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THE INFLUENCE OF CHROMATIN DOMAINS ON THE REPAIR OF DOUBLE-STRAND BREAKS

Writing Assignment, 25.11.2021

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Layman's Summary

Cells store their genetic information, which contains the instructions required to carry out all cellular activities, in the form of DNA. Inside the cells, the DNA is wrapped around special proteins, called histones, to form a complex structure called chromatin. Chromatin is more compacted than naked DNA, which allows the DNA to be stored inside the restricted volume of the nucleus. Different chromatin domains exist around the genome, characterized by differences in function and structural elements. The two main chromatin domains are euchromatin and heterochromatin. Euchromatin is assembled in areas of the genome that are rich in genes, which are sequences of DNA that contain information to guide the production of proteins. Euchromatin is characterized by an open structure that allows the access of gene-reading proteins to the DNA. Proteins that compose euchromatin contribute to the regulation of this open structure. On the other hand, heterochromatin is a much denser structure, containing very few genes. Heterochromatin is enriched for repetitive DNA sequences which are potentially dangerous for DNA integrity, and its compacted nature represents a strategy to maintain control over this possible threat. Similarly to euchromatin, heterochromatin components are important for the proper performance of its functions.

Many cellular processes and external cues can cause the formation of DNA damage. For instance, UV radiations and DNA duplication can lead to the breakage of both DNA strands, an event termed double-strand break (DSB). DSBs are serious lesions that need to be repaired in order to prevent events such as DNA mutations and structural defects of chromosomes, which can lead to cancer. Cells can choose between different processes to repair DSBs, and while some of them result in the perfect restoration of the original DNA molecule, others can introduce some errors. It is not completely clear how cells decide to use one repair process instead of the others. Recently, however, it was proposed that DSBs arising in euchromatin and heterochromatin might be repaired differently. In particular, it was shown that proteins that are present in euchromatin or heterochromatin can act as guides to select a repair pathway that works best for the characteristics of their particular chromatin domain. In this review, we summarize our current knowledge on the role of chromatin components during DSB repair. We examine how specific euchromatic- and heterochromatic- proteins contribute to the repair of DSBs, focusing on crucial repair steps such as the choice between different repair processes. We conclude by discussing crucial questions as well as future perspectives of the field.

THE INFLUENCE OF CHROMATIN DOMAINS ON THE REPAIR OF DOUBLE-STRAND BREAKS

Abstract

Double-strand breaks (DSBs) represent particularly dangerous forms of DNA damage that can result in the formation of mutations and chromosomal rearrangements when unrepaired or misrepaired. In order to preserve genome integrity, DSBs can be processed and repaired through several pathways, including homologous recombination (HR) and non-homologous end joining (NHEJ). As the final repair products depend on the pathway utilized to restore the damage, the process of repair pathway choice constitutes a crucial step of DSB repair. In eukaryotes, chromatin is classified into two main domains, euchromatin and heterochromatin, each exhibiting differences in protein composition and function. While both euchromatin and heterochromatin can be subjected to DSB formation, it has recently been proposed that the chromatin environment surrounding the DSB site might contribute to the repair pathway choice process. Both pre-existing and damage-induced histone marks, histone variants and chromatin-associated proteins of euchromatin and heterochromatin have been found to be involved in several steps of DSB repair in their respective domains, including the selective recruitment of certain repair proteins to promote the usage of specific repair pathways. This suggests that the role of euchromatin- or heterochromatin- proteins during DSB repair is to promote the usage of repair pathways that suit best the characteristics of their respective domains. Here, we discuss the influence of chromatin components on DSB repair. More specifically, we will focus on the roles covered by histone marks, histone variants and non-histone proteins associated with euchromatin and heterochromatin in different steps of DSB repair.

1. Introduction

A wide variety of exogenous and endogenous agents, including UV radiations and products of oxidative metabolism, can cause the formation of different kinds of DNA damage (**Hoeijmakers, 2009; Lindahl & Barnes, 2000**). Double-strand breaks (DSBs), which are generated when both strands of the DNA are broken, represent a particularly threatening lesion that must be repaired in order to preserve the integrity of the DNA molecule. Failure to properly repair DSBs can lead to mutations or chromosomal rearrangements, such as acentric or dicentric chromosomes and translocations, which in turn can promote the onset of tumorigenesis (**Cannan & Pederson, 2016; Kasparek & Humphrey, 2011**). The two major pathways used by the cell to repair DSBs are homologous recombination (HR) and non-homologous end joining (NHEJ) (**Scully et al., 2019**). While HR has traditionally been described as a safe process, that is able to restore the original sequence of the damaged DNA molecule, NHEJ can lead to small insertions or deletions, and is therefore considered mutagenic (**Scully et al., 2019**). Additional error-prone pathways, termed alternative end-joining (Alt-EJ) and single strand annealing (SSA), have also been shown to contribute to DSB repair, albeit more modestly than HR and NHEJ (**Bhargava et al., 2016; Frit et al., 2014**).

Whether a DSB will be repaired by HR or NHEJ is dependent on various factors, including the cell cycle and the cell type where the damage was induced (**Ceccaldi et al., 2016; Scully et al., 2019**). Interestingly, in recent years it has been shown that the repair pathway choice process can also be influenced by the chromatin environment surrounding the damage (**T. Clouaire & Legube, 2015; Ferrand et al., 2021**). Chromatin is generally classified into euchromatin, characterized by the presence of coding sequences and high rates of transcription, and heterochromatin, mainly composed of repetitive and silenced DNA (**Allshire & Madhani, 2018**). These two chromatin domains display distinct patterns of histone marks and are enriched with specific chromatin proteins involved in the regulation of chromatin structure and function (**Morrison & Thakur, 2021; Zhou et al., 2010**). Increasing evidence suggests that pre-existing and damage-induced histone marks and chromatin proteins present in euchromatin and heterochromatin can directly promote the use of certain DSB repair pathways by acting as recruitment signals for specific repair proteins (**T. Clouaire & Legube, 2015; Ferrand et al., 2021**). In addition, it has been shown that chromatin components can contribute to other processes during the DNA damage response (DDR). These include the initiation of signaling pathways that trigger the recognition and the repair of newly formed DSBs, and the re-organization of chromatin to facilitate the access of repair machineries (**Ferrand et al., 2021**). Through these mechanisms, histone marks and chromatin proteins contribute to adapting the DNA damage response to the characteristics and needs of their respective chromatin domains (**Ferrand et al., 2021**).

In this review, we summarize our current knowledge on the roles of euchromatic and heterochromatic components, such as histone marks, histone variants and chromatin-associated proteins, during DSB repair. In the next two chapters, we respectively introduce the general principles of chromatin organization and DSB repair pathways. In the following chapter we will discuss the general chromatin response to DSB induction and how this process is regulated by ataxia–telangiectasia mutated (ATM), an essential DNA repair kinase. Subsequently, we will focus on the roles covered by specific heterochromatic and euchromatic proteins in the DSB repair process in their respective domains. Finally, we end by discussing open questions and future perspectives in the field.

2. Basic Principles of Chromatin Organization

In order to store their genomes inside the limited nuclear space, eukaryotic cells organize their DNA in the form of chromatin (**Kornberg, 1977**). The building block of chromatin is the nucleosome, which consists of 147 basepairs (bp) of DNA wrapped around two copies of each core histone protein (H2A, H2B, H3 and H4) and one copy of the H1 linker histone **(Fyodorov et al., 2018; Luger et al., 1997**). Nucleosomes and various non-histone proteins assemble to form a higher-level, more complex, structure termed chromatin. Chromatin ensures that the DNA is tightly packaged inside the nucleus, and, in addition, it regulates all processes involving DNA. With the deposition of post-translational modifications (PTMs), like methyl and acetyl groups, to histone tails or the substitution of core histones with different variants, the compaction of chromatin and the factors recruited therein can be dramatically altered (**Martire & Banaszynski, 2020; T. Zhang et al., 2015)**. For instance, acetylation of histones can causes the weakening of contacts between histones and DNA, making the DNA accessible to the transcriptional machinery (**T. Zhang et al., 2015**). Moreover, chromatin structure is regulated by the action of chromatin remodelers, which are enzymes able to affect chromatin accessibility by mediating nucleosome eviction, histone variant deposition and nucleosome spacing (**Tyagi et al., 2016**). The combination of histone-PTMs and -variants, together with the action exerted by chromatin remodelers, define differential chromatin states: euchromatin and heterochromatin (**Figure 1**) (**Martire & Banaszynski, 2020; T. Zhang et al., 2015**).

The fraction of chromatin containing active genes refers to as “euchromatin”. Euchromatin is located on chromosome arms and displays an open conformation, with histone PTMs and histone variants promoting the access of transcriptional factors and therefore facilitating the process of transcription (**Ernst et al., 2011; Kharchenko et al., 2011; Venkatesh & Workman, 2015**). These include the trimethylated lysine 4 on histone H3 (H3K4me3), the acetylated lysine 27 on histone H3 (H3K27ac), the methylated lysine 36 on histone H3 (H3K36me) and the histone variant H2A.Z (**Creyghton et al., 2010; Draker et al., 2012; Santos-Rosa et al., 2002; Strahl et al., 2002**).

Heterochromatin, which can cover large percentages of the genome in eukaryotes, is instead characterized by a dense nucleosomal pattern, a scarce presence of genes and a modest transcriptional activity (**Allshire & Madhani, 2018; Grewal & Jia, 2007; Hoskins et al., 2002; Lander et al., 2001**). Heterochromatin that is enriched in repeated DNA sequences and constantly maintained in a silenced state is referred to as “constitutive chromatin” (c-Het). Although much of what constitutes c-Het had previously been labelled as “junk DNA”, it is now clear that c-Het covers roles in a variety of relevant processes, such as centromere assembly, chromosomal segregation, sister chromatid cohesion and the regulation of genome architecture (**Bernard et al., 2001; Dernburg et al., 1996; Folco et al., 2008; Mizuguchi et al., 2014; Ono, 1972**). Moreover, heterochromatinization of recombination-prone repeated sequences and transposable elements contributes to the maintenance of genome integrity by avoiding the formation of aberrant chromosomal structures as well as the disruption of genes (**Peng & Karpen, 2008; Slotkin & Martienssen, 2007**). The characteristic PTMs of c-Het include the di- and trimethylated lysine 9 on histone H3 (H3K9me2 and H3K9me3), the trimethylated lysine 20 on histone H4 (H4K20me3) and the trimethylated lysine 56 on histone H3 (H3K56me3) (**Jack et al., 2013; Riddle et al., 2011; Schotta et al., 2004**). Additionally, the DNA underlying heterochromatin is enriched with methylation marks (**Bird, 2002**). Importantly, H3K9me3 recruits the heterochromatin protein 1 (HP1), which participates in the compaction of heterochromatin through the formation of polymers (**Canzio et al., 2013; James et al., 1989; Lachner et al., 2001**). Spreading of c-Het is also dependent on HP1, which interacts with the methyltransferase SUV-39, generating a positive-feedback loop in which new H3K9me3 marks, established by SUV-39, recruit additional HP1 molecules (**Aagaard et al., 1999; Rea et al., 2000**) Interestingly, it has been observed that HP1 can also drive heterochromatin formation and expansion through phase-separation (**Larson et al., 2017; Strom et al., 2017**). When phosphorylated or bound to DNA, HP1 is able to induce the formation of liquid droplets which can combine to generate a heterochromatic, dense and membrane-less compartment (**Larson et al., 2017; Strom et al., 2017**). Factors that are compatible with this phase-separated domain can access the compartment and interact with heterochromatin, whereas proteins displaying different physical properties are excluded (**Larson et al., 2017; Strom et al., 2017**). While providing a new mechanism for the

formation of heterochromatin, this model might also explain how the access of proteins to heterochromatic regions is regulated.

The uncontrolled expansion of heterochromatin is counteracted by the existence of various mechanisms that help to confine heterochromatinization to gene-poor regions (**Allshire & Madhani, 2018**). For instance, the presence of specific DNA sequences, such as the tRNA genes and the recruitment sites for the transcriptional factor TFIIIC, functions as a border between euchromatic and heterochromatic regions in humans and yeast (**Noma et al., 2006; Raab et al., 2012; Scott et al., 2006**). Another mechanism employed to restrict chromatin expansion involves the action of specialized proteins able to counteract the deposition of typical heterochromatic histone marks (**Ayoub et al., 2003; Trewick et al., 2007; Zofall & Grewal, 2006**). Finally, the balance between euchromatin and heterochromatin can be regulated through histone turnover (**Aygün et al., 2013; Sadeghi et al., 2015; Verrier et al., 2015**).

Another major type of heterochromatin is facultative heterochromatin, which covers genes that are maintained silenced until their expression is needed for developmental purposes or for processes taking place at specific phases of the cell cycle (**Trojer & Reinberg, 2007**). This domain is enriched for the trimethylated lysine 27 on histone H3 (H3K27me3), which is deposited by the polycomb group (PcG) proteins. PcG proteins also contribute to the maintenance of heterochromatin together with additional factors such as HP1 and long non-coding RNAs (lncRNAs) (**Bayne & Allshire, 2005; Maison & Almouzni, 2004**). Facultative heterochromatin can occupy large genomic regions, or be restricted to small domains, as in the case of the Hox gene clusters (**Forlani et al., 2003; Gendrel & Heard, 2014**). A classic and extensively studied example of facultative heterochromatin in mammals is the inactivated X chromosome (Xi) of females, which is established to guarantee dosage compensation between the sexes (**Gendrel & Heard, 2014**). Central to the establishment of Xi is the transcription of the X-inactive specific transcript lncRNA (Xist), which accumulates in cis to the Xi, leading to the recruitment of Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2) and the deposition of specific histone PTMs, such as H3K27me3 and the mono-ubiquitinated lysine 119 on histone H2A (H2AK119ub1) (**de Napoles et al., 2004; Plath et al., 2003; Zylicz et al., 2020**). These histone marks create a chromatin environment that is inaccessible to additional transcriptional factors, thereby promoting the silencing of the X chromosome.

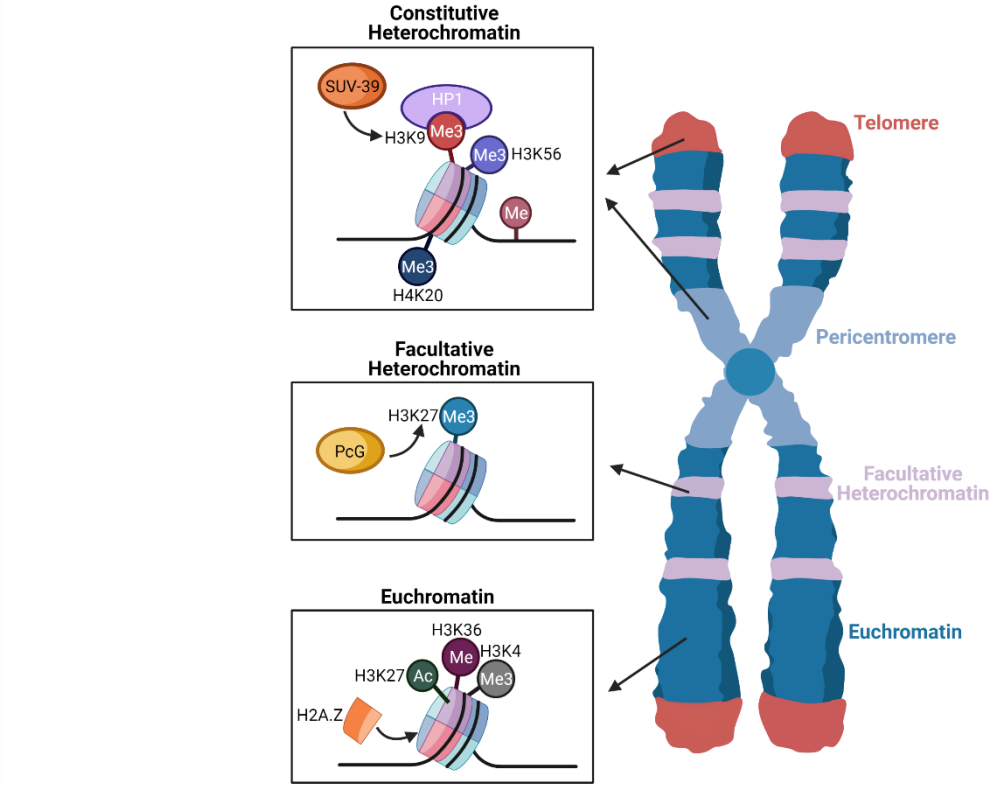

FIGURE 1. Histone Marks Characteristic of Euchromatin and Heterochromatin. Euchromatin localizes to the chromosome arms and is enriched for the H3K27ac, H3K36me and H3K4me3 histone marks. Additionally, the histone variant H2A.Z can be found in euchromatic nucleosomes. Constitutive heterochromatin is observed at the subtelomeric and pericentromeric regions of chromosomes. Histone marks typical of constitutive heterochromatin include H4K20me3, H3K56me3 and H3K9me2/3, of which the latter is deposited by the methyltransferase SUV-39 and associates with the heterochromatic protein HP1. DNA packaged into constitutive heterochromatin is enriched for methyl groups. Facultative heterochromatin is distributed along the chromosome arms and is characterized by the presence of the histone mark H3K27me3, deposited by the proteins of the Polycomb group (PcG). This figure was created on Biorender.com.

Different heterochromatin domains display distinct localizations inside the nucleus (**Padeken & Heun, 2014; van Steensel & Belmont, 2017**). Facultative heterochromatin, for instance, can be observed in the form of nuclear clusters, termed "Polycomb bodies", or in association with the nuclear periphery and the nucleolus, as in the case of the X chromosomes (**Lanzuolo et al., 2007; Saurin et al., 1998; L. F. Zhang et al., 2007**). Specifically, it has been observed that the localization of the silenced X chromosome alternates between the nuclear periphery and the peri nucleolar region, depending on the cell cycle phase (**L. F. Zhang et al., 2007**). In contrast, the active X chromosome stably associates with the nuclear periphery (**L. F. Zhang et al., 2007**). Constitutive heterochromatin can be organized inside the nucleus through different strategies. These include the assembly of one or multiple nuclear clusters of heterochromatin, known as "chromocenters", as seen in mouse cells and *Drosophila*, and the formation of heterochromatin domains in close proximity to the nucleolus or the nuclear periphery (**Guelen et al., 2008; James & Elgin, 1986; Németh et al., 2010; Pickersgill et al., 2006; Van Koningsbruggen et al., 2010; Wreggett et al., 1994**). In the latter case, the heterochromatin regions are respectively referred to as nucleolus-associated domains (NADs) and lamina-associated domains (LADs). NADs and LADs are repressive domains and display similar features. NADs are particularly enriched for repetitive regions exhibiting low gene density, such as centromeres, and are often silenced (**Németh et al., 2010; Van Koningsbruggen et al., 2010**). LADs, similarly, are composed of transcriptionally repressed heterochromatin and are defined by the presence of H3K9me3 and H3K27me3, which are histone marks of constitutive and facultative heterochromatin (**Guelen et al., 2008; Harr et al., 2015; Peric-Hupkes et al., 2010; Wen et al., 2009**). Amongst the various heterochromatin regions, pericentromeres and telomeres have been observed in LADs (**Guelen et al., 2008**). However, some heterochromatic domains can alternate their localization between NADs and LADs, indicating that they partially overlap (**Kind et al., 2013; Németh et al., 2010; Solovei et al., 2004; Van Koningsbruggen et al., 2010**).

3. DNA Double Strand Break Repair Pathways

Exogenous and endogenous mutagens constantly threaten the DNA by causing lesions that could compromise its structural integrity and functionality (**Klungland et al., 1999; Lindahl & Barnes, 2000; Phillips et al., 1988**). The formation of DNA double-strand breaks (DSBs), where both strands of the DNA are severed, is particularly dangerous, as it could eventually result in mutations or chromosomal rearrangements, such as acentric/dicentric chromosomes and translocations, which in turn can promote the onset of tumorigenesis (**Cannan & Pederson, 2016; Kasparek & Humphrey, 2011**). To avoid these events, cells have developed specialized pathways to efficiently detect and repair DSBs (**Ciccia & Elledge, 2010**). Four major different DSB repair pathways are currently known: Non-Homologous End Joining (NHEJ), Homologous Recombination (HR), Single Strand Annealing (SSA) and Alternative End-Joining (Alt-EJ) (**Figure 2** and **Figure 3**) (**Ceccaldi et al., 2015**).

During Non-Homologous End Joining (NHEJ), the two ends of a DSB are processed until they are ready to be ligated (**Figure 2**, right panel) (**Pannunzio et al., 2018**). Albeit kinetically fast, this pathway is considered to be mutagenic, since the modifications introduced to the DSB ends by the processing enzymes can cause the addition or loss of several nucleotides (**Pannunzio et al., 2018**). At the beginning of NHEJ, the DSB ends are bound by the heterodimer Ku70/80, leading to the recruitment of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the formation of the DNA-PK complex (**Liang et al., 1996; Meek et al., 2008**). This complex phosphorylates itself and several factors taking part in the repair process, contributing to their recruitment and activation. If the broken DSB ends are blunt, they can be directly ligated by the complex formed by the DNA ligase 4 and the X-ray cross-complementing protein 4 (LIG4-XRCC4) (**Grawunder et al., 1997**). This step is promoted by XRCC4-like factor (XLF) and Paralogue of XRCC4 and XLF (PAXX), which are recruited by LIG4-XRCC4 (**Brouwer et al., 2016; Ochi et al., 2015**). Specifically, PAXX contributes to end ligation by promoting the stability of the NHEJ components at the DSB site, whereas XLF interacts with XRCC4 to maintain the DSB ends close together (**Brouwer et al., 2016; Ochi et al., 2015**). Alternatively, if the ends contain overhangs or adducts that would prevent efficient ligation, they can be processed by different enzymes, such as the kinase Polynucleotide kinase 3'-phosphatase (PNKP) and the nuclease Artemis, of which the latter is recruited by the DNA-PK complex (**Bernstein et al., 2005; Goodarzi et al., 2006**). Moreover, in order to produce ends that can be properly sealed, the DNA polymerase λ and μ can be utilized to add nucleotides (**Gu et al., 2007; McElhinny et al., 2005**). Importantly, the DSB ends can be subjected to multiple rounds of processing by these enzymes before eventually being ligated. It is therefore possible that the repaired DNA molecule will display small insertions or deletions, and thus a different sequence from the original molecule (**Pannunzio et al., 2018**).

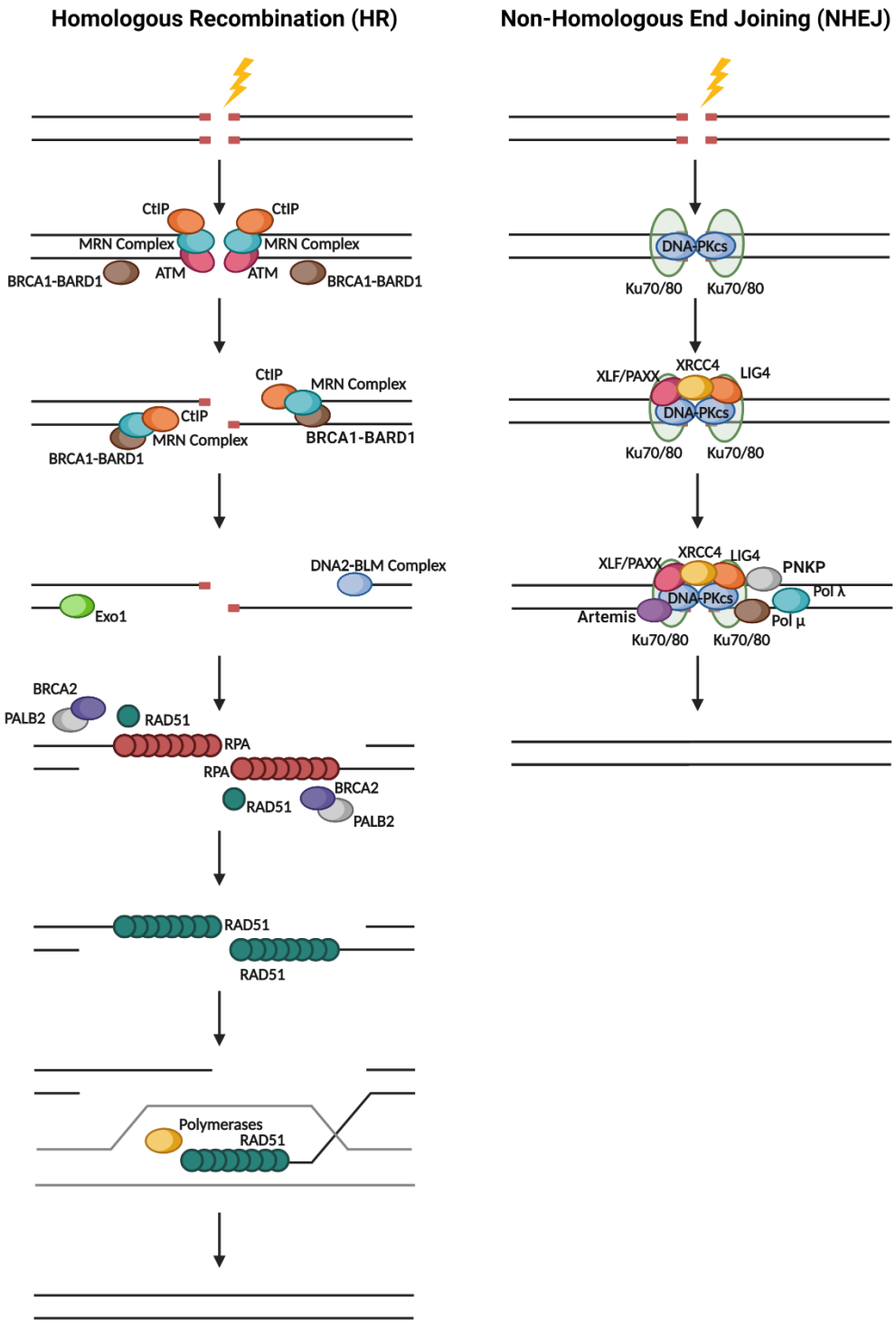

FIGURE 2. Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) Repair Pathways. **Left**. Homologous recombination is initiated by the recruitment of the MRN complex to the DSB site, which leads to the association of CtIP and ATM to the break. ATM triggers the arrival of the BRCA1-BARD1 complex, which, together with CtIP, promotes the 5' DSB end resection activity of MRN. Exo1 and the DNA2-BLM complex continue the end resection activity started by MRN, resulting in the production of two 3'-ssDNA strands. RPA molecules rapidly recognize and associate to the newly formed 3'-ssDNA, however they are subsequentially replaced by RAD51 molecules with the aid of BRCA2 and its partner PALB2. The RAD51-coated 3'-ssDNA conducts a search for a homologous sequence and, once this has been found, anneals to it. Next, the translesion and δ polymerases extend the RAD51-associated 3'-ssDNA using the complementary annealed strand as template. Finally, the D-loop is resolved, the RAD51-associated 3'-ssDNA re-associates with its complementary strand in the damaged molecule, and the DNA double strand is re-sealed. **Right**. During NHEJ, the DSB ends are recognized by the heterodimer Ku70/80, which associates with DNA-PKcs to form the DNA-PK complex. LIG4-XRCC4 is recruited to the damage site to ligate the DSB ends together with XLF and PAXX, which maintain DSB ends in the correct position during ligation. However, DSB ends can require processing, and therefore various enzymes, such as the nuclease Artemis, the kinase PNKP and the polymerases λ and μ are recruited to produce ends that can be ligated by LIG4-XRCC4. This figure was created on Biorender.com.

Homologous recombination (HR) is the other main DSB repair pathway. Contrary to NHEJ, HR has been described as a slower but conservative process since it aims at repairing the DSB by using an identical DNA sequence as a template (**Wright et al., 2018**). For this reason, HR is predominantly utilized during the S and G2 phases, when the sister chromatid is available, whereas NHEJ can be employed throughout the cell cycle (**Hustedt & Durocher, 2017**). Although considered an accurate pathway, in certain cases the results of HR can be dramatic. If the homologous chromosome is used as a template, for instance, the repair process could result in loss of heterozygosity (LOH) (**Moynahan & Jasin, 2010**). The repair of repeat-rich regions also represents a challenge for HR, since similar sequences could engage in aberrant recombinant events and lead to the loss or gain of repeats (**Peng & Karpen, 2008**).

The completion of HR requires additional processing steps in comparison to NHEJ (**Figure 2**, left panel). First, the DSB is recognized by the heterotrimeric complex MRE11-RAD50-NBS1 (MRN), which in turn recruits CtBP-interacting protein (CtIP) and the ataxia telangiectasia mutated (ATM) kinase (**Limbo et al., 2007; Sartori et al., 2007; Syed & Tainer, 2018; Uziel et al., 2003**). By phosphorylating serine 139 on histone H2AX (also named γH2A.X), ATM triggers a signaling cascade that leads to chromatin remodeling events (discussed in the next chapter) resulting in the recruitment of the Breast cancer type 1 susceptibility protein-BRCA1 Associated RING Domain 1 complex (BRCA1-BARD1) (**H. Kim et al., 2007; Rogakou et al., 1998; Sobhian et al., 2007; B. Wang et al., 2007**). MRE11, stimulated by BRCA1-BARD1 and CtIP, then initiates a short-range resection of the 5' DSB end, which is later completed by the exonuclease EXO1 and the nuclease-helicase complex DNA2-BLM (**Nimonkar et al., 2008; Yun & Hiom, 2009**). This results in the formation of a long 3'-ssDNA end, which is rapidly bound by the replication protein A (RPA) complex (**H. Chen et al., 2013**). The RPA molecules are subsequently exchanged with RAD51 molecules, which coat the 3'-ssDNA and guide the search for a homologous sequence (**Renkawitz et al., 2014; J. Xu et al., 2017**). The RPA-RAD51 exchange is promoted by a complex consisting of Partner and localizer of BRCA2 (PALB2) and the Breast cancer type 2 susceptibility protein (BRCA2) (**Carreira & Kowalczykowski, 2011; Esashi et al., 2007**). Once a homologous sequence has been identified, the RAD51-associated 3'-ssDNA anneals to its complementary strand, causing the displacement of the other strand in the invaded molecule (named "D-loop") (**Zhao et al., 2017**). Next, the δ and translesion polymerases are recruited to carry out DNA synthesis at the 3'-ssDNA, employing the complementary sequence as a template (**Kane et al., 2012**). Finally, the D-loop is dismantled, the 3'-ssDNA anneals to the complementary strand of the damaged DNA molecule and the two DSB ends are ligated to repristinate DNA integrity.

Single strand annealing (SSA) and alternative end-joining (Alt-EJ) are less characterized pathways whose mechanisms are, similarly to HR, based on resection of the 5' DSB end (**Chang et al., 2017**). Unlike HR, however, they have been described as intrinsically mutagenic (**Mendez-Dorantes et al., 2018; Simsek & Jasin, 2010**). It is still under investigation whether SSA and alt-EJ represent secondary mechanisms that are employed when the main repair pathways are defective, or if certain cellular contexts specifically rely on their activation to repair DSBs (**Deriano & Roth, 2013**).

During Alt-EJ, similarly to HR, the 5' ends of the DSB are resected by the MRN complex and CtIP (**Figure 3**, left) (**Myler et al., 2017**). After this step, short homologous regions in the 3'-ssDNA strands can bind to each other (**Chang et al., 2017**). The protruding 3' ends adjacent to the annealed region can be processed and removed by several nucleases, such as the xeroderma pigmentosum group F-Excision Repair Cross-Complementation Group 1 (XPF-ERCC1) and Artemis (**Chang et al., 2017**). Next, the gaps created by the processing are reconstituted by Pol Θ, which is recruited by Poly(ADP-Ribose) Polymerase 1 (PARP1), and the dsDNA molecule is ligated by DNA ligase 1 or by the DNA ligase 3-X-ray repair cross-complementing protein 1 (LIG3-XRCC1) complex (**Kent et al., 2016; Masani et al., 2016; Ray Chaudhuri & Nussenzweig, 2017**). Similarly, in single strand annealing (SSA), the extensive 5' end resection mediated by MRE11 and EXO1 leads to the annealing of homologous sequences in the 3'-ssDNA (**Figure 3**, right) (**Scully et al., 2019**). These homologous regions are longer than those exposed in Alt-EJ, which is why this pathway is thought to occur especially in repeat-rich domains (**Scully et al., 2019**). In yeast and mammals, the annealing is promoted by RAD52 through the eviction of RPA from the 3'-ssDNA (**Aleksandrov et al., 2020; Bennardo et al., 2008; Symington, 2002**). The excess nucleotides on the 3'-ssDNA are removed by XPF-ERCC1 (**Motycka et al., 2004**). During both Alt-EJ and SSA, the action of nucleases such as XPF-ERCC1 can cause the deletion of nucleotides. For this reason, these repair pathways are considered error-prone (**Mendez-Dorantes et al., 2018; Aleksandrov et al., 2020**).

Different factors, including the complexity of the damage, cell cycle phase and transcriptional activity all participate in the complex regulation of DSB-repair pathway choice (**Scully et al., 2019; Ceccaldi et al., 2016**). For instance, while end-resection during G2 and S phase is promoted by cyclin dependent kinase (CDKs) -dependent phosphorylation of CtIP, ATM and EXO1, in G1 phase the recruitment of p53-binding protein 1 (53BP1) to DSB sites impairs the binding of BRCA1, favoring NHEJ (**Bunting et al., 2010; Escribano-Díaz et al., 2013; Huertas & Jackason, 2009; Jazayeri et al., 2006;**

Tomimatsu et al., 2014). The abundance of Ku throughout the cell cycle also promotes repair through NHEJ, since its binding to DSB ends blocks the recruitment and activation of resection factors (**Mimitou & Symington, 2010**).

During DSB repair, changes in chromatin organization and dynamics are carried out to promote the efficient and timely processing of damage (**Hauer & Gasser, 2017**). Recently, it has been proposed that chromatin, through its marks, its compaction state and its nuclear position can also cover an active role in the repair pathway choice (**Ferrand et al., 2021; Kalousi & Soutoglou, 2016**). Specific histone PTMs, histone variants and non-histone proteins associated with different chromatin domains might strategically promote or limit the access of certain repair factors to the DSB site, channeling the repair towards the pathway that suits best the characteristics of that chromatin region. After discussing the role of ATM in DSB repair in the next chapter, chapter number 5 will discuss the existence of this "histone code", with a special focus on the existing and the damage-induced chromatin marks that have been reported to influence the response to DSB formation in euchromatin and heterochromatin.

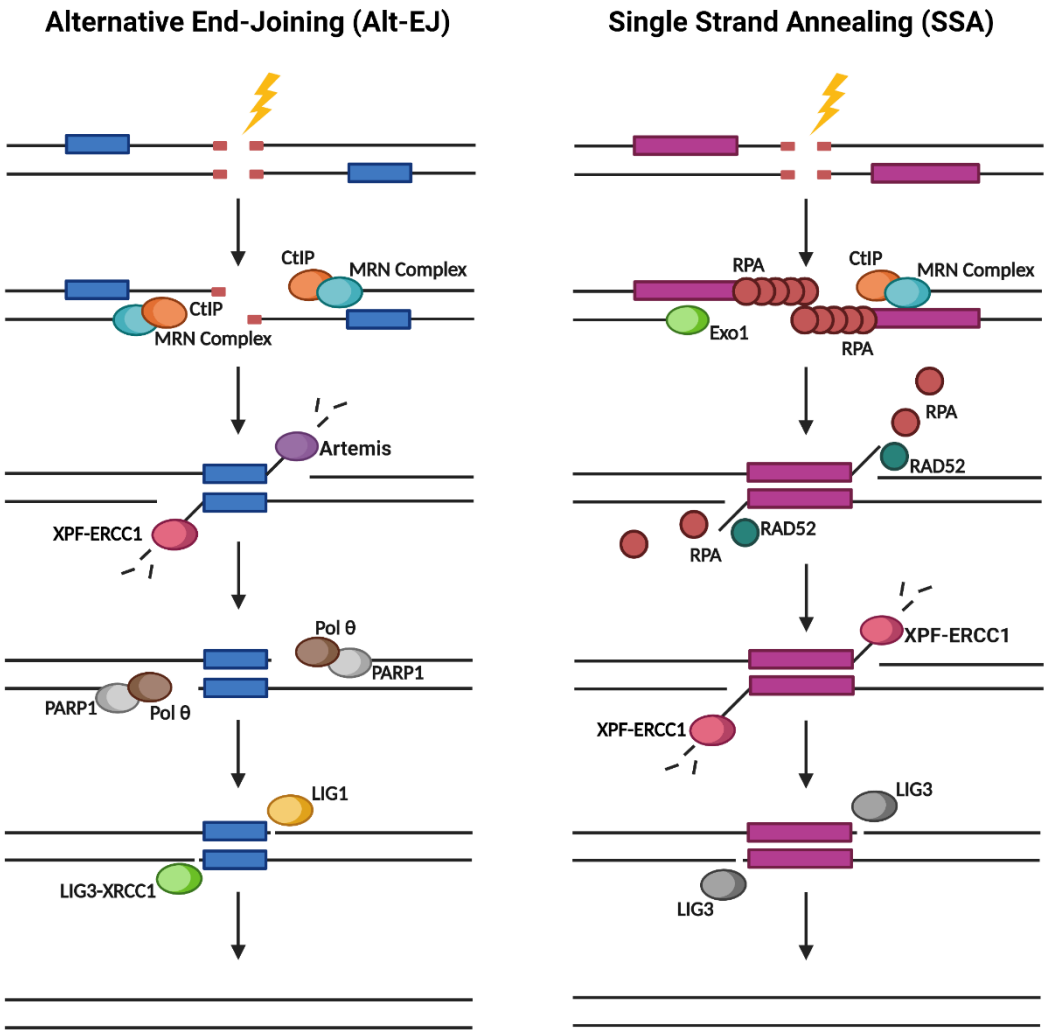

FIGURE 3. Alternative End-Joining (Alt-EJ) and Single Strand Annealing (SSA) Repair Pathways. Left. During alternative end-joining, 5' DSB end resection performed by the MRN complex and CtIP leads to the annealing of complementary sequences contained in the ss-DNA strands. Next, the XPF-ERCC1 and Artemis nucleases process the ss-DNA strands to remove the excessive nucleotides. This results in gaps that are filled in by Polymerase Θ, which is recruited to the DSB site by PARP1. Lastly, ligation of the dsDNA molecule is carried out by LIG1 and LIG3-XRCC1 ligases. **Right**. Similarly to Alt-EJ, single strand annealing starts with MRN-, CtIP- and Exo1-mediated 5'DSB end resection. Annealing of homology regions contained in the resulting ssDNA strands is facilitated by RAD52, which is required to trigger the eviction of RPA molecules from the ssDNA strands. Finally, the nuclease XPF-ERCC1 reduces the ssDNA strands length, and the nicks of both DNA strands are ligated by ligase LIG3. This figure was created on Biorender.com.

4. ATM is the Master Regulator of the General Response to DSBs

In order to create an environment that is accessible to the DSB repair machineries, the activation of the HR or NHEJ pathway is preceded by various chromatin remodeling events (**Aleksandrov et al., 2020**). These are indispensable to allow the recruitment of the repair factors at the site of a broken DNA molecule, and establish the conditions that permit DSB repair pathway choice (**Lee & Paull, 2021**).

The chromatin remodeling process is orchestrated by the ataxia–telangiectasia mutated (ATM) kinase, a member of the PIKK protein family, which in the absence of damage can be found in an inactive and polymeric form (**Aleksandrov et al., 2020; Bakkenist & Kastan, 2003; Savitsky et al., 1995**). Upon the formation of DSBs, ATM is converted in active monomers and recruited to the DSB site through its interaction with the MRN complex (**Andegeko et al., 2001; Bakkenist & Kastan, 2003; Uziel et al., 2003**). The activation of ATM has been proposed to be dependent on different factors, such as its binding to the MRN complex, the acetylation carried out by the TIP60 histone acetyltransferase on ATM and the presence of chromatin marks such as H3K9me3 on chromatin surrounding the DSB (**Ayrapetov et al., 2014; Carson et al., 2003; Sun et al., 2005, 2009**). After multiple auto-phosphorylation events, ATM catalyzes the addition of phosphate groups to hundreds of target proteins, leading to their recruitment and activation at DSBs (**Kozlov et al., 2006, 2011; Matsuoka et al., 2007**). Through this extensive kinase activity, ATM promotes efficient repair of damage, while also regulating cell cycle checkpoint activation, cell cycle arrest and, in case of failing repair attempts, apoptosis (**Shiloh & Ziv, 2013**).

The chromatin response to DSB formation, which is described by the "access-repair-restore" model, entails that transient chromatin re-organization events have to be carried out in order to establish conditions that are favorable to the repair process (**Polo & Almouzni, 2015**). These chromatin events, which include the displacement or eviction of histones to allow the recruitment of repair factors, are reverted after repair is completed so that the original chromatin composition is restored. ATM contributes to the chromatin response to DSBs by promoting chromatin accessibility and by recruiting HR and NHEJ repair factors (**Figure 4**) (**Kakarougkas et al., 2014; Shanbhag et al., 2010; Ziv et al., 2006**). Chromatin accessibility is increased by ATM through the phosphorylation of repressor KRAB-domain associated protein 1 (KAP1), which leads to its dispersion throughout chromatin (**Ziv et al., 2006**). Interestingly, specifically in highly compacted heterochromatin regions, chromatin accessibility is achieved through a specialized mechanism, as phosphorylation of KAP1 promotes chromatin relaxation by weakening the interaction between KAP1 and Chromodomain Helicase DNA Binding Protein 3 (CHD3), thus causing the removal of CHD3 from heterochromatin, which on its turn promotes chromatin decondensation (**Goodarzi et al., 2008, 2011**). Moreover, the formation of ubiquitinated lysine 120 on histone H2B (H2BK120ub), mediated by the ATM substrate RNF20-RNF40, has also been described as a player involved in chromatin decondensation, since H2BK120ub can recruit SNF2H, a remodeling complex able to regulate the spacing of nucleosomes to allow access of repairing factors (**Klement et al., 2014; Moyal et al., 2011; Zhu et al., 2005**).

After promoting the decondensation of the damaged chromatin, ATM participates in the subsequent repair step by recruiting DDR factors at the site of damage. This is achieved through a cascade of chromatin modifications that starts with the phosphorylation of Ser139 on the histone variant H2A.X across chromatin surrounding the break, termed γH2A.X (**Burma et al., 2001; Rogakou et al., 1998**). This event leads to the binding of another ATM substrate, MDC1, which is required to propagate γH2A.X to the neighboring chromatin regions and to recruit the E3 ubiquitin ligase enzyme RNF8 (**Kolas et al., 2007; Lukas et al., 2004; Stucki et al., 2005**). RNF8 ubiquitinates lysine 63 on the H1 histones, triggering the binding of another E3 ubiquitin ligase, RNF168 (**Thorslund et al., 2015**). The modifications carried out by RNF168 on the histones H2A and H2A.X, such as ubiquitination of lysines 13 and 15 (H2AK13/15ub), recruit factors of the HR and NHEJ pathways (**Becker et al., 2021; Doil et al., 2009; Mattiroli et al., 2012**). For instance, NHEJ can be stimulated by the loading of 53BP1 on the site of damage, an event promoted by the ubiquitinated lysine 15 on histone H2A (H2AK15ub) together with the trimethylated lysine 79 on histone H3 (H3K79me3) and dimethylated lysine 20 on histone H4 (H4K20me2) (**Fradet-Turcotte et al., 2013; Huyen et al., 2004; Sanders et al., 2004**). Similarly, the ubiquitination events carried out by RNF8 and RNF168 seem to facilitate the loading of factors involved in HR. In particular, it was recently found that the BRCA1-BARD1 complex is recruited at DSB sites through the interaction with H2AK13/15ub (**Becker et al., 2021; Dai et al., 2021; Hu et al., 2021**). By catalyzing the deposition of ubiquitin molecules on lysines 125, 127 and 129 on histone H2A (H2AK125/127/129ub), BRCA1-BARD1 further triggers the association of the remodeler SMARCAD1, an event that is crucial to remove 53BP1 and proceed with repair through HR (**Densham et al., 2016; Kalb et al., 2014**).

In addition to modifying chromatin components, ATM directly phosphorylates repair proteins to contribute to the regulation of their function during DSB repair. ATM targets include NHEJ proteins such as 53BP1, DNA-PKcs, Artemis,

XRCC4 and Pol λ, and HR proteins, including CtIP, EXO1, BLM, MRE11, BRCA1 and PALB2 (**Ababou et al., 2000; Ahlskog et al., 2016; Bolderson et al., 2010; Bothmer et al., 2011; B. P. C. Chen et al., 2007; Cortez et al., 1999; Imamichi et al., 2014; Riballo et al., 2004; Sastre-Moreno et al., 2017; H. Wang et al., 2013**). The interplay between many of the proteins involved in DSB repair reveals the existence of a more complex system for repair pathway choice.

In conclusion, evidence suggests that ATM kinase activity plays a general supporting role for both HR and NHEJ by favoring the recruitment and activation of many factors involved in the two pathways. ATM's duty is therefore to assist the repair pathway choice by organizing an environment that is open to the access of a multitude of repair proteins.

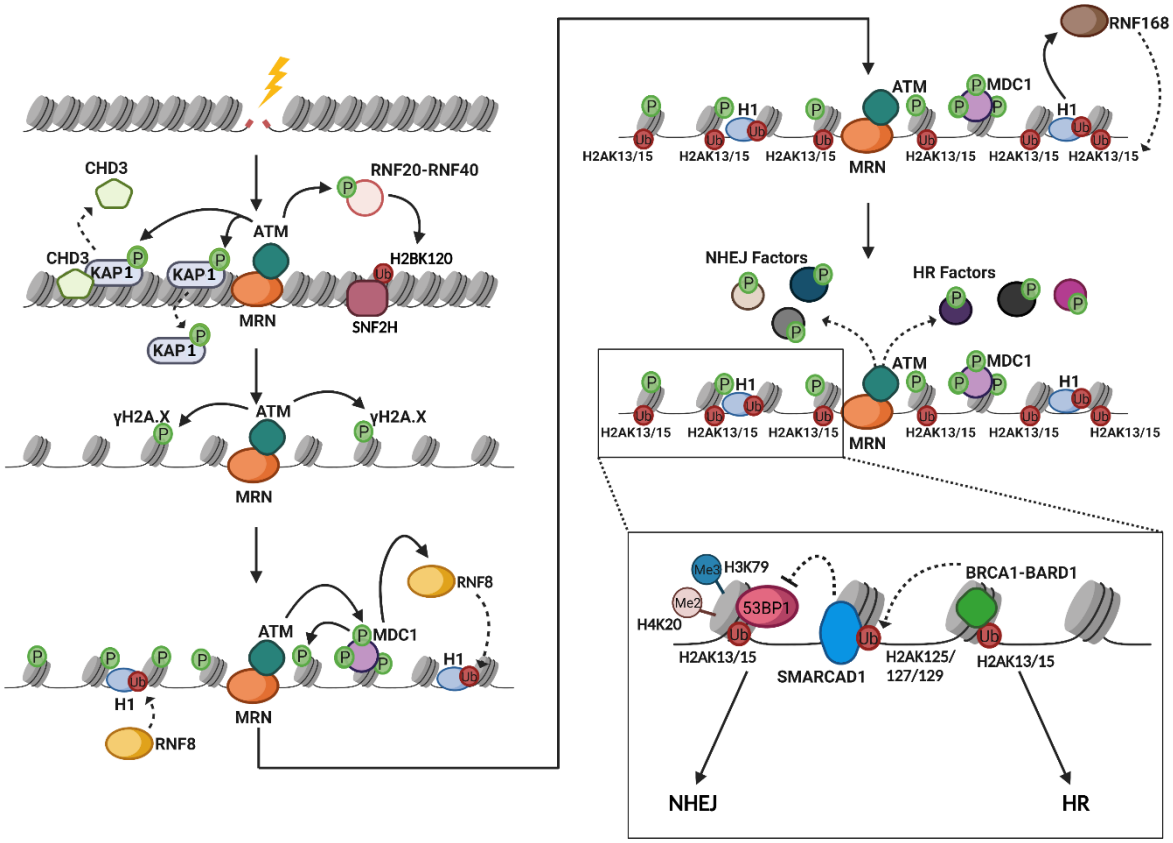

FIGURE 4. ATM orchestrates the general chromatin response to DSBs. After DSB induction, the MRN complex recruits ATM at the site of damage. ATM facilitates chromatin decondensation by phosphorylating KAP1, leading to its displacement from chromatin. In heterochromatin, KAP1 phosphorylation results in CHD3 eviction. Moreover, phosphorylation of RNF20-RNF40 by ATM promotes ubiquitination of H2BK120, allowing the association of SNF2H and the consequent chromatin relaxation. In addition to promoting chromatin relaxation, ATM triggers chromatin modifications that allow the recruitment of repair factors. ATM phosphorylates the histone variant H2A.X (γH2A.X), leading to the recruitment of another ATM substrate, MDC1, which deposits additional γH2A.X marks and recruits RNF8. RNF8 ubiquitinylates the linker histone H1 causing the association of RNF168, which in turn ubiquitinylates lysines 13 and 15 on H2A. This histone mark represents a recruitment signal for NHEJ and HR proteins alike. The NHEJ-promoting protein 53BP1 recognizes H2AK13/15ub, H4K20me2 and H3K79me3, while the interaction between BRCA1-BARD1 and H2AK13/15ub promotes the ubiquitinylation of lysines 125, 127 and 129 on histone H2A. The deposition of these histone marks triggers the association of SMARCAD1 which inhibits 53BP1's recruitment. Promotion of DSB repair by ATM is also achieved through the direct phosphorylation of HR and NHEJ proteins. This figure was created on Biorender.com.

5. Influence of Heterochromatic and Euchromatic Proteins on DSB Repair

5.1. Impact of Heterochromatin Histone Marks and Proteins on DSB Repair in Heterochromatin

The repetitive and compact nature of heterochromatin affects its susceptibility to DNA damage and the efficiency of DNA repair in this domain. The centromeric and pericentromeric regions, in fact, seem to be particularly prone to the formation of DSBs associated with replication stress, reflecting the difficulty of successfully replicate repeat-rich regions (**Chakraborty et al., 2020; Crosetto et al., 2013**). Moreover, heterochromatin compaction has been described as an obstacle for fast and efficient DNA damage repair (**Fortuny & Polo, 2018**). Although access of repair factors is not blocked

by heterochromatin, several studies have in fact showed that both Nucleotide Excision Repair (NER) and Mismatch Repair (MMR) are slower in this domain (**Adar et al., 2016; Han et al., 2015; Jiricny, 2013**). It is not clear whether repair of DSBs is also carried out with different kinetics in euchromatin versus heterochromatin, since supporting evidence originally obtained in mouse cells has not been confirmed in subsequent studies conducted in *Drosophila* (**Chiolo et al., 2011; Goodarzi et al., 2008; Janssen et al., 2016**).

HR has been described as a major repair pathway in heterochromatin in both *Drosophila* and mammalian cells (**Beucher et al., 2009; Chiolo et al., 2011; Kakarougkas et al., 2013**). However, NHEJ can also be employed - even in higher percentages than HR, as shown in *Drosophila* somatic cells and mouse (**Janssen et al., 2016; Tsouroula et al., 2016**). The mutagenic repair pathway Alt-EJ has also been found to be used to repair CRISPR-Cas9 DSBs in heterochromatin (**Schep et al., 2021**). Interestingly, Alt-EJ repair pathway usage seems to be higher in heterochromatin than euchromatin (**Schep et al., 2021**). Lastly, heterochromatin does not seem to heavily rely on the alternative DSB repair pathway SSA. The use of this alternative repair pathway has been hypothesized to be reserved for repairing events in the case of defective canonical repair pathways, such as HR or NHEJ repair (**Janssen et al., 2016; Tsouroula et al., 2016**).

Interestingly, research has established that DSB repair pathway usage varies amongst the different heterochromatin compartments. For instance, in mouse cells, the repeat-rich centromeric region relies on HR throughout interphase (**Tsouroula et al., 2016**). On the contrary, HR in heterochromatin surrounding the centromere, known as "pericentromere", is restricted to the S/G2 phase (**Tsouroula et al., 2016**). Telomeres, the heterochromatic domains located at the ends of chromosomes, are preferentially repaired by HR and Alt-EJ (**Doksani & de Lange, 2016**). Finally, heterochromatin sequences associated with the nuclear lamina (LADs) recruit NHEJ and Alt-EJ factors and display a higher Alt-EJ-to-NHEJ ratio compared to euchromatin (**Lemaître et al., 2014; Schep et al., 2021**).

Research focusing on the influence of chromatin on the DNA damage response has revealed that several heterochromatin components, such as histone marks, chromatin remodelers and heterochromatin-associated proteins are involved in the organization and the completion of DSB repair in this domain (**Caron et al., 2021**). While chromatin proteins can affect several heterochromatin properties, including its position in the nuclear space, its compaction and its transcriptional state, they can also participate in DSB processing more directly by modulating DDR signaling and the association of repair factors to the damaged heterochromatin. In this way, heterochromatin proteins can contribute to the repair pathway choice process and influence the outcome of DSB repair.

5.1.1. Role of Heterochromatin Histone Marks and Proteins in Repair Pathway Choice in Heterochromatin

Several features of heterochromatin have been identified as involved in the regulation of DSB repair pathway choice in this domain (**Figure 5A**). For instance H3K27me3, which characterizes facultative heterochromatin, has recently been linked to the regulation of the Alt-EJ-to-NHEJ ratio in heterochromatin (**Schep et al., 2021**). The authors demonstrated that the use of Alt-EJ in DSB repair decreases when H3K27me3 is impaired, suggesting that this histone mark facilitates Alt-EJ and/or negatively regulates NHEJ.

Studies have also investigated the influence of the other typical heterochromatin histone mark, H3K9me3, on the process of repair pathway choice. H3K9me3 is enriched at heterochromatic DSBs in both *Drosophila* and murine cells, and appears to promote DSB repair through HR (**Janssen et al., 2019; Tsouroula et al., 2016**). Depletion of H3K9 methyltransferases results in the reduction of cells with Tosca (the homolog of resection factor Exo1 in *Drosophila*) enriched at pericentromeric DSBs, hinting that methylation of H3K9 could favor HR repair through the recruitment of associated factors (**Janssen et al., 2019**). The mechanism through which H3K9me3 promotes HR might involve TIP60, a factor that has been shown to interact with H3K9me3 in *in vitro* and *in vivo* experiments and that can favor BRCA1 recruitment at the sites of damage (**Sun et al., 2009; Tang et al., 2013**). A role for H3K9me3 in HR regulation was also suggested in mouse cells, where the deposition of this histone mark by SET domain bifurcated histone lysine methyltransferase 1 (SETDB1) contributes to alternative lengthening of telomeres (ALT), a process that depends on HR (**Gauchier et al., 2019**).

Enrichment of monomethylated lysine 9 on histone H3 (H3K9me1) and monomethylated lysine 56 on histone H3 (H3K56me1) was further detected upon DSB generation in heterochromatin (**Janssen et al., 2019**). This accumulation of H3K9me1 and H3K56me1 results from the demethylation of H3K9me2/me3 and H3K56me2/me3 at heterochromatic DSBs, which is mediated by the demethylase dKDM4A (**Colmenares et al., 2017; Janssen et al., 2019**). While dKDM4A is important to guarantee the proper repair kinetics in heterochromatin, its demethylation activity also impacts the process of repair pathway choice in this domain. Since dKDM4A depletion caused increased recruitment of HR factors and decreased NHEJ usage in damaged heterochromatin, the authors have hypothesized that H3K9me1 and H3K56me1 could

represent recruitment signals for NHEJ proteins, in opposition to the HR-prone role that H3K9me2/3 might cover (**Janssen et al., 2019**).

In addition to the heterochromatic histone marks, the H3K9me3-binding protein HP1 might affect DSB repair pathway decision in heterochromatin. Although accumulation of HP1 has been documented both in euchromatin and heterochromatin (see "Impact of Heterochromatin Histone Marks and Histone Variants on DSB Repair in Euchromatin and Heterochromatin"), in heterochromatin the homolog HP1γ might specifically promote the usage of NHEJ for DSB repair (**Tsouroula et al., 2016**).

Overall, several components of heterochromatin, including histone marks and their ligands, are emerging as key players in the repair pathway choice process in heterochromatin. Further research focused on defining the mechanisms underlying the contributions of heterochromatin proteins will improve our understanding of their roles during DSB repair and determine whether these roles are conserved across different species.

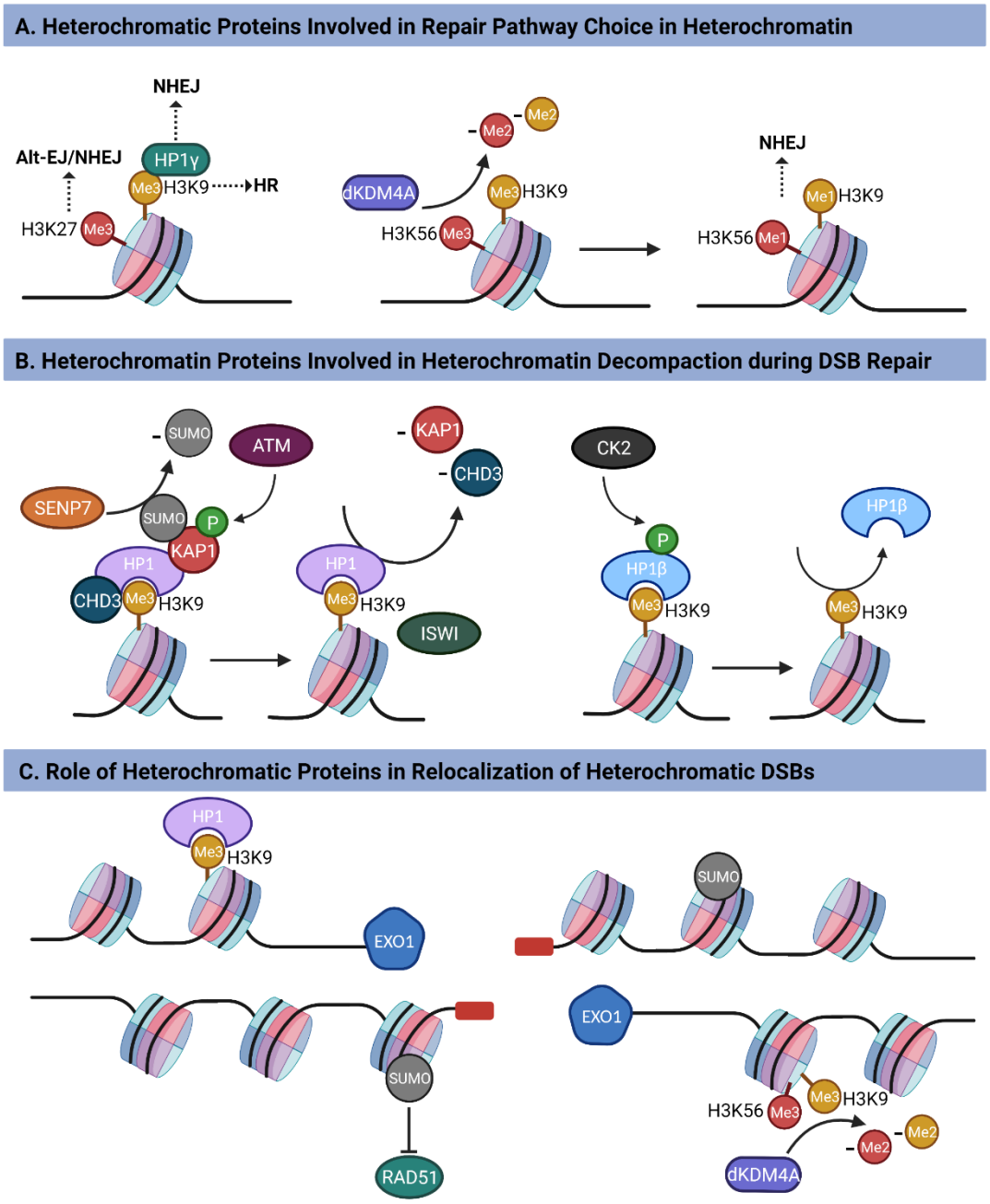

Figure 5. Heterochromatic Proteins Involved in Heterochromatic DSB Repair. A. Various heterochromatic proteins participate in the repair pathway choice in heterochromatin. H3K27me3 contributes to the regulation of Alt-EJ or NHEJ, while enrichment of H3K9me3 at DSBs promotes repair through HR. HP1γ, which binds to H3K9me3, facilitates NHEJ. Demethylation of H3K56 and H3K9 by dKDM4A promotes usage of NHEJ for DSB repair. **B.** Heterochromatin proteins stimulate heterochromatin decompaction during DSB repair.

KAP1, which binds to HP1, is phosphorylated by ATM and de-sumoylated by SENP7, leading to its displacement and the removal of CHD3. This in turn stimulates recruitment of the chromatin remodeler ISWI, which mediates chromatin relaxation. HP1β is released from heterochromatin upon CK2-dependent phosphorylation, contributing to heterochromatin decondensation. **C.** Various heterochromatic proteins are required for the relocalization of heterochromatic DSBs, including HP1 and dKDM4A. Relocalization is also promoted by the processes of end-resection and sumoylation, which additionally suppress the recruitment of RAD51 inside the heterochromatic domain before DSB relocalization. This figure was created on Biorender.com.

5.1.2. Role of Heterochromatin Histone Proteins in Heterochromatin Decompaction during DSB Repair

While some heterochromatin proteins can affect the process of repair pathway choice, others, including remodelers and structural proteins, can participate in earlier phases of DSB repair in heterochromatin (**Caron et al., 2021**). Specifically, before the actual repair takes place, several heterochromatin proteins take part in the re-organization of heterochromatin, a process that aims at creating a chromatin environment accessible to the repairing factors and suitable for the repairing process. Since heterochromatin is characterized by high nucleosomal density, it has in fact been proposed that this domain requires to undergo decompaction in order for DSB repair to occur (**Goodarzi et al., 2008; Noon et al., 2010**). In support of this theory, experimental evidence has demonstrated that chromatin relaxation takes place in pericentromeric heterochromatin in different species (**Chiolo et al., 2011; Jakob et al., 2011; Tsouroula et al., 2016**). However, this might not be the case for all heterochromatin regions, since decondensation is not a prerequisite for repair in telomeres (**Timashev et al., 2017; Vancevska et al., 2017**).

One of the proteins involved in the promotion of relaxation is HP1β, a component of heterochromatin which associates with the histone mark H3K9me3 (**Figure 5B**) (**Ayoub et al., 2008**). HP1β-mediated promotion of chromatin decompaction depends on its phosphorylation by casein kinase 2 (CK2) upon the induction of DSBs, an event that leads to its detachment from the damage site and to consequent chromatin relaxation (**Ayoub et al., 2008**). A central role in promoting heterochromatin relaxation is also covered by the phosphorylation of KAP1 by ATM. KAP1 is a component of heterochromatin which associates with HP1 (**Sripathy et al., 2006**). After DSB induction, KAP1 is phosphorylated by ATM and desumoylated by SUMO1/Sentrin Specific Peptidase 7 (SENP7), which leads to its removal and the displacement of the remodeler CHD3 from damaged chromatin (**Garvin et al., 2013; Goodarzi et al., 2008, 2011; Ziv et al., 2006**). This event, in turn, stimulates the binding of the antagonistic remodeler Imitation Switch (ISWI) to the DSB site, which results in chromatin relaxation (**Klement et al., 2014**).

In conclusion, various heterochromatin proteins promote efficient DSB repair by alleviating the chromatin compaction typical of heterochromatin. It will be interesting, in the future, to investigate whether different heterochromatic domains are decompacted through similar mechanisms during DSB repair, or if the specificities of heterochromatin relaxation vary across different domains.

5.1.3. Role of Heterochromatin Proteins in Heterochromatin Movement during DSB Repair

The presence of repeated sequences in heterochromatin represents a challenge for efficient repair of DSBs arising in this domain, since their tendency to undergo illegitimate recombination events can result in the formation of aberrant products, including dicentric/acentric chromosomes, translocations and deletions (**Peng & Karpen, 2008**). It is therefore surprising that repair in this domain often relies on the HR pathway, given that NHEJ might appear as a safer choice. Usage of NHEJ could result in small insertions and deletions, but the impact of these DNA changes would likely be very modest, considering that heterochromatin contains relatively few genes when compared to euchromatin (**Hauer & Gasser, 2017**). In order to safely repair DSBs through HR in heterochromatin, cells have adopted a number of strategies that minimize the chance of aberrant recombination.

One such strategy involves the relocalization of DSBs, which has been shown to take place in *Drosophila* and mouse heterochromatin. In these two species, repair of DSBs through HR is initiated inside the heterochromatin domain, where all the early repair steps are carried out. After end resection is completed, however, repair is temporarily halted and the damaged heterochromatin is relocalized to the periphery of the heterochromatin compartment, where the subsequent steps of HR can occur and repair can be finalized (**Chiolo et al., 2011; Jakob et al., 2011; Janssen et al., 2016; Ryu et al., 2015; Tsouroula et al., 2016**). In particular, in *Drosophila*, a fraction of the heterochromatic DSBs are transported to the nuclear periphery through the contribution of the motor protein myosin and actin filaments (**Caridi et al., 2018; Ryu et al., 2015**).

Several heterochromatic proteins are involved in the promotion of DSB relocalization, including HP1 and dKDM4A (**Figure 5C**) (**Chiolo et al., 2011; Colmenares et al., 2017**). Additionally, the processes of end-resection and SUMOylation have also been demonstrated to contribute to the DSB movement to the nuclear periphery (**Chiolo et al., 2011; Ryu et al., 2015, 2016**). SUMOylation appears to be particularly important to promote safe HR in heterochromatin,

since it also blocks RAD51 recruitment inside the damaged heterochromatic domain and thereby suppresses aberrant recombination events between heterochromatic repeats (**Ryu et al, 2015, 2016**). Consistently, RAD51 recruitment and strand invasion can be performed only upon DSB relocalization to the nuclear periphery and ubiquitination of SUMOylated proteins (**Ryu et al., 2015**). Relocation of damaged heterochromatin has been observed in mouse as well, where centromeric and pericentromeric DSBs migrate to the periphery of their heterochromatic regions in order to be repaired by HR (**Jakob et al., 2011; Tsouroula et al., 2016**). Similarly to what has been described in *Drosophila*, DSB relocation in mouse cells depends on end-resection, and RAD51 is excluded from the center of the heterochromatic damaged domain (**Tsouroula et al., 2016**).

It has been shown that interfering with the relocation process results in erroneous recombination and rearrangements events (**Chiolo et al., 2011; Ryu et al., 2015**). Therefore, the separation in space and time between the resection and the homology search during HR, as reported in the two species, is thought to inhibit dangerous recombination events between repeated sequences present in heterochromatin surrounding the damage. By confining the DSB domain to a peripheral region, the use of proper donor sequences, such as homologous chromosomes or sister chromatids, is encouraged, and the safe repair of DSBs in repetitive DNA is promoted.

Together, current models of heterochromatin repair suggest the presence of specialized processes of heterochromatin decompaction, DSB movement and repair. Future work will likely elucidate how these dynamics are intertwined and how these processes are conserved in other heterochromatin domains, such as LADs and NADs.

5.2. Impact of Euchromatin Histone Marks and Histone Variants on DSB Repair in Euchromatin

Studies focusing on the genome-wide distribution of DSBs have identified active transcribed regions as particularly susceptible to the generation of DNA breaks (**Canela et al., 2017, 2019; Crosetto et al., 2013; Yan et al., 2017**). Late-replicating long genes, together with early-replicating active genes are considered fragile sites (CFSs and ERFSs, respectively), which are sites of breakage, caused by replication stress, visible on metaphase chromosomes (**Glover et al., 1984; LeTallec et al., 2013; Tubbs et al., 2018**). CFSs have been extensively studied as sites prone to the formation of chromosomal rearrangements, however our current understanding of their characteristics is still incomplete (**Hirsch, 1991; Saayman & Esashi, 2021**). Nonetheless, evidence suggests that DSB formation is more common in actively transcribed regions (**Canela et al., 2017, 2019; Crosetto et al., 2013; Yan et al., 2017**). Several mechanisms have been proposed to explain the link between transcriptional activity and damage formation. These include replication-transcription conflicts and the presence of secondary structures (R-loops, G-quadruplex etc.), which could accumulate and cause the formation of stalled replication forks (**Bochman et al., 2012; Gan et al., 2011; Helmrich et al., 2011; Prado & Aguilera, 2005**). In addition, these structures could promote the collision between the replication and the transcriptional machineries, resulting in an increased chance of DSB formation (**Voineagu et al., 2008**). Topoisomerases, which are normally utilized to induce DNA breaks and relieve topological tension during several processes of DNA metabolism, have also been described as a possible source of DSBs during transcription (**Marnef et al., 2017**). It has been shown that DSBs are generated by Topoisomerase II to resolve DNA supercoils that could impede RNA polymerase during transcription elongation (**Joshi et al., 2012**). In addition, Topoisomerase I and II can induce the formation of single-strand breaks (SSBs) and DSBs to promote transcription initiation (**Puc et al., 2015; Trotter et al., 2015**). While topoisomerase-mediated DSBs are intermediates that should be resolved, failure to do so might transform them into stable DSBs requiring the activation of the DNA damage response (**Marnef et al., 2017**).

The accurate repair of DSBs in euchromatin is particularly important to preserve the integrity of crucial genomic sites such as genes and their regulatory elements. Accordingly, HR appears to be the preferential repair pathway used in actively transcribed regions. Indeed, DSBs in actively transcribed genes are specifically associated with the HR factor RAD51, when compared to other regions of the genome (**Aymard et al., 2014; Wei et al., 2015**). However, HR is only prevalent during the G2 and S phases of the cell cycle and the recruitment of NHEJ proteins in euchromatin has been documented, indicating that transcriptionally active sites are repaired by other pathways as well (**Aymard et al., 2014; Schep et al., 2021; Ui et al., 2015; Wei et al., 2015**).

Similarly to heterochromatin, the combination of histone marks, histone variants and chromatin-interacting proteins in the euchromatic environment all participate in the regulation of DSB repair.

5.2.1. Role of Euchromatin Histone Marks in Repair Pathway Choice in Euchromatin

Both the pre-existing chromatin marks as well as the DNA-damage induced PTMs on histones promote the recruitment of crucial DSB repair factors. These histone marks can directly influence the accessibility of break sites as well as direct the repair pathway choice process.

A crucial role in the process of repair pathway choice is covered by lysine 20 on histone 4 (H4K20) (**Figure 6A**). Depending on its methylation state, H4K20 can channel repair to HR or NHEJ. Dimethylated H4K20 (H4K20me2) represents a very common histone mark associated with most H4 histones in undamaged chromatin, however conflicting evidence exists on its accumulation upon the formation of DSBs (**Botuyan et al., 2006; Hsiao & Mizzen, 2013; Pei et al., 2011; Schotta et al., 2008; Tuzon et al., 2014**). Nevertheless, the combination of H4K20me2 and the DNA damage-induced histone mark H2AK15ub is necessary for the enrichment of 53BP1, a key NHEJ factor that blocks end resection and HR-dependent DSB repair (**Botuyan et al., 2006; Fradet-Turcotte et al., 2013; Mattiroli et al., 2012**). However, the interaction between H4K20me2 and 53BP1 is regulated by the presence of many antagonists which compete for H4K20me2. For example, the acetyltransferase TIP60 acetylates H2AK15 and H2AK16 to inhibit 53BP1's association with H4K20me2, with acetylated H2AK16 further supporting HR by serving as a binding site for BRCA1 (**Hsiao & Mizzen, 2013; Jacquet et al., 2016; Tang et al., 2013**).

Moreover, the distribution of H4K20me2 throughout the cell cycle regulates repair pathway choice. While H4K20me2 is abundant in G1, it becomes sparser after S phase, when new unmethylated H4 histones are incorporated in post-replicative chromatin (**Pellegrino et al., 2017; Pesavento et al., 2008; Saredi et al., 2016**). As a result, NHEJ is especially promoted before replication, when H4K20me2 levels are higher (**Pellegrino et al., 2017**). In line with this, in S/G2 phases, unmethylated H4K20 (H4K20me0) is recognized by the TONSL-MMS22L and the BRCA1-BARD1 complexes to ensure DSB repair through the HR pathway (**Nakamura et al., 2019; Saredi et al., 2016**).

In contrast to H4K20me2-marked chromatin, other euchromatic regions enriched for the transcription-associated trimethylated lysine 36 on histone H3 (H3K36me3) have been shown to rely on HR for DSB repair. Specifically, H3K36me3 promotes HR by recruiting LEDGF, which is associated with the resection factor CtIP (**Aymard et al., 2014; Bleuyard et al., 2017; Carvalho et al., 2014; Daugaard et al., 2012; Pfister et al., 2014**). Consistent with this, damage arising in H3K36me3-poor euchromatin was found to employ mainly NHEJ (**Aymard et al., 2014; Pfister et al., 2014**). Interestingly, in humans, dimethylation of H3K36 (H3K36me2) could potentially play a different role than H3K36me3, as it was reported that this mark is enriched close to sites of DSB induction, where it enhances the binding of NHEJ factors such as Ku70 (**Fnu et al., 2011**). Finally, it has been described that certain histone mark associated with transcription need to be removed for repair to proceed (**Gong et al., 2017**). For instance, it was proposed that removal of the methyl groups from trimethylated lysine 4 on histone H3 (H3K4me3) by the demethylase enzyme KDM1A is essential to promote transcriptional silencing of damaged chromatin by ZMYND8–NuRD (**Gong et al., 2017**).

Collectively, these studies suggest that during the response to DNA damage, several histone marks typical of euchromatin positively contribute to the repair of DSB, especially by regulating the usage of the different repair pathways.

5.2.2. Role of Euchromatin Histone Variants in DSB Repair in Euchromatin

Euchromatic elements such as gene bodies, promoters and enhancers normally contain the histone variants H3.3 and H2A.Z (**Adam et al., 2001; Chow et al., 2005; Goldberg et al., 2010; Jin et al., 2009**). In addition to their other functions linked to gene regulation, several lines of evidence suggest that these histones play direct roles in DSB repair.

While H3.3 is observed in undamaged chromatin, this histone variant can also be enriched at sites of DSB induction, where it impacts both the processes of NHEJ and HR (**Figure 6B**) (**Juhász et al., 2018; Luijsterburg et al., 2016**). Since the depletion of H3.3 causes the impairment of NHEJ, it has been proposed that effective repair through this pathway requires the presence of this histone variant (**Luijsterburg et al., 2016**). Although the underlying mechanism has not been defined yet, it is possible that H3.3 contributes to the generation of a chromatin context that is favorable to the recruitment of NHEJ components, in part through the binding of Ku80 (**Luijsterburg et al., 2016**). In line with this, H3.3-containing nucleosomes have been demonstrated to be more mobile than nucleosomes containing canonical histones, suggesting that H3.3 incorporation at DSB sites might facilitate histone displacement to render chromatin more permissive to the recruitment of repair proteins (**Jin & Felsenfeld, 2007**). It has also been reported that the incorporation of H3.3 can be mediated by Alpha Thalassemia/Mental Retardation Syndrome X-linked (ATRX) and Death domain associated protein (DAXX) during HR-mediated repair (**Juhász et al., 2018**). In this case, H3.3 inclusion is carried out at a later stage of repair, after resection and strand invasion, and supports DNA synthesis, favoring sister chromatid exchange (SCEs) as an outcome of repair (**Juhász et al., 2018**). The authors have hypothesized that the incorporation of H3.3 during DNA synthesis might be important to relieve the negative supercoiling of the DNA located behind the polymerase, a

topology that promotes the deposition of new nucleosomes and the structural integrity of the stressed DNA (**Juhász et al., 2018**).

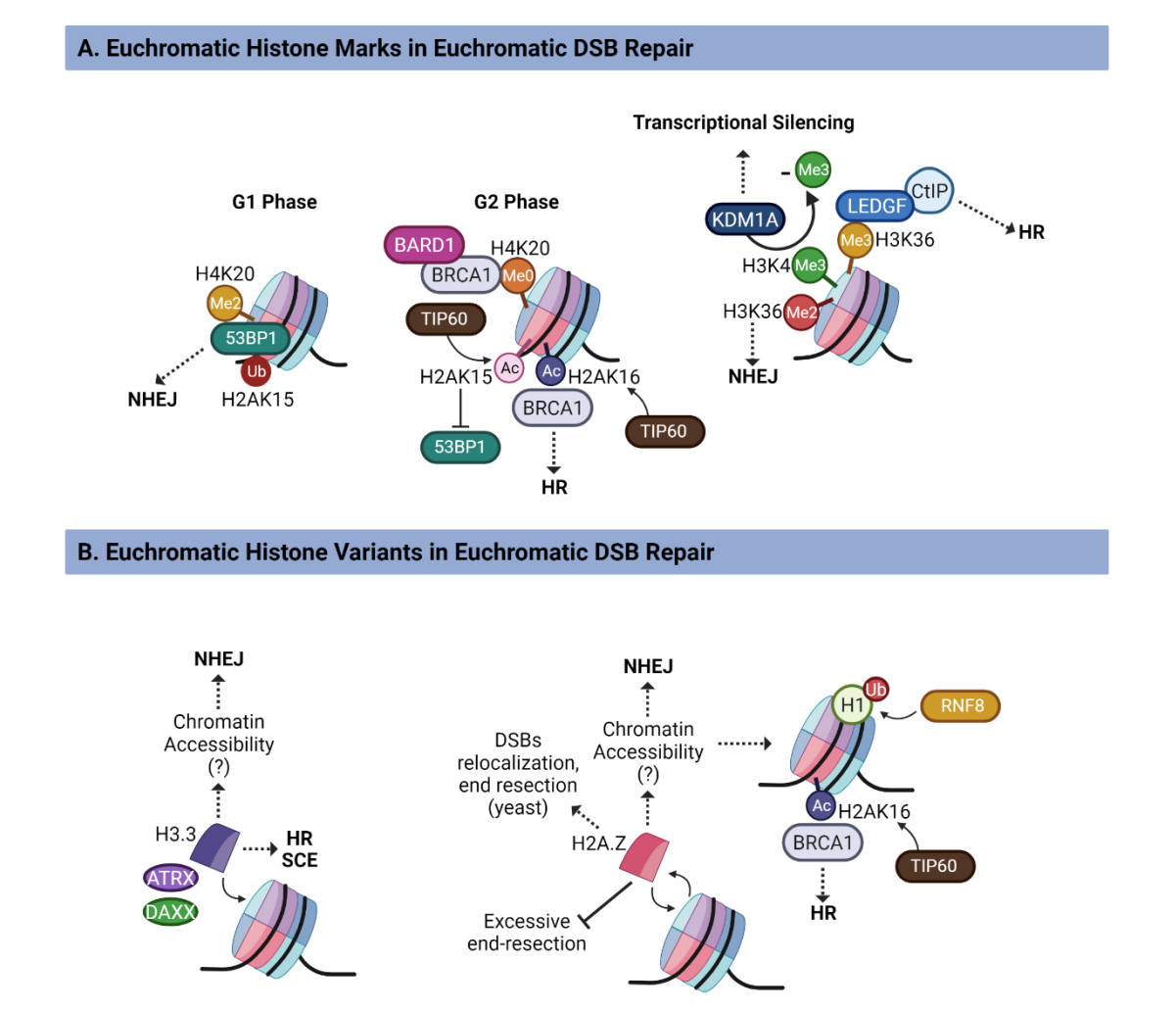

FIGURE 6. Euchromatic Histone Marks and Variants Involved in Euchromatic DSB Repair. A. Role of Euchromatic Histone Marks in DSB Repair. During G1, 53BP1 recognizes H4K20me2 and H2AK15ub at sites of DSBs, channeling repair to NHEJ. During G2, the absence of H4K20me2 triggers the association of the BRCA1-BARD1 complex, which is involved in HR. Additionally, during this cell cycle phase, TIP60 deposits H2AK15ac and H2AK16ac at DSBs to inhibit 53BP1's recruitment and promote the recruitment of BRCA1, facilitating repair through HR. The LEDGF-CtIP complex promotes HR through the interaction between H3K36me3 and LEDGF, while H3K36me2 facilitates NHEJ. Finally, removal of H3K4me3 by KDM1A influences DSB repair by stimulating transcriptional repression in euchromatin. **B.** Role of Euchromatic Histone Variants in DSB Repair. Histone variant H3.3 channels DSB repair through NHEJ, possibly by rendering chromatin more accessible to NHEJ proteins. Additionally, deposition of H3.3 by ATRX and DAXX at sites of damage during HR repair influences the repair outcome by promoting sister chromatid exchanges (SCE). Deposition of H2A.Z at sites of DSB leads to acetylation of H2AK16 and ubiquitinylation of histone linker H1, promoting the HR pathway. H2A.Z can also stimulate NHEJ. The effects of H2A.Z on HR and NHEJ might be mediated by its ability to promote chromatin accessibility. Moreover, H2A.Z regulates end resection, while in yeast it facilitates the relocalization of persistent DSBs and end resection. This figure was created on Biorender.com.

In addition to H3.3, research has identified the histone variant H2A.Z as an important regulator of DSB repair. This is supported by data showing that H2A.Z depletion causes genomic instability and sensitivity to DNA-damaging compounds, and by the observation that H2A.Z is assembled at DSB sites (**Alatwi & Downs, 2015; Gursoy-Yuzugullu et al., 2015; Kalocsay et al., 2009; Kusch et al., 2004; Morillo-Huesca et al., 2010; Papamichos-Chronakis et al., 2011; Y. Xu et al., 2012**).

H2A.Z has been proposed to be involved in the regulation of both NHEJ and HR pathways. First, it has been reported that deposition of H2A.Z is necessary for several key steps of DSB repair to take place, including TIP60-dependent acetylation of histone H4 and ubiquitination events mediated by RNF8 (**Kolas et al., 2007; Mailand et al.,**

2007; Murr et al., 2005; Y. Xu et al., 2012). These acetylation and ubiquitination events, in turn, support HR by promoting BRCA1 recruitment (**Kolas et al., 2007; Mailand et al., 2007; Murr et al., 2005**). In agreement, it was shown that depletion of H2A.Z leads to reduced BRCA1 levels at DSBs and decreased HR usage (**Y. Xu et al., 2012**). Surprisingly, upon H2A.Z knock-down a decline in NHEJ usage and a decrease in the levels of NHEJ factor Ku70/80 at DSBs were also observed (**Y. Xu et al., 2012**). This indicates that H2A.Z might also positively affect the NHEJ pathway by facilitating the recruitment of NHEJ-associated proteins (**Y. Xu et al., 2012**). Since H2A.Z, similarly to H3.3, confers to nucleosomes a lower degree of stability, it is possible that H2A.Z promotes the association of HR and NHEJ factors by stimulating the re-organization of the damaged chromatin into a more relaxed and accessible form (**Jin et al., 2009**). Additionally, it was proposed that H2A.Z could participate in DSB processing by regulating the resection step during repair (**Alatwi & Downs, 2015; Y. Xu et al., 2012**). Xu and colleagues reported that depletion of H2A.Z results in accumulation of RPA binding and impaired association of Ku70/80 to the DSB ends, suggesting that in the absence of H2A.Z excessive end resection occurs (**Y. Xu et al., 2012**). Accordingly, when CtIP was depleted to impede end resection in cells lacking H2A.Z, Ku70/80 was again observed at DSB sites (**Y. Xu et al., 2012**). These results indicate that the presence of H2A.Z at DSBs is required to prevent extreme resection of DSB ends, an event that would impede the recruitment of NHEJ factor Ku70/80 to the sites of damage (**Y. Xu et al., 2012**). It has also been proposed that deposition of H2A.Z is beneficial for DSB repair only if closely followed by its removal (**Alatwi & Downs, 2015; Gursoy-Yuzugullu et al., 2015; Lademann et al., 2017**). Indeed, interfering with H2A.Z removal leads to hypoacetylated H4, impaired RAD51 loading, reduced recruitment of NHEJ factors and enhanced usage of the Alt-EJ pathway (**Alatwi & Downs, 2015; Gursoy-Yuzugullu et al, 2015**). It is however not clear if excessive resection is prevented by H2A.Z or by its removal (**Alatwi & Downs, 2015; Gursoy-Yuzugullu et al, 2015; Lademann et al., 2017**). Interestingly, in yeast, H2A.Z is involved in the repair of persistent DSBs through the promotion of the relocation of damaged chromatin to the nuclear periphery and the resection of the damaged DNA (**Horigome et al., 2014; Kalocsay et al., 2009**). This result suggests that H2A.Z could operate through different mechanisms inside the cell, depending on the context of the DSB.

Overall, the evidence discussed above demonstrates that repair of DSBs in euchromatin relies on the contributions of many histone marks and variants during various steps, including transcriptional silencing, which is crucial in euchromatin, and the process of repair pathway choice. The roles of euchromatic histone marks in DSB repair are better characterized than the ones covered by histone marks in heterochromatin, however the underlying mechanisms are not fully understood yet. For example, it would be interesting to clarify how euchromatic histone marks are regulated during different phases of the cell cycle. This might be especially relevant since it has been proposed that, during G1, DSBs arising in transcriptionally active genes are not repaired, but clustered and processed by the HR machinery only after S phase (**Aymard et al., 2017**). Additionally, it will be important to determine how transcription is recovered after DSB repair, and whether euchromatin proteins are involved in this process.

5.3. Impact of Heterochromatin Histone Marks and Histone Variants on DSB Repair in Euchromatin and Heterochromatin

Interestingly, research has uncovered that several heterochromatic proteins are involved in the repair of DSBs arising both in heterochromatin and euchromatin. These heterochromatic histone marks, histone variants and heterochromatin-associated proteins have been shown to contribute to various processes during DSB repair, including the initiation of DDR signaling, transcriptional silencing and the promotion of repair through HR or NHEJ (**Caron et al., 2021**).

Typical heterochromatic histone variants have been found to be involved in the general response to DSBs (**Figure 7**). In particular, several studies have investigated the role covered by macroH2A, a H2A histone variant that is observed in constitutive and facultative heterochromatin, where it contributes to transcriptional silencing (**Buschbeck et al., 2009; Douet et al., 2017**). MacroH2A exists in two isoforms (macroH2A1 and macroH2A2) and it has been reported to accumulate at DSB sites (**Thomas Clouaire et al., 2018; Costanzi & Pehrson, 2001; C. Xu et al., 2012**). Interestingly, it has been proposed that the two splicing variants of macroH2A1 exert opposite effects on the process of DSB repair (**Khurana et al., 2014; Kustatscher et al., 2005; Sebastian et al., 2020**). MacroH2A1.2, whose enrichment on damaged DNA depends on ATM, stimulates the recruitment of BRCA1 and thus favors repair by HR (**Khurana et al., 2014**). Consistently, this histone variant is accumulated at sites of replication fork stalling, where it similarly functions to facilitate HR by increasing the presence of BRCA1 (**J. Kim et al., 2018**). In line with the HR-promoting function, macroH2A.1.2 might also take part in transcriptional silencing at DSBs (**Kumbhar et al., 2021**). In contrast, the other macroH2A.1 variant (macroH2A1.1) interacts with the Alt-EJ factors LIG3 and PARP1, and likely covers a role in Alt-EJ, as suggested by the decrease in Alt-EJ usage upon macroH2A1.1 inhibition (**Sebastian et al., 2020**).

The centromere-specific H3 histone variant CENP-A has also been linked to DSB repair. CENP-A is recruited to DSB sites together with various other centromeric proteins in human and mouse cells (**Zeitlin et al., 2009**). However, several aspects of its role in the repair process are not clear yet, including the mechanism of CENP-A's association to the damaged chromatin and its function therein (**Zeitlin et al., 2009**). Given that the presence of CENP-A at DSBs was found to be unaffected by the cell cycle phase or H2A.X, and that its recruitment was fast and comparable to factors involved in DSB processing, the authors have suggested that CENP-A participates directly in the repair process.

In addition to heterochromatic histone variants, it has been reported that the histone marks H3K9me3 and H3K27me3, typically observed in heterochromatin, are transiently accumulated at sites of damage in euchromatin (**Figure 7**) (Ayrapetov et al., 2014a; Chou et al., 2010; O'Hagan et al., 2008). The deposition of H3K27me3 was shown to be mediated by the Polycomb Repressive Complex 2 (PRC2), which is in turn recruited at the site of damage by chromodomain Y-like (CDYL1) (**Abu-Zhayia et al., 2018; Y. Zhang et al., 2011**). The authors proposed that the H3K27me3 accumulation is carried out to strengthen transcriptional silencing at the site of damage (**Abu-Zhayia et al., 2018; Y. Zhang et al., 2011**). The accumulation of H3K9me3 at euchromatic DSBs was instead shown to facilitate the activation of ATM through the activation of TIP60, which is responsible for the acetylation and activation of ATM (Ayrapetov et al., 2014a; Burgess et al., 2014). Many heterochromatic proteins involved in the regulation of H3K9me3 have also been observed at sites of DSBs in euchromatin (**Alagoz et al., 2015; Baldeyron et al., 2011; Soria & Almouzni, 2013; J. Tang et al., 2013; Wu et al., 2015**). The H3K9me3-binding protein HP1, together with the H3K9 methyltransferases SUV39H1/H2 and SetDB1, have been proposed to participate in the regulation of end resection during HR, specifically by influencing the spatial organization of BRCA1 relative to 53BP1 (**Alagoz et al., 2015**). Accordingly, other studies found that both isoforms α and β of HP1 could favor DSB repair by HR, perhaps through the interaction with BARD1, which could stimulate resection (**Wu et al., 2015; Soria & Almouzni, 2013; Baldeyron et al., 2011**). Finally, structural factors normally residing at heterochromatic domains might also cover roles in the general response to DSB. For instance, the ATPase Structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1), which localizes to the X chromosome and telomeres, has been observed to accumulate at sites of damage (**Coker & Brockdorff, 2014; Grolimund et al., 2013; Nozawa et al., 2013; M. Tang et al., 2014**). Depletion of this factor is associated with increased usage of the HR pathway and inhibition of NHEJ, suggesting that its presence at DSBs might regulate the repair pathway choice process by promoting the use of NHEJ (**M. Tang et al., 2014**).

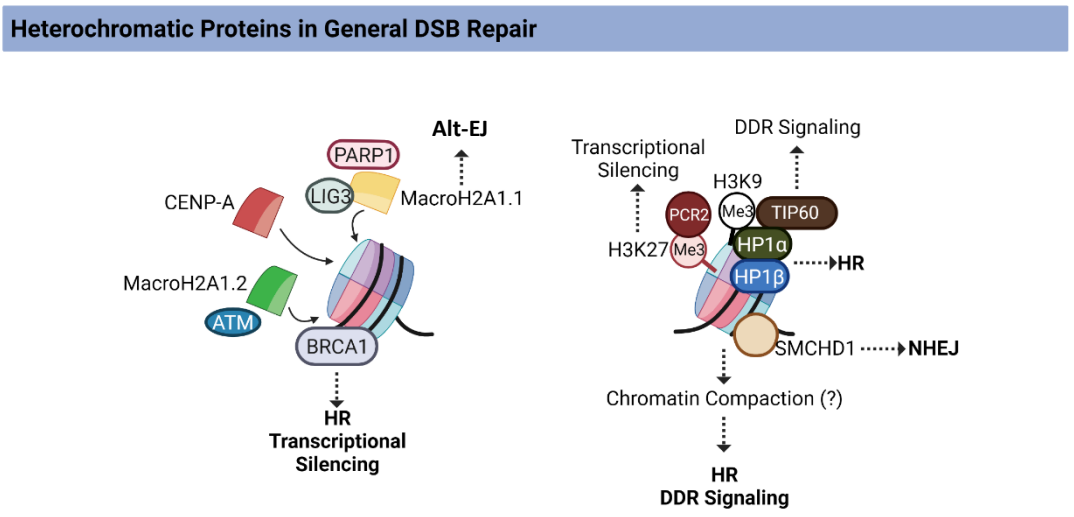

FIGURE 7. Role of Heterochromatic Proteins in General DSB Repair. Heterochromatic histone variants, histone marks and heterochromatin-associated proteins are involved in DSB repair arising in both euchromatin and heterochromatin. Histone MacroH2A1.1 interacts with Alt-EJ-associated factors LIG3 and PARP1, while MacroH2A1.2, which is deposited by ATM, promotes transcriptional silencing and HR by triggering the recruitment of BRCA1. Accumulation of CENP-A has been observed at DSB sites, however its function in DSB repair is still unknown. H3K27me3 is deposited at DSBs by PCR2 and takes part in transcriptional silencing. Similarly, H3K9me3 transiently accumulates at DSBs, where it stimulates HR by recruiting HP1α and HP1β, and DDR signaling through TIP60. The heterochromatin remodeler SMCHD1 channels repair of DSBs through NHEJ. It is possible that heterochromatic proteins promote repair at euchromatin by inducing a compact chromatin state, which has been suggested to be important for DDR and HR progression. This figure was created on Biorender.com.

Interestingly, even though heterochromatin might represent a barrier to DSB repair initiation, it has been proposed that the roles covered by heterochromatic proteins at euchromatic DSBs might depend on their ability to induce a transient re-organization of the damaged euchromatin towards a more condensed state (**Burgess et al., 2014; Kalousi & Soutoglou, 2016; Khurana et al., 2014**). This event, in turn, has been suggested to be required for the activation of the DRR signaling and the subsequent association of HR-associated factors (**Burgess et al., 2014; Kalousi & Soutoglou, 2016; Khurana et al., 2014**). This is supported by data showing that the histone variant MacroH2A and the H3K9 methyltransferase PRDM2 are recruited to DNA breaks to facilitate the ATM-mediated chromatin condensation and the binding of BRCA1 (**Khurana et al., 2014**). In other studies, chromatin condensation was found to be important to induce ATM signaling (Ayrapetov et al., 2014a; Burgess et al., 2014). In agreement, Burgess and colleagues found that DDR induction could be obtained in an undamaged compartment by forcing the enrichment of heterochromatin factors (HP1α/β/γ) (**Burgess et al., 2014**). Although the heterochromatin proteins in euchromatic DSBs appear to be important for the progression of repair, their accumulation has to be followed by a controlled dismantling step (**Alagoz et al., 2015**). Failure to do so could impair subsequent phases of repair, as suggested by the defective end resection observed in cells unable to remove KAP1 and HP1 from DSBs (**Kalousi et al., 2015**).

In conclusion, many heterochromatic proteins can participate in the repair of DSBs arising in both euchromatin and heterochromatin. These proteins can positively impact many steps of the DNA damage response, including signaling, repair pathway choice and transcriptional silencing. In order to further expand our comprehension of these events, future research will have to determine the different roles covered by heterochromatin proteins during the repair of euchromatic DSBs and heterochromatic DSBs.

6. Discussion and Future Perspectives

The study of the DSB repair process in the context of chromatin organization has revealed that chromatin marks and chromatin-associated proteins contribute to the regulation of the DNA damage response in both euchromatin and heterochromatin. Specifically, pre-existent and damage-induced chromatin marks and chromatin-associated proteins of euchromatin and heterochromatin have been proposed to participate in many steps of the DSB repair process, including the initiation of the DNA damage response, the re-organization of the chromatin environment to promote repair and the recruitment of repair proteins. Despite these results, several questions on the influence of euchromatin- and heterochromatin-associated proteins on DSB repair remain to be answered.

First, it is still unclear how euchromatin and heterochromatin factors specifically impact the DSB response in their respective domains. Although research has uncovered that many heterochromatin and euchromatin proteins cover a role in DNA damage repair, few studies have so far investigated how these factors differently behave in different chromatin regions. This is in part due to the methods used to induce the formation of DSBs, which in many cases do not allow to distinguish the DSBs generated in euchromatin from the DSBs induced in heterochromatin. The use of ionizing radiations, for instance, causes the stochastic formation of damage all over the genome, making it difficult to highlight differences in how the DSB response in euchromatin and heterochromatin is managed. For this reason, the mechanisms underlying the contribution to DSB repair of several euchromatin and heterochromatin proteins remains to be clarified. For example, it has been shown that H3K9me2/3, a histone mark typical of heterochromatin, is enriched at damage sites in euchromatin and heterochromatin, where it promotes repair of DSBs through HR (**Ayrapetov et al., 2014; Janssen et al., 2019; Tsouroula et al., 2016**). It has also been reported that H3K9me3 could channel DSB repair to HR by interacting with TIP60, which in turn promotes BRCA1 recruitment to the DSB site (**Sun et al., 2009**). However, this study did not induce DSB formation specifically in heterochromatin, and therefore it is currently unknown whether H3K9me3 promotes HR differently in euchromatin and heterochromatin. Other histone marks and histone variants, such as H3K36me3 and H3.3, are present both at euchromatic and heterochromatic DNA (**Chantalat et al., 2011; Chow et al., 2005; Jha & Strahl, 2014; Udugama et al., 2015**). However, since the contribution of these proteins has only been studied in euchromatin, future research should aim at clarifying their role in heterochromatic DSB repair. To this end, it would be beneficial to employ DSB-inducing methods that allow the generation of single DSBs in specific chromatin compartments, as shown in recent studies that successfully utilized such methods to examine the response to DSBs in specific chromatin contexts (**Schep et al., 2021; Janssen et al., 2019**).

While the mechanisms regulating the participation of many chromatin proteins in DSB repair are still obscure, the role of some chromatin components in the DDR has not been assessed yet. Pericentromeric heterochromatin is characterized by the histone mark H3K56me3, and only recently its enrichment at DSB sites in heterochromatin was reported (**Janssen et al., 2019**). Similarly to H3K9me3, it has been proposed that H3K56me3 can promote repair of DSBs through HR in heterochromatin, but the mechanism through which this histone mark contributes to the DDR remains to

be determined (**Janssen et al., 2019**). Interestingly, in *Drosophila,* H3K56me3 and H3K9me3 are also demethylated by dKDM4A at heterochromatic DSB sites (**Janssen et al., 2019**). Demethylation promotes DSB repair by NHEJ through an unknown mechanism, in contrast to the pro-HR role of H3K9me3 and H3K56me3 (**Janssen et al., 2019**). Thus, it would be interesting to investigate how monomethylated H3K9/H3K56 and trimethylated H3K9/H3K56 relate to each other functionally and temporally during DSB repair. In addition, while in pericentric heterochromatin the presence of H3K56me3 is regulated by the same enzymes that control H3K9me3, it is not clear whether H3K56me3 could cover a separate role from H3K9me3 during DSB repair (**Jack et al., 2013**). However, a possible indication comes from H3K56me3's role during DNA replication. During the S phase, H3K56me3 levels decrease and demethylation of H3K56 is required to promote the association of PCNA to the replication fork (**Jack et al., 2013; Yu et al., 2012**). Therefore, it is possible that the demethylation of H3K56me3 might aid repair by promoting the accessibility of repair proteins at the site of DSBs (**Janssen et al., 2019**). Finally, H3K9me3 and H3K56me3 do not colocalize in all heterochromatin compartments. For instance, at subtelomeres, H3K9me3 is enriched, whereas H3K56me3 is absent (**Chadwick, 2007; Jack et al., 2013**). Telomeres are however enriched with acetylated lysine 56 on histone H3 (H3K56ac), whose levels during the cell cycle are in part regulated by the deacetylase SIRT6 (**Michishita et al., 2009**). Various studies have shown that SIRT6 is involved in DNA repair through different mechanisms, including H3K56 deacetylation and interaction/deacetylation of protein partners (**Gao et al., 2018; Geng et al., 2020; Mao et al., 2011; McCord et al., 2009; Mostoslavsky et al., 2006**). Moreover, deacetylation of H3K56ac has been reported at DSBs sites in euchromatin (**Miller et al., 2010; Tjeertes et al., 2009**). Certain aspects of DNA repair at telomeres are controlled by SIRT6, such as the telomere mobility after oxidative damage (**Gao et al., 2018**). Although this function is carried out by SIRT6 independently from its H3K56 deacetylation ability, it would be interesting to further assess whether deacetylation of H3K56 is involved in the regulation of DSB repair at telomeres (**Gao et al., 2018**).

Although heterochromatin exists in two different states, constitutive and facultative, characterized by specific histone marks and chromatin proteins, most studies examining the role of heterochromatic factors in DSB repair have only focused on constitutive heterochromatin. Our knowledge on the DDR in facultative heterochromatin is still incomplete, as well as the role covered by the chromatin features of this domain in the repair process. Recently it was proposed that in facultative heterochromatin the mutagenic pathway Alt-EJ is more commonly used than in euchromatin, and that the histone mark H3K27me3 might be involved in the repair pathway choice process by regulating the usage of NHEJ and Alt-EJ through an unknown mechanism (**Schep et al., 2021**). Interestingly, the influence of chromatin components on alternative pathways such as Alt-EJ and SSA has not been explored yet. Therefore, getting new insights into the DDR in facultative heterochromatin might also lead to uncovering how chromatin proteins and histone marks participate in the regulation of Alt-EJ and SSA.

Similarly to what was reported for facultative heterochromatin, DSB repair in the context of lamina-associated domain (LADs) has been understudied. LADs are gene-depleted heterochromatin domains, characterized by the presence of H3K9me3 and H3K27me3, which can be repaired by NHEJ and Alt-EJ (**Lemaître et al., 2014; Schep et al., 2021; van Steensel & Belmont, 2017**). It is currently unclear how the chromatin proteins and marks of LADs affect the DSB response, however it has been shown that the use of Alt-EJ is particularly enhanced in late-replicating chromatin regions enriched with H3K9me2 that localize at the nuclear lamina (**Schep et al., 2021**). This suggests that distinct histone marks of a chromatin region, together with its position inside the nuclear space, can promote the repair of DSBs through a specific pathway. While it is becoming apparent that the response to DNA damage is influenced by the location of the damaged chromatin inside the nucleus, it would be interesting to assess how chromatin marks and proteins participate in this process, and how the interplay between chromatin composition and spatial organization regulates the repair of DSBs in different regions of the genome (**Kalousi & Soutoglu, 2016**).

One exciting discovery regarding the DSB repair process in heterochromatin is that in *Drosophila* and mouse cells, the heterochromatic DSBs are relocalized to a peripheral compartment to ensure the safe completion of repair through HR (**Chiolo et al., 2011; Jakob et al., 2011; Janssen et al., 2016; Ryu et al., 2015; Tsouroula et al., 2016**). Surprisingly, it is unclear whether this process is active in human cells as well. Although the relocalization of heterochromatic DSBs in human cells has been reported, this evidence has not been followed up yet (**Jakob et al., 2011**). However, it is possible that a similar process occurs in human cells, since it has been shown that relocation of different types of DNA breaks is a conserved mechanism occurring in several organisms. For instance, in yeast the induction of DSBs in the repetitive ribosomal DNA (rDNA) cluster triggers the relocalization of the damaged site to the nuclear pore complex (NPC), where HR can be performed without the risk of promoting excessive recombination events, which could compromise the integrity of the cluster (**Torres-Rosell et al., 2007**). Moreover, DSBs that arise at telomeres move to the

NPC for their repair, as do collapsed replication forks and DSBs that show resistance to the repair process (**Chung et al., 2015; Nagai et al., 2008; Oza et al., 2009; Su et al., 2015; Therizols et al., 2006; Whalen et al., 2020**).

Interestingly, SUMOylation, which is required for the movement of heterochromatic DSBs in *Drosophila*, has been shown to be involved in the regulation of many of the relocalization pathways in yeast (**Horigome et al., 2014; Nagai et al., 2008; Ryu et al., 2015, 2016; Torres-Rosell et al., 2007**). This indicates the existence of a conserved pathway for the repair of particularly challenging types of damage as well as DSBs arising in delicate genomic regions. Many of these processes have also been described in human cells, including the relocalization of damaged rDNA, collapsed forks and persistent DSBs (**Kalocsay et al., 2009; Lamm et al., 2020; Sluis & McStay, 2015**). In addition, telomeres undergoing faulty replication can be relocalized to the NPC, possibly to promote the restart of stalled replication forks while protecting the integrity of the repetitive telomeric sequences (**Pinzaru et al., 2020**). Although a role for SUMOylation in this pathway has not been reported, the authors revealed that telomere relocalization relies on actin filaments, similar to what was observed for heterochromatic DSBs in *Drosophila* (**Caridi et al., 2018; Pinzaru et al., 2020**). As various relocalization pathways active in human cells appear to be similar to those identified in *Drosophila* and yeast, it is worth investigating whether in humans the repair of heterochromatic DSBs also requires the relocalization to a peripheral compartment, such as the NPC or other alternative sites. Likewise, the association between relocalization of DSBs and HR repair, as found in *Drosophila*, should be examined.

While in *Drosophila* and mouse cells the repair of heterochromatic DSBs though HR is associated with relocalization, it remains to be established whether NHEJ in heterochromatin also requires specific steps in comparison to NHEJ in euchromatin. It is currently unclear if relocalization of heterochromatic DSBs occurs during NHEJ, as conflicting evidence has been reported. In mouse cells, it has been observed that 53BP1 and the LINC complex, which resides at the nuclear envelope, promote the mobility of damaged telomeres and the repair through NHEJ (**Dimitrova et al., 2008; Lottersberger et al., 2015)**. However, in another study conducted in mouse cells, DSBs occurring in pericentromeric heterochromatin did not relocate to the periphery of their domain during the G1 phase of the cell cycle (**Tsouroula et al., 2016**). Since the usage of NHEJ is prevalent during this phase, this result could indicate that relocalization of DSBs is strictly associated with the HR pathway (**Tsouroula et al., 2016**). This is in contrast with results obtained in *Drosophila*, where it was shown that knock-down of HR or NHEJ proteins did not impair DSBs relocalization (**Janssen et al., 2019**). Thus, future research will have to clarify whether NHEJ repair in heterochromatin is associated with relocalization, and if this process is conserved across different species.

In addition to clarifying these aspects related to the DSB repair in heterochromatin, many questions remain to be answered to fully understand how chromatin responds to the generation of DNA damage. For example, while various chromatin marks and proteins have been reported to be involved in the processing of DSBs, many have been described to have opposite effects. The interplay between these marks and proteins therefore remains to be determined to completely understand how these processes shape the response to DNA damage. To clarify this, it will be important to define how the chromatin marks and proteins involved in the DDR are managed temporally and spatially during the repair process. Moreover, future studies will have to explain how the pre-existing chromatin features that participate in DSB repair are activated to become recruitment signals solely after damage induction and, subsequently, how this function is reverted to be able to reconstitute the pre-damage chromatin composition. Finally, it will be interesting to examine the role of chromatin during newly described repair-associated processes, such as the transcription of non-coding RNAs during DSB repair (**D'Alessandro & d'Adda di Fagagna, 2017**).

Overall, pursuing an in-depth understanding of the DSB repair process in the context of chromatin organization will allow us to precisely define the specificities of the DNA damage response in different genomic regions and to identify the key players required to maintain genomic stability in different chromatin contexts. Resolving these fundamental biological questions will ultimately offer new perspectives not only to understand how genomic instability can promote the onset of pathologies such as cancer, but also to facilitate the development of new therapeutic treatments.

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