-methylation on lysine 9 of histone H3 (H3K9me2 and H3K9me3) [23,25]. Heterochromatin can be further divided into constitutive and facultative heterochromatin [23,25]. Constitutive heterochromatin is the same heterochromatin region shared by all cells of a given species, and is usually associated with repetitive DNA [26]. For instance, the centromeric, pericentric, and telomeric regions in the genome are prevalently constitutive heterochromatin. In contrast, the heterochromatic state in facultative heterochromatin can be reversed between the cell types or under different conditions [23]. A gene that is transcriptionally repressed in facultative heterochromatin in one cell could become transcriptionally active due to chromatin decondensation in another cell. This decondensation would alter DNA damage induction, signaling, and repair in facultative heterochromatin.

## Chromatin Response to DSBs and their Repair

Upon DSBs, the DNA ends generated are bound with either the Mre11/Rad50/NBS1 (MRN) complex or the Ku70/Ku80 heterodimer. Single-strand DNA (ssDNA) can be generated from the DNA ends undergoing long-range resection and recognized by ssDNA binding protein replication protein A (RPA). MRN, Ku70/Ku80,

and RPA are thus designated as ‘sensors’ [1,5,27]. Following this DNA end–protein binding, three members of the phosphoinositide 3-kinase-related protein kinase (PIKK) family, ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and ataxia telangiectasia- and Rad3-related kinase (ATR), are, respectively, recruited to the sites of the breaks and activated (Fig. 1) [1,5,27,28]. Activated kinases phosphorylate hundreds of overlapping and unique substrates, and orchestrate a network of molecular events involved in DSB signaling and repair [1,5,28–32]. Some of these substrates are located at the ends of DNA breaks, some are on chromatin including histone H2A variant H2AX, and some, such as MRN, ATM, p53 binding protein 1 (53BP1), and breast cancer 1 (BRCA1), even appear in both fractions [33–37]. Phosphorylation of H2AX on S139 of the C-terminal SQEY motif generates  $\gamma$ H2AX, which recruits and retains many DSB-signaling/repair proteins including mediator of DNA damage checkpoint protein 1 (MDC1), MRN, ATM, 53BP1, and BRCA1 on the damaged chromatin up to megabases, forming the  $\gamma$ H2AX chromatin [9,38–40]. Factors that transduce damage signaling, such as ATM,  $\gamma$ H2AX, MDC1, MRN, and tat-interacting protein 60 (Tip60), are called ‘transmitter’ [5,41]. The  $\gamma$ H2AX chromatin induced by ATM extends up to megabases with high  $\gamma$ H2AX density, whereas the DNA-PKcs-mediated  $\gamma$ H2AX chromatin response is weaker and less extensive [40]. As activated ATR can phosphorylate H2AX in response to hydroxyurea, ATR may also mediate  $\gamma$ H2AX chromatin formation in the cells [42,43].

Since a part of the cellular response to DSBs involves chromatin and the other part of it does not, we therefore divide this response into two domains of response (Fig. 1): the ‘DNA domain’, which is active locally on ‘naked’ DNA ends of the breaks and does not involve the neighboring chromatin, and the adjacent ‘chromatin domain’, which mobilizes hundreds of protein factors including chromatin components to transduce DNA damage signaling on chromatin over kilobases or even megabases away from the breaks [34,44]. The formation of the chromatin domain is partly initiated by  $\gamma$ H2AX. Paradoxically, despite the apparent complexity and significant extension of the  $\gamma$ H2AX-dependent chromatin domain, H2AX-null mice are viable, and deletion of H2AX has only mild effect on DNA damage sensitivity, genome stability, and cell-cycle checkpoint regulation [34,45,46]. As many of DSBs induce phosphorylation of H2AX inefficiently or not at all, it is possible that the  $\gamma$ H2AX chromatin may only play a critical role in sensing and



**Figure 1. Two-domain response to a DSB** Cellular response to a DSB is activated when the ends of the break are detected by any of the three complexes: MRN/ATM, RPA/ATR, and Ku70/Ku80/DNA-PKcs. This response can be divided into a two-domain response: the DNA domain response and the chromatin domain response. Because these three complexes can phosphorylate histone H2AX to generate  $\gamma$ H2AX, initiating  $\gamma$ H2AX chromatin cascade of the damage signaling. This part of the chromatin response is called the  $\gamma$ H2AX chromatin domain. The DNA domain, however, does not involve H2AX. Although the DNA domain response appears to be responsible for most of DSB repair, the  $\gamma$ H2AX chromatin domain extending up to megabases of chromatin also plays an important role in DSB repair.

repairing a small subset of DSBs. As numerous studies have indicated that heterochromatin generates even weaker or no  $\gamma$ H2AX signaling upon DSBs [47–49], the chromatin response to DSBs in heterochromatin may involve different players, engage different mechanisms, and lead to different outcomes in DNA damage signaling and repair.

The purpose of the DNA damage response is to organize proper ‘effectors’ to execute repair of DSBs or other cellular activities such as cell cycle, senescence, and apoptosis and protect the integrity of the genome [1,5]. To repair DSBs, cells have evolved two major conserved but distinct pathways: homologous recombination (HR) and non-homologous end joining (NHEJ) [50–53]. HR requires a homologous sequence as the template to repair DSBs and is primarily required for repair of some subsets of DSBs during the S and G2 phases of the cell cycle. Predominant use of the neighboring sister chromatid available in the S and G2 phases as a repair template for HR allows for potentially error-free repair [51,52]. Single-strand annealing (SSA) is a unique form of HR that can repair DSBs between two repeat sequences in a single DNA duplex, and causes DNA deletion between them [51,52]. While general HR relies on the Rad51 recombinase, SSA is mostly independent of Rad51, but uses Rad52 instead. In contrast, NHEJ, which rejoins DNA ends of a DSB through ligation, requires little or no homology between them and is regulated by a largely distinct set of genes [50,53]. It is active in all phases of the cell cycle. Several core NHEJ factors, required for ‘canonical NHEJ’, have long been identified, including DNA-PKcs, Ku70/Ku80, and XRCC4/DNA ligase 4 [50,53]. ‘Alternative NHEJ’ also operates, independent of any of these core components, and exhibits increased engagement of end resection and increased use of microhomology [50,53]. Due to the processing of the damaged ends before the ligation step, NHEJ has a propensity to lose or insert a few nucleotides or even more at the ends after completion and is considered an error-prone repair mechanism [50]. However, depending on the type of the DNA cleavage, this pathway can repair a DSB without any deletion or insertion [44,54].

In the model of the two-domain response, what are the contributions of the DNA domain and the chromatin domain to DSB repair? The DNA domain of the DNA damage response appears to play a major role in DSB repair, but we and others have shown that the  $\gamma$ H2AX chromatin domain is also required for efficient HR and NHEJ [33,34,44]. Given the compacted nature of heterochromatin, the chromatin domain may be more important for DSB repair in heterochromatin than in euchromatin. Heterochromatic response to DSBs will be further discussed in the following sections.

### Induction of Heterochromatic DSBs

In heterochromatin, DNA replication is slower and transcription is less active than in euchromatin [18,20,21]. Heterochromatin ‘opening’ for these occasions seems to provide a limited opportunity for attack on heterochromatic DNA by DSB-inducing agents. However, replication and transcription proceeding into heterochromatin could convert damage on single strand of the DNA duplex such as nick and base modifications into DSBs, or replication of this region of DNA may encounter replication stress, thus stalling replication forks and causing DSBs arising from fork collapse. Indeed, the repeated sequences enriched in heterochromatin have a tendency to cause replication fork stalling and subsequent collapse of stalled fork [55], inducing replication-associated DSBs. Even with no replication or transcription, heterochromatin is not static. Other cellular activities occurring within heterochromatin can expose heterochromatic

DNA to attacks initiated by DSB-inducing agents. Nevertheless, due to the highly condensed chromatin structure, it is apparent that heterochromatin is less accessible by DSB-inducing agents and has lower sensitivity to DSB induction by radiation [11,56,57].

Early works attempted to assess the sensitivity of heterochromatin to radiation-induced DSBs by counting damage-inducing chromosomal aberrations in a particular chromatin region [58–64], but this approach is indirect and questionable. Recent use of  $\gamma$ H2AX focus formation appears to be more direct. Several groups have shown that radiation-induced  $\gamma$ H2AX foci form mostly on euchromatic regions [47,48,65–68]. However, many DSBs can be repaired quickly enough without activating the formation of  $\gamma$ H2AX or may induce little phosphorylation of H2AX. For instance, endonuclease cleavage of a specific site of heterochromatin induced inefficient phosphorylation of histone H2AX on the chromatin near the break [67]. Measuring  $\gamma$ -radiation-induced  $\gamma$ H2AX focus formation frequencies, in combination with ImmunoFISH to locate chromosomal territories, Falk *et al.* [48] found less  $\gamma$ H2AX foci in territories of transcriptionally inactive chromosomes than those with ‘open’ chromatin structure. These results suggest that heterochromatin is less sensitive to radiation damage.

By causing DNA damage to kill cancer cells, radiation and radiomimetic drugs are widely used in cancer treatment. In the study of how DNA damage is induced by radiation and radiomimetic drugs, two mechanisms are proposed to explain the refractory nature of heterochromatin to DNA damage [11]. First, due to condensation of chromatin structure, heterochromatin contains a smaller amount of water as compared with euchromatin. As a result, in heterochromatin, radiation-induced radiolysis of water is less efficient, generating fewer reactive radicals and causing less DNA damage [69]. Second, in addition to histones, a higher abundance of chromatin-binding proteins such as HP1, SUV39, and HDACs in heterochromatin may be more able to sequester free radicals, shielding DNA within heterochromatin from damage by radical-mediated chemical reaction [60,70,71]. However, it is unclear whether protein modifications specific to heterochromatin can protect its DNA from DSB-inducing agents.

### Heterochromatic Response to DSBs

It is known that repair of DSBs between heterochromatin and euchromatin is different. This discrepancy is partly attributed to the DNA damage signaling specific to either chromatin structure [8,10,14]. No evidence, however, suggests that sensing DSBs in heterochromatin is much different from that in euchromatin. The same DSB sensors, such as MRN, Ku70/Ku80, and RPA, are used in the cells to detect heterochromatic DSBs [8,10,14]. Due to tight compaction of chromatin structure and limited movement of nucleosomes, the DNA ends of DSBs exposed from heterochromatin may be shorter and less heterogeneous than those from euchromatin. Combined with the abundance of repeated DNA sequences, these end structures may impose a bias towards certain sensors and require different transmitters and effectors to execute. For example, it is possible that MRN is not able to compete efficiently with Ku70/Ku80 in binding to heterochromatic DNA ends; as a result, the occurrence of DNA end resection over a long distance is less frequent in heterochromatin. This may help explain why the HR repair pathway is generally avoided in heterochromatic DSB repair.

In order for DSB signaling and DSB repair to be efficient in heterochromatin, one early step is to relax the highly compacted

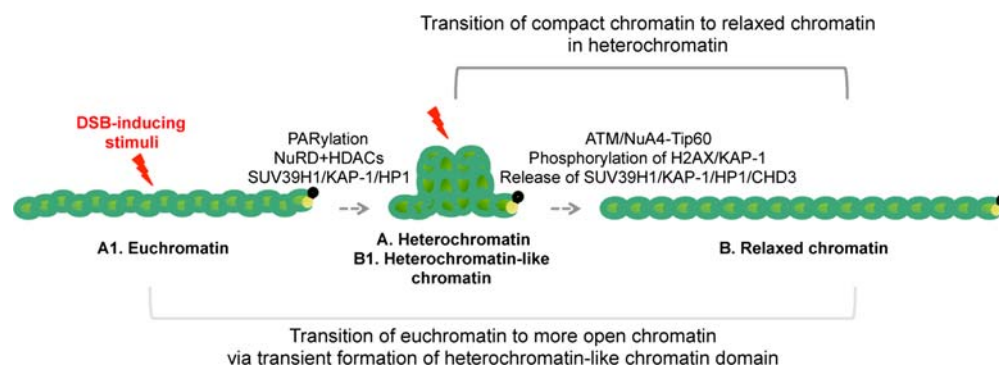
chromatin structure of heterochromatin [8,10,14]. This relaxation requires additional factors that specialize in ‘opening’ repressive chromatin structure of heterochromatin. Indeed, several factors, including HP1, KRAB domain-associated protein (KAP-1), and the Tip60 acetyltransferase, have recently been identified as critical mediators that assist decondensation of heterochromatin upon DSBs (Fig. 2) [71–82].

HP1 is an important chromatin-binding protein which helps to pack and maintain heterochromatin [18,77]. In particular, it plays a key role in the assembly and maintenance of heterochromatin at centromeres and telomeres [83,84]. This protein has three isoforms in mammalian cells: HP1 $\alpha$ , HP1 $\beta$ , and HP1 $\gamma$  [85]; all three of them have inhibitory effects on the repair of heterochromatic DSBs, as depletion of them removes the requirement of ATM in this repair [75]. HP1 binds to H3K9me3 and the histone methyltransferase SUV39H1 which can trimethylate H3K9 [18,77,85]. In response to irradiation, HP1 $\alpha$  and HP1 $\beta$  exhibit an initial and transient disassociation from damaged heterochromatin, suggesting possible DNA damage-induced relaxation of heterochromatin [8,77,78]. As irradiation-induced phosphorylation of HP1 $\beta$  at Thr51 by casein kinase 2 (CK2) disrupts the interaction of HP1 $\beta$  with H3K9me3, this phosphorylation may drive the transient release of HP1 $\beta$  from the damaged heterochromatin [78].

Surprisingly, HP1 can also be recruited to the sites of heterochromatic DSBs, as accumulation of HP1 is found at the sites of laser-induced DNA lesions on heterochromatin [76–78,86]. It is thought that this recruitment event precedes the initial release of HP1 and both together help to transduce damage signaling in heterochromatin in a spatiotemporal manner [77,78,86]. The accumulation of HP1 at heterochromatic DSBs is found to be dependent upon the C-terminal chromoshadow domain, but independent of the N-terminal H3K9me3-interacting chromodomain and H3K9me3 [71,78]. The three HP1 proteins can form homodimers and heterodimers through their C-terminal chromoshadow domain [85]. The dimerization of two chromoshadow domains creates a binding surface for KAP-1 and p150, a subunit of the histone chaperone CAF-1 complex [85,87,88]. Through this interaction, KAP-1 and CAF-1 may recruit HP1 to the sites of DSBs in heterochromatin [85,89]. But it is not

known whether this HP1–KAP-1 interaction helps to dock KAP-1 onto heterochromatin. DSB-induced recruitment of HP1 exhibits a different kinetics between euchromatin and heterochromatin, suggesting the involvement of different players or different mechanisms [8,10]. At present, the functions of this HP1 accumulation at heterochromatic DSBs are not clear; however, three possibilities have been proposed: (1) to enhance damage signaling; (2) to stabilize damaged chromatin; and (3) to provide additional binding epitopes for transmitters and effectors in the DNA damage response [8,10,14].

Phosphorylation of KAP-1 at Ser824 is among the key molecular events in heterochromatic response to DSBs (Fig. 2) [72–75]. KAP-1 was originally identified as a nuclear corepressor for the transcription factor class KRAB domain-containing zinc finger proteins [90,91]. Its direct involvement in the DNA damage response was revealed when Ziv *et al.* [72] observed DNA damage-induced phosphorylation of KAP-1 and a role of this phosphorylation in ATM-dependent chromatin relaxation. In fact, following DSBs, MRN binds to DNA ends of DSBs and recruits ATM [28,92,93]. ATM is then activated to phosphorylate its substrates including the heterochromatin-associated protein KAP-1 at Ser824 [72]. This phosphorylation at the sites of heterochromatic DSBs requires  $\gamma$ H2AX, MDC1, ring finger protein 8 (RNF8), RNF168, and 53BP1 [74]. By concentrating MRN and ATM at the sites of irradiation-induced DSBs, 53BP1 may enhance ATM-mediated phosphorylation of KAP-1 [74]. The phosphorylation of the chromatin-associated KAP-1 can disrupt the interaction of SUMOylated KAP-1 with CHD3 on chromatin [94,95]. CHD3, a catalytic subunit of the NuRD chromatin-remodeling complex, inhibits heterochromatin decondensation possibly by opposing the chromatin-remodeling activities of an imitation switch (ISWI) complex [95]. The disassociation of CHD3 from SUMOylated KAP-1 releases this inhibitory effect, thus promoting the relaxation of heterochromatin [74,94,95]. As the DNA ends in heterochromatin can also be bound by Ku70/Ku80 and RPA, their respective PIKK partner DNA-PKcs and ATR could be recruited and activated to phosphorylate KAP-1. Indeed, both kinases have been shown to phosphorylate KAP-1 [73]. It is therefore conceivable that the phosphorylation of KAP-1 can mediate DNA-PKcs- and ATR-dependent relaxation of heterochromatin.



**Figure 2. DSB-induced decondensation of heterochromatin and heterochromatin-like chromatin transiently formed in euchromatic regions** In heterochromatin (A), a DSB can initiate a transition of the highly compacted structure of heterochromatin to a more open, flexible chromatin structure (B) in order to facilitate the ATM-mediated DSB signaling involving a number of protein factors (the NuA4–Tip60 complex, H2AX, KAP-1, SUV39H1, and CHD3) and related molecular events such as phosphorylation of H2AX and HP1. Recent studies have also indicated that DSB induction in euchromatin (A1) induces a transient establishment of a repressive, heterochromatin-like chromatin domain (B1) partly via PARylation of chromatin components and recruitment of proteins that help to form and maintain heterochromatin. These proteins include NuRD, HDACs, SUV39H1, KAP-1, and HP1, etc. Transition of this transient repressive chromatin to a more relaxed chromatin (B) may employ a similar mechanism that underlies DSB-induced heterochromatin decondensation except extra requirement for the removal of PARylation. The euchromatic response to a DSB mimics the response of a worm to exogenous stimuli: initial body contraction (A1 to B1) and subsequent body relaxation (B1 to B).



But it is unknown whether DNA-PKcs- and ATR-dependent relaxation of heterochromatin can be induced by heterochromatic DSBs.

Recruitment of Tip60 to DSBs is another key step in relaxing heterochromatin in response to DSBs (Fig. 2) [8,10,81,82,96]. Tip60 is a member of the MYST family of histone acetyltransferases and a subunit of mammalian NuA4 complex, which contains at least 15 other subunits including the PIKK Trrap and the p400 ATPase [97]. When ATM is recruited by MRN to the sites of DSBs, Tip60 may be recruited together, as Tip60 was found to be in complex with ATM and MRN [80,82,98,99]. However, it is unknown whether the DSB-induced recruitment of Tip60 and its subsequent action in heterochromatin occur in the 'DNA domain' of the response or in the 'chromatin domain' of the response. The chromodomain of Tip60 can also bind to H3K9me3, and the activation of Tip60 requires this binding [81]. Hence, DNA damage-induced transient release of HP1 from H3K9me3 on the damaged heterochromatin may allow Tip60 to bind the vacated H3K9me3 and help to activate Tip60 in response to heterochromatic DSBs. In fact, DSB-induced CK2-dependent phosphorylation of HP1 $\beta$  and subsequent disassociation of HP1 $\beta$  from damaged chromatin were shown to be critical for the activation of the acetyltransferase activity of Tip60 [81]. Tip60 can acetylate ATM, and this acetylation is important for the full activation of ATM [80,82]. Tip60 also acetylates histones such as H4 and H2A/H2AX and other non-histones such as DNA-PKcs [96,97,100]. Hyperacetylation of H4 by Tip60 promotes chromatin decondensation from 30-nm fiber to 11-nm chromatin structure following DSBs [97]. Acetylation of H2AX by Tip60 may initiate ubiquitination of H2AX and the eviction of  $\gamma$ H2AX from the damaged chromatin, thus facilitating chromatin reorganization [96]. Whether or not this ubiquitination is mediated by RNF8 and RNF168 is not clear, although these two ubiquitin ligases can ubiquitinate H2A and H2AX [101–103].

Like Tip60, Trrap and p400 can also be recruited to sites of DSBs, likely as components of the NuA4 complex [97]. The enzymatic activities of Trrap and p400 are required for DNA damage-induced transition of repressive chromatin structure to open chromatin structure [10,97]. Both Trrap and p400 can promote the exchange of histone H2A variant H2A.Z in nucleosomes and are required for Tip60-mediated hyperacetylation of H4, which is assisted by the removal of H2A.Z from nucleosomes [10,97,104,105]. H2A.Z has ~60% homology to H2A, but contains an extended acidic domain as compared with H2A [106]. The C-terminal tail of H2A, along with several residues of H2B, forms an acidic patch on the nucleosome surface [106]. The binding of the N-terminal tail of H4 to this acidic patch promotes the compaction of chromatin [107,108]. The presence of H2A.Z in the nucleosome increases this compaction of chromatin due to its extended acidic domain, whereas acetylation of the N-terminal tail of H4 may relax this compacted chromatin by abrogating the interaction of H4 with the acidic patch on nucleosomes [10,106–110]. The relaxation of the compacted chromatin in turn induces more acetylation of the N-terminal tail of H4, generating a cascade of further chromatin relaxation [10]. Therefore, both the exchange of H2A.Z and hyperacetylation of H4 provide a means to regulate the relaxation of heterochromatin in response to DSBs. The exchange of H2A.Z at DSBs by Trrap and p400 and acetylation of H4 by Tip60 may be coordinated to promote the decondensation of the damaged heterochromatin.

Recent studies have indicated that DNA damage in euchromatin can induce a transient compaction of chromatin prior to the relaxation of chromatin (Fig. 2) [10,81,111], and this transient compaction

of chromatin around DSB may be required for efficient initiation of DNA damage signaling [112]. Upon DSBs, the chromatin surrounding the breaks can be rapidly PARylated by poly(ADP-ribose) polymerases. This modification allows the recruitment of several repressive complexes containing HP1, KAP-1, SUV39H1, PRDM2, CHD3/CHD4, and HDACs onto the damaged chromatin, creating a heterochromatin-like chromatin domain associated with decreased acetylation of histones, increased H3K9me2/3, and increased binding of HP1 and KAP-1 [113–117]. The formation of this heterochromatin-like domain may serve several purposes in euchromatic response to DSBs: (i) to repress transcription around the breaks; (ii) to limit mobility to keep the ends of the breaks close; (iii) to prevent unwanted end processing/end resection; (iv) to prepare proper engagement of the DSB repair pathways; and (v) to enhance initiation DNA damage signaling. For whatever purpose, it has to be achieved quickly because the heterochromatin-like state is transient from seconds to minutes. Transition to a more open and flexible chromatin structure may involve the removal of PARylation, recruitment of Tip60 phosphorylation of KAP-1, demethylation of H3K9me2/3, and disassociation of HP1 on chromatin, partly mirroring DSB-induced relaxation of heterochromatin [10,118,119].

## DSB Repair in Heterochromatin

Despite significantly improved understanding of DSB repair in general, our knowledge of heterochromatic DSB repair remains quite limited. Given the significant difference in chromatin structure between heterochromatin and euchromatin, it is, however, no surprise that repair of DSBs in heterochromatin is different from that in euchromatin [8,10,14]. In DSB repair, the compacted structure of heterochromatin can be a barrier. Thus, the damaged heterochromatin may need to undergo decondensation, exposing the DNA ends of heterochromatic DSBs for access by repair factors. Indeed, heterochromatic DSBs are repaired with slower kinetics compared with euchromatic lesions, and the factors known to mediate heterochromatin decondensation, either through the release from or the binding to heterochromatin, are implicated in the repair of these DSBs [8,10,14,68,75]. These factors have been identified to include components of the ATM DSB-signaling pathway, such as ATM, MDC1, MRN, RNF8, RNF168, 53BP1, and Tip60, and heterochromatin-associated proteins, such as KAP-1 and CHD3 [8,10,14]. When heterochromatin is disorganized due to deficient activities of heterochromatin-associated proteins such as KAP-1, HP1, SUV39H1, and HDAC2, the need for ATM in repair of heterochromatic DSBs is relieved [75,120]. Furthermore, ICFA (immunodeficiency, centromeric region instability, facial anomalies syndrome type a) and HGPS (Hutchinson–Guilford progeria syndrome) are two syndromes associated with defects in heterochromatin formation [121]. It has been shown that both ICFA cells and HGPS cells do not require ATM for heterochromatic DSB repair [75,120].

However, in the studies above, most results were obtained by analyzing the decay of  $\gamma$ H2AX foci in the context of heterochromatin. This approach has several limitations [8,11]. First, the formation of  $\gamma$ H2AX may not be efficient or necessary upon some subsets of heterochromatic DSBs. Any implication derived from these studies cannot be applied directly to the repair of these subsets of heterochromatic DSBs. Second, as mentioned before, in addition to ATM, which is recruited to heterochromatic DSBs by MRN, DNA-PKcs and ATR can be recruited and activated by different DNA end-protein complex structures, such as the DNA end–Ku70/Ku80 complex and the ssDNA–RPA complex [1,5,27]. But it is unclear

whether DNA-PKcs- or ATR-dependent signaling becomes activated in response to heterochromatic DSBs, and if so, how it regulates heterochromatic DSB repair. Finally, details of DSB repair in heterochromatin are scarce from these studies and have a limited scope; therefore, many important questions are yet to be answered: What proportions of heterochromatic DSBs are repaired by HR and NHEJ, respectively? Does the abundance of repetitive DNA elements promote SSA? Is microhomology-mediated end joining (also called MMEJ) active? What is the pattern or frequency of deletion and insertion in heterochromatic NHEJ as compared to euchromatic NHEJ?

The abundance of repeated DNA sequences in heterochromatin poses a challenge for cells to accurately repair heterochromatic DSBs [8,10,11]. These repeated sequences can be mistakenly used as a homologous template for HR or encourage homology-mediated SSA and microhomology-mediated MMEJ for DSB repair in heterochromatin [10,122,123]. These repair mechanisms can induce deletions, translocations, and other chromosomal rearrangements, thus causing instability in heterochromatic domains in chromosomes [122,123]. The early studies have indicated a higher frequency of irradiation-induced translocations in heterochromatin than in euchromatin, although this observation is debatable [11,62]. Facing this challenge, cells may have evolved a control over the selection of repair pathways for heterochromatic DSB repair. NHEJ may be a preferred mechanism for repair of heterochromatic DSBs, whereas HR and SSA are apparently suppressed in heterochromatic DSB repair [10]. Consistently, DSB repair in H3K9me2/3-rich regions (heterochromatin-like) is preferentially mediated by NHEJ [124]. But HR is not excluded for heterochromatic DSB repair. When HR occurs, DSBs in heterochromatin appear to be relocated to the heterochromatin periphery for HR-directed repair [125,126]. This approach may help to restrain the action of unregulated DSB repair pathways and avoid recombination/joining with unwanted DNA sequences. Whatever mechanism is selected, the abundance of repeated DNA sequences in heterochromatin must be taken into consideration in making the selection for repair of heterochromatic DSBs.

## Concluding Remarks

DSBs are among the most deleterious type of DNA damage which poses a serious threat to genome integrity and cell survival. By coordinating with DSB detection, DNA damage signaling and other cellular activities, proper repair of this type of lesions is ensured to help maintaining the fidelity of the genome of a eukaryote. While the further packing of DNA into heterochromatin in a cell increases the complexity in the cellular activities of eukaryotes, this condensation can be a structural barrier in initiating the very same cellular activities including the DNA damage response [8,10,11,14]. Although it may be a natural selection for an excess of repetitive DNA elements being compacted into heterochromatin, DNA damage in heterochromatic regions of a chromosome seems to be far more harmful to cells than DNA damage in euchromatic regions. Therefore, it is important that cells have evolved additional mechanisms in dealing with DSBs in heterochromatin. On the one hand, heterochromatin may exploit its compacted structure and its associated proteins to protect heterochromatic DNA from attacks of the DSB-inducing agents. On the other hand, heterochromatin must be relaxed to respond to DSBs and carry out proper repair. In particular, as repeated DNA sequences can serve as an unwanted but convenient template or substrate for all DSB repair pathways including HR, SSA, NHEJ, and MMEJ, causing genomic instability especially in

heterochromatic regions of a chromosome, heterochromatic DSB repair must be tightly controlled in a spatiotemporal manner [10,122,123].

DSBs in heterochromatin can be divided into at least two groups depending on repair kinetics: one is hard to repair and the other easy to repair. DSBs that can initiate a microscopically observable cascade of response on chromatin may indicate their resistance to repair. As discussed in this review, this cascade involves many factors including MRN, ATM,  $\gamma$ H2AX, KAP-1, and Tip60 [8,10,14]. The ability to identify these factors is largely attributed to the accumulation of these factors at the sites of heterochromatic DSBs and the detectability of the modifications of these accumulated factors. However, the easy-to-repair group of DSBs may have been ignored, possibly due to the limitation of the current detection approaches. It is possible that the DNA ends of a large subset of DSBs are maintained at proximity by the static nature of heterochromatin structures prior to initiation of the DNA damage signaling. The abundance of Ku70/Ku80 may allow the rapid binding of Ku70/Ku80 to the DNA ends in heterochromatin even before the relaxation of heterochromatin [50,127]. This binding not only prevents end resection, but also activates the NHEJ repair pathway. As a result, this subset of heterochromatic DSBs could be quickly repaired by direct ligation of DNA ends even without activating the ATM-KAP-1-HP1 signaling. The supposed simplicity of this repair likely requires no additional end processing/end resection and only involves a small number of factors. As this repair causes little perturbation in heterochromatin including DNA and chromatin structure, promotion of this repair in the context of heterochromatin may be beneficial for maintaining genomic stability and preventing human diseases associated with heterochromatic defects. It could also be exploited to limit the toxicity of irradiation and chemotherapy to heterochromatic regions of chromosomes in cancer treatment [7,11]. Therefore, it is important to make an effort to determine the existence of such repair, and if it exists, to elucidate how the repair is engaged and regulated. Such an effort can be expedited by recent development in technologies, such as chromosome conformation capture [128] and the clustered regularly interspaced short palindromic repeats (CRISPR) gene editing technology [129].

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