# Impact of chromatin organization on the repair of double-strand breaks in DNA

## Writing Assignment

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# Layman's summary

The instructions for all cellular activities are stored in genes which are made up of DNA. DNA is present in all human body cells and each cell contains roughly two meters of DNA. To fit the DNA in the cell nucleus it must be compacted. To do so, DNA is wrapped around special proteins called histones. A set of 8 histones together with DNA form a nucleosome which can be seen as one chain link. All these links together form a complex structure called chromatin. Chromatin can be in a very compacted state called heterochromatin in which the links are tightly packed together. Heterochromatin does not store many genes and it contains a lot of repetitive DNA. Chromatin can also be present in a loose form called euchromatin in which more space between the links exists. This loose structure is required because euchromatin contains a lot of genes that must be accessible to special proteins that use this information for cellular activities. The state of chromatin is determined by the usage of different histones, modifications to histones, and proteins that bind to chromatin.

DNA is continuously damaged by many factors such as errors in cellular processes or environmental factors such as smoking or UV radiation. One of the most dangerous types of DNA damage is a double-strand break (DSB). In a DSB, both DNA strands are broken, and the DNA strand is cut into two pieces. This type of damage can cause cancer or can be so dangerous that the cell decides to activate cell death. The cells can choose between different mechanisms to repair a DSB; some repair mechanisms can repair the DNA back to its original state while others leave small errors. The mechanism that is used to repair a DSB depends on many factors such as cell type, age of the cell, location in the DNA, complexity of the DSB, and the cell cycle phase.

Recently more effort has been put into investigating the role of the chromatin environment in the choice of a DSB repair pathway. For example, it was shown that a specific histone-binding protein named HP1 plays an important role in DSB repair in heterochromatin where it is involved in relocating DSBs away from repetitive DNA. In addition, histone H3K27me3 which is only present in a specific type of heterochromatin plays an important role in DSB repair in this specific type of heterochromatin.

In this review, I summarize the current knowledge on the role of the chromatin environment in DSB repair. I examined how histone modifications, histone variants, and DSB repair proteins in a specific chromatin environment play a role in DSB repair. I also highlight the differences between DSB repair in heterochromatin between humans, mice, and fruit flies. I conclude the report by discussing open questions and future perspectives in the field.

# Abstract

Cells are continuously exposed to endogenous and exogenous sources of damage causing DNA double-strand breaks (DSBs). Unrepaired DSBs represent to most dangerous form of DNA damage that can result in cell death, mutations, or genomic instability, one of the hallmarks of tumorigenesis. In reaction to DNA damage, the cell activates the DNA damage response which senses the DSB and recruits DSB repair factors. DSBs can be repaired through several repair pathways but the main pathways are homologous recombination (HR) and non-homologous end joining (NHEJ). The choice of DSB repair pathway depends on the cell cycle phase but also the location of the DSB. In eukaryotes, there are two main chromatin domains; Heterochromatin and euchromatin, each defined by specific histone modifications, histone variants, and chromatin binding proteins. These pre-existing chromatin factors, as well as damage-induced chromatin factors that surround a DSB, have been found to play an important role in the choice of DSB repair pathway. The surrounding factors can selectively interact with or recruit DSB repair factors of a certain pathway thereby promoting the usage of that specific pathway. It has therefore been suggested that these heterochromatin and euchromatin-specific factors, together with the cell cycle, help to promote the DSB repair pathway that suits that specific DSB the best. In this report, I discuss the influence of chromatin environment on DSB repair. More specifically I focus on the difference in DSB repair in constitutive heterochromatin between humans, *Drosophila,* and mouse cells, DSB repair in facultative heterochromatin, and DSB repair in centromeres and euchromatin.


# 1. Introduction

The genetic information that defines the identity and function of a cell is stored in the DNA. A single cell is confronted with an estimated $10^5$ lesions a day (Hoeijmakers, 2009). Endogenous or exogenous influences such as UV radiation, toxic agents, or environmental stress can cause DNA damage. Double strand breaks (DSBs), which are generated by damage to both strands of the DNA helix at the same location represent one of the most dangerous types of DNA damage. Proper repair of DSBs is critical for maintaining genome integrity and preventing the accumulation of DNA mutations, which is one of the hallmarks of tumorigenesis (van Bueren & Janssen, 2024). Eukaryotic cells mainly use two pathways for repairing DSBs. Non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ repairs DSBs by directly ligating the broken ends of the DNA. NHEJ is a fast process that can be used throughout the interphase of the cell cycle but sometimes comes at the cost of losing genetic information at the break site (van Bueren & Janssen, 2024). HR uses a homologous DNA sequence as a template for repairing DNA damage and is mainly active in the S and G2 phases of the cell cycle. Although HR is more accurate, it is a slower repair process than NHEJ (van Bueren & Janssen, 2024). Besides the two main pathways, additional pathways also contribute to the repair of DSBs such as single-strand annealing (SSA) and alternative end-joining (a-EJ). However, these pathways are employed less frequently than NHEJ and HR (Scully et al., 2019).

The repair pathway used by the cell to repair the DSB depends on numerous factors, including the phase of the cell cycle and cell type. Besides these factors, chromatin context also influences the repair pathway choice (Scully et al., 2019). Chromatin is a structure consisting of DNA, proteins, and RNA. The smallest unit of chromatin is the nucleosome formed by short stretches of DNA wrapped around histones (Luger, 2006). Chromatin can be classified into euchromatin, characterized by a higher transcription rate, and accessible DNA, or heterochromatin, which generally has a low transcription rate and highly condensed DNA (Lammerding, 2011). Whether chromatin is classified as euchromatin or heterochromatin depends on factors such as histone post-translational modifications, histone variants, and histone-binding proteins.

The chromatin context plays an important role in determining the repair pathway used to repair a DSB. For example, constitutive heterochromatin consists of areas with high levels of repetitive DNA (Mitrentsi et al., 2022). HR repair between homologous repetitive sequences in these areas could cause genome rearrangements. Via a complex mechanism of histone modifiers and DNA repair proteins, DSBs

are relocated to the heterochromatin periphery where HR can safely take place (Chiolo et al., 2011). Interestingly, similar dynamics have recently been found in facultative heterochromatin. H3K27me3 and polycomb group proteins characterize facultative heterochromatin. Via demethylation of H3K27me3, DSB can be relocated outside the polycomb bodies (Wensveen et al., 2023).
Another example of how chromatin context influences repair is the fact that the euchromatic histone mark H3K36me3, usually involved in directing transcription, can help recruit RAD51, an important factor in DSB repair by HR (Aymard et al., 2014).

In this review, I summarize the current knowledge on the role of chromatin organization in DSB repair. First, I introduce the general principles of chromatin organization including histone variants, post-translational modifications (PTMs), and chromatin-associated proteins. I highlight some of the factors that help to define chromatin into heterochromatin or euchromatin. In the next chapter, I will describe the principles and factors involved in DSB repair pathways such as HR, NHEJ, and alternative repair pathways SSA and A-EJ. In the last chapter, I will discuss the impact of chromatin organization in the repair of DSBs focusing on specific factors in heterochromatin and euchromatin. Finally, I will discuss open questions and future perspectives in the field.

## 2. Organization of chromatin

In eukaryotes, the nucleus houses the cell's genetic information and acts as a site for DNA transcription and processing. The nucleus is a spherically shaped organelle with a diameter between 5 to 20 µm (Lammerding, 2011). To fit all the DNA into the nucleus the DNA must be highly compacted. The compaction is achieved by wrapping the DNA around histones and chromosomal proteins. This compacted structure of DNA is known as chromatin (Lammerding, 2011). Chromatin consists of repeating building blocks called nucleosomes. To form these nucleosomes ~147 base pairs (bp) of double-stranded DNA wrap around an octamer of histone proteins (Klein & Hainer, 2020). This octamer is comprised of two histone H3-H4 pairs that define a tetrameric inner core and two outer H2A/H2B histone dimers. These nucleosomes are arranged into higher chromatin structures by the binding of histone H1 linker proteins and other non-histone proteins (Luger, 2006; Martire & Banaszynski, 2020). The core and linker histones mainly interact with the DNA by the formation of electrostatic interactions between their positively charged Arg and Lys residues and the negatively charged backbone phosphates of the DNA (Fyodorov et al., 2018).

The synthesis of histones is often coupled to the replication of DNA to ensure proper propagation of the parental chromatin structure. New histones are directly bound to the DNA by histone chaperones that work together with DNA polymerases (Martire & Banaszynski, 2020). The genes for histones are present as multiple copies and are often intronless. These histone genes are clustered within the genome and their expression is tightly regulated. However, for some histones, the genes are located outside these clusters and their expression is not regulated by the cell cycle. These genes are often present as one or two copies and contain introns resulting in splice variants (Martire & Banaszynski, 2020). These histones, termed histone variants, have slightly different structural properties and fulfill distinct functions in cell division, DNA repair, transcription, or chromatin remodeling (Martire & Banaszynski, 2020).

The N-terminal tails of the histones can undergo PTMs such as acetylation, methylation, phosphorylation, and ubiquitination (Sullivan & Karpen, 2004). Histone acetylation often reduces the affinity of the histone with the DNA and is therefore associated with actively transcribing DNA (Morrison & Thakur, 2021). The combination of histone modifications, incorporation of histone variants, presence of chromatin-binding proteins, and chromatin remodelers define the organization of the chromatin and therefore the accessibility of the DNA and transcription (Martire & Banaszynski, 2020; Morrison & Thakur, 2021)

In 1928, Emil Heitz improved a cytological staining method and discovered that certain parts of the chromosome were more densely stained than others during the cell cycle. He defined heterochromatin

as the more densely stained and euchromatin as the less stained. In 1929 he suggested that due to the compacted state of heterochromatin, it is most likely genetically inactive (Berger, 2019; Morrison & Thakur, 2021). Nowadays chromatin is distinguished into three groups as is seen in Figure 1: Euchromatin, heterochromatin, and centromeric chromatin.

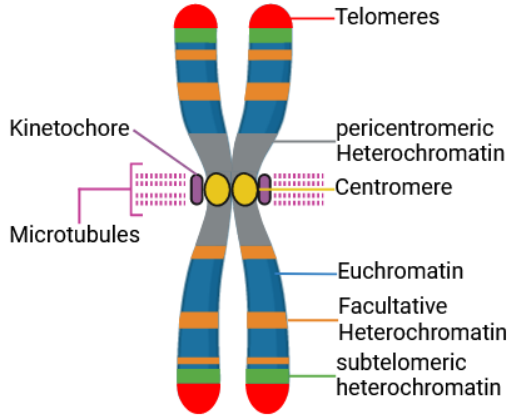



**Figure 1: Different chromatin domains in a eukaryotic chromosome:** constitutive heterochromatin is located nearby telomeres (subtelomeric heterochromatin), and surrounding centromeres (pericentromeric heterochromatin). Facultative heterochromatin and euchromatin are distributed along the chromosome arms. Centromeric sequences pair the two sister chromatids in mitosis where they facilitate microtubule attachment via the kinetochore. This figure was created using BioRender.com.

***Euchromatin***

Euchromatin is a less compacted configuration of chromatin containing transcriptionally active genes. The less compacted state of the chromatin is achieved by more spacing between the nucleosomes caused by histone variants and specific histone modifications. Histone modifications that are associated with the open structure include the acetylation of histone lysine 27 on histone 3 (H3K27ac), methylation of lysine 36 on histone 3 (H3K36me), and di- or tri-methylation of lysine 4 on histone 3 (H3K4me2/3) (Miller & Grant, 2013). Besides histone modifications, histone variants such as H2A.Z, H2A.B, and H3.3, specific histone chaperones, and chromatin remodelers can promote the euchromatin state (Morrison & Thakur, 2021).

***Heterochromatin***

Heterochromatin is defined as a condensed chromatin structure with modest transcriptional activity and is often present in gene-poor regions, pericentromeric regions, and sub-telomeres (Morrison & Thakur, 2021). Heterochromatin is highly conserved, is critical for proper genome function, and can be categorized into constitutive- and facultative-heterochromatin (Janssen et al., 2018).

***Constitutive heterochromatin***

Constitutive heterochromatin (cHC) mainly encompasses regions containing high concentrations of repetitive DNA such as satellite sequences and transposable elements (TEs) located at the peri-centromeres and sub-telomeres as seen in (Figure 1). TEs can threaten genomic stability through their ability to relocate within the genome. TEs can be classified using their mechanism of transposition. Retrotransposons are RNA intermediates that are reverse transcribed into DNA which subsequently is inserted into the host genome (Di Stefano, 2022). DNA transposons encode for a transposase enzyme that can insert itself elsewhere in the genome through a cut-and-paste mechanism (Di Stefano, 2022). Although it is rare for TEs to be randomly distributed into the genome, insertion into regulatory elements or genes can dramatically impact genome structure or gene expression, which could subsequently lead to disease development (Bourque et al., 2018; Di Stefano, 2022). Besides active insertion into other locations, another genomic TE threat arises from the possibility of aberrant recombination between TE

sequences of the same family, which can cause chromosomal translocations, deletions, or inversions which can be harmful (Di Stefano, 2022). To maintain proper genomic integrity, cHC silences the transcription of these TE regions and thereby prevents TE activity or ectopic recombination from occurring (Di Stefano, 2022). cHC also silences the repetitive DNA sequences to prevent the formation of R-loops (Zeller et al., 2016). These R-loops can be created through aberrant transcription of repetitive sequences and can cause genomic instability by colliding with the replication fork or creating supercoils that interfere with the progression of the replication fork (Petermann et al., 2022). Finally, another role for heterochromatin in the maintenance of genome stability is the fact that pericentric heterochromatin is also responsible for the cohesion of the sister chromatid via the stabilization of the cohesin complex, thereby promoting mitotic fidelity (Schalch & Steiner, 2017).

Heterochromatin is characterized by histone H3 lysine 9 di- and tri-methylation (H3K9me2/3) which is catalyzed by the methyltransferases SETDB1 and SUV39H1/2. Histone modifications such as trimethylated lysine 56 and 64 on histone 3 (H3K56me3 and H3K64me3) and trimethylated lysine 20 on histone 4 (H4K20me3) are also enriched and have been implicated to play a role in modulation of heterochromatin structure (Janssen et al., 2018). A crucial factor in the formation of heterochromatin is Heterochromatin Protein 1 (HP1) which directly binds to H3K9me2/3. HP1 consists of two structured domains, the chromodomain and the chromoshadow domain, and an unstructured hinge region that connects the chromodomain and chromoshadow domain (Canzio et al., 2013). The chromodomain (CD) binds to H3K9me2/3. The hinge region participates in the binding with DNA/RNA, and the chromoshadow domain (CSD) participates in the homodimerization (Canzio et al., 2013). The ability of HP1 to dimerize via its CSD and bind to H3K9me via its CD makes it possible to multimerize, thereby making HP1 the central regulator of spreading, as well as compaction of the heterochromatin domain (Canzio et al., 2013; Hamali et al., 2023).

The spreading of heterochromatin can either be to re-establish heterochromatin (maintenance) or to establish a newly repressed domain (typically the first nucleation event) (Hamali et al., 2023). The process for heterochromatin maintenance is different from promoting the first nucleation event. In the maintenance context, surrounding nucleosomes already carry repressive marks such as the H3K9me2/3 which are partially inherited and function as a positive feedback loop to help re-establish the heterochromatin state (Hamali et al., 2023). In the newly specified heterochromatin, the establishment starts from a nucleation center via histone modifications, and non-coding RNA or transcription factors (Wang et al., 2014). While it is not yet completely understood it has been suggested that via repetitive cycles of histone modifications, heterochromatin spreads from the nucleation center (Wang et al., 2014). Endless spreading of heterochromatin could have negative consequences by for example silencing active genes in neighboring euchromatin regions. Therefore, elements that flank the heterochromatin regions play a key role in limiting the spreading (Janssen et al., 2018). Examples of boundary mechanisms are nucleosome-free regions, the protection and presence of active gene chromatin markers such as H3K4me3 or H3K36me3 which act antagonistically to methylation enzymes, high histone-turnover rate, and tRNA genes (Hamali et al., 2023; Janssen et al., 2018; Wang et al., 2014).

It has been shown that a liquid-like population of HP1 can generate phase transition compartments that are surrounded by a chromatin-bound fraction (Larson et al., 2017; Strom et al., 2017). Using large-scale molecular simulations, it has been shown that the key kinetics and thermodynamic features of heterochromatin condensates may be reconciled with a liquid-liquid phase-separation (LLPS) model of organization. The model is driven by HP1 self-attraction and the specific affinity of HP1 for methylated chromatin which was ratified by live-microscopy experiments in early fly embryos (Tortora et al., 2023). The *in vivo* role of HP1a LLPS in heterochromatin -structure and -dynamics remains largely unresolved. However, it has been suggested that these compartments could serve as a mechanism to enrich phase-compatible proteins that help stabilize the chromatin structure or help to concentrate DDR factors to form DNA repair centers (Chen et al., 2023; Larson et al., 2017; Strom et al., 2017)

***Facultative heterochromatin***

Facultative heterochromatin (fHC) is present on DNA loci that are regulated during development. Depending on the developmental- or differentiation- state of the cell, chromatin switches between transcriptionally active and inactive (Morrison & Thakur, 2021). fHC is the transcriptional inactive state and common features are the exclusion of many of the active histone modifications by histone deacetylation either via passive processes such as chromatin replication or actively via histone deacetylases (Żylicz & Heard, 2020). fHC is marked by trimethylation of lysine 27 on histone 3 (H3K27me3). H3K27 methylation is regulated by polycomb group (PcG) proteins, a set of conserved long-term transcriptional gene repressors (Bogliotti & Ross, 2012). In *Drosophila,* Polycomb Response Elements (PRE) recruit PcG proteins to the chromatin. PREs can recruit Polycomb Repressive Complex 2 (PRC2), which in turn tri-methylates H3K27. H3K27me3 is recognized by proteins of the Polycomb Repressive Complex 1 (PRC1) which silences genes (Cheutin & Cavalli, 2012). PRC2 is the central methylase but requires PRC1, which catalyzes the ubiquitination of histone 2A at lysine 119 (H2AK119ub), necessary for efficient repression of certain genes, which subsequently promotes differentiation in embryonic stem cells (Hamali et al., 2023; Klein & Hainer, 2020). Polycomb proteins bound to silenced genes can cluster within the nucleus where they form structures called polycomb bodies. The size of polycomb bodies depends on the exchange of PcG proteins between the nucleoplasm and polycomb bodies, and the size of the genomic region coated by H3K27me3 and PcG proteins (Cheutin & Cavalli, 2012). Polycomb bodies can mediate condensation of chromatin thereby limiting transcription (Cheutin & Cavalli, 2012).

fHC can be present on an entire chromosome, as is seen in the silencing of one of the X chromosomes in female mammals but also smaller domains distributed on the chromosome such as the homeobox genes (Hox). The Hox genes participate in specifying the body plan of an embryo along the head-tail axis of the body (Morrison & Thakur, 2021). Although these genes are only active for a brief time, they fulfill the definition of fHC as the Hox clusters are regulated by the widespread addition of PcG components (Żylicz & Heard, 2020). Similar to cHC it was shown that PcG proteins can also undergo LLPS which suggests that LLPS could also play a role in facultative heterochromatin (Tatavosian et al., 2019).

While there is much similarity between fHC and cHC, the presence of H3K27me3 and PcG proteins versus H3K9me3 and HP1 proteins respectively forms a significant difference between these two types of heterochromatin. These heterochromatin-specific factors play an important role in the choice of repair pathway in DSB, which will be discussed in Chapter 4.

***Centromeric chromatin***

The centromere is a unique chromosomal locus that plays a role in kinetochore formation, which serves as an attachment site for microtubules to separate the chromosomes during mitosis and meiosis (Sullivan & Karpen, 2004). Although the overall chromosome segregation machinery is highly conserved across eukaryotes, centromeric DNA sequences and its protein components are fast evolving. The centromeric DNA is characterized by tandemly arranged DNA (Morrison & Thakur, 2021). The core of the centromere is defined by a centromere-specific histone H3 variant named centromere protein A (CENP-A) that is present in almost all eukaryotes (Schalch & Steiner, 2017). CENP-A is required for the formation of the kinetochore complex (Morrison & Thakur, 2021). While many protein components of the centromere are conserved, the organization of CENP-A nucleosomes and the interaction with the DNA varies widely between taxa (Schalch & Steiner, 2017). Upon assembly of CENP-A nucleosomes, the centromeric DNA undergoes positive supercoiling which likely reduces the stress on the DNA during the pulling of the sister chromatids (Morrison & Thakur, 2021). The centromeric core region is flanked by pericentric heterochromatin which is characterized by H3K9me2/3 bound by heterochromatin proteins. During mitosis, a three-dimensional structure is formed by the centromeric chromatin and pericentromeric heterochromatin which exposes the CENP-A to the surface (Schalch & Steiner, 2017). This allows for the assembly of kinetochores which act as a capture device for microtubules from the

spindle poles (Schalch & Steiner, 2017). Besides that, it provides elasticity and resistance to tension created during segregation (Schalch & Steiner, 2017).

The organization of chromatin is defined by many factors. Heterochromatin plays an important role in silencing gene-poor or repetitive regions critical for maintaining genome integrity. Euchromatin plays an important role in the regulation of transcription by allowing the recruitment of transcription factors to the DNA. While chromatin plays an important role in maintaining genome integrity, repair of DNA damage also plays an important role in maintaining genome integrity. In the next chapter, I discuss several repair mechanisms present in cells to repair damaged DNA.

# 3. DNA damage response

***How does damage occur?***

Maintaining genomic stability is essential for the survival of an organism. Mutagenesis plays an essential role in evolution but also contributes to cancer, aging, and human diseases. DNA is known as a molecule that is highly susceptible to modification. DNA damage can occur due to endogenous factors such as replication errors, oxidative DNA damage, or DNA demethylation but also due to exogenous factors such as ionizing or ultraviolet radiation, chemical agents, toxins, or environmental stress (Chatterjee & Walker, 2017). Although all DNA damage can have negative consequences for genomic stability, DSBs represent one of the most significant ones. In DSBs, both strands of the DNA helix are damaged at the same location. Unrepaired DSBs can lead to deletions, translocations, or amplifications in the genome and can therefore dramatically alter genomic integrity (Chatterjee & Walker, 2017). To prevent these events from occurring, eukaryotic cells respond to DNA damage by activating the DNA damage response (DDR). The DDR is a complex response that includes DNA repair, cell cycle checkpoints, and cell death pathways that collectively reduce the dangerous consequences of DNA damage (Chatterjee & Walker, 2017). This response also involves the modification of chromatin at the damage site, facilitating the assembly of DDR proteins. It also triggers cell-cycle checkpoints during DNA replication such as G1/S, intra-S, and G2/M. These checkpoints help to prevent duplication or segregation of damaged DNA and the modification of gene expression (Chatterjee & Walker, 2017; Groelly et al., 2023). Defects in the DDR are a hallmark of cancer as mutations involving upregulation of proto-oncogenes, downregulation or loss of tumor suppression genes, and the delay of apoptosis are drivers of tumorigenesis (Groelly et al., 2023; Pilié et al., 2019).

***The dynamics of DDR in DSB repair***

The DDR signaling cascade is a complex system of coordinated events that requires the action of many proteins. The function of these proteins can be categorized as DNA damage sensors, transducers, mediators, and effectors (Polo & Jackson, 2011). DNA damage sensors find broken DNA in a sequence-independent manner. Some of the most important sensors are poly ADP-ribose polymerase 1 (PARP1), SIRT6, the MRN complex (MRE11, RAD50, NBS1), and the Ku70/80 complex (Onn et al., 2020). These sensors initiate downstream signaling which leads to the phosphorylation of the histone variant H2AX (γH2AX) by the phosphoinositide 3-kinase-related kinase family (PIKK), including Ataxia Telangiectasia Mutated (ATM), Ataxia Telangiectasia and Rad3-related protein (ATR), and DNA-dependent Kinase Catalytic subunit (DNA-PKCs) (Aricthota et al., 2022). These kinases generate γH2AX chromatin domains that spread up to megabases around the break site. Phosphorylation of H2AX generates a carboxyl-terminal phospho-motif which acts as a site to recruit repair factors (mediators) to the chromatin. One of these repair factors is MDC1, which can recruit various downstream DNA repair factors such as P53 binding protein 1 (53BP1) and Breast Cancer type 1 susceptibility protein (BRCA1) (Blackford & Jackson, 2017). The metabolic state and cell cycle stage can affect how a DSB is repaired and can lead to changes in the recognition of the DNA damage, which can subsequently lead to different repair pathway choices. For example, recognition of the damaged

DNA by Ku70/80 leads to activation of DNA-PKcs which leads to the recruitment of specific factors involved in NHEJ, while DNA recognized by the MRN complex leads to recruitment of ATM which in turn recruits factors involved in HR such as BRCA1 (Blackford & Jackson, 2017). Many factors such as competition between NHEJ and HR factors, pH, and chromatin landscape influence DSB repair pathway choice (Aricthota et al., 2022).

***DSB repair pathways***

DSBs can be repaired via two main pathways, NHEJ, or HR (Figure 2). Besides these two main pathways, there are several alternative pathways such as a-EJ and SSA (Figure 3).

***Non-Homologous End- Joining***

NHEJ (Figure 2 left panel) is initiated by the formation of a Ku70/80 heterodimer at the ends of the DSB. Ku70/80 is a ring-shaped molecule that tightly encircles 3-4 bp of DNA. Ku70/80 acts as a hub for the recruitment of NHEJ downstream factors such as DNA-PK(cs), DNA ligase IV (LIG4), and associated scaffolding factors such as X-ray repair cross-complementing protein 4 (XRCC4), XRCC4-like factor (XLF), and the paralogues XRCC4 and XLF (PAXX) which together can form the DNA-PK complex (Scully et al., 2019; Stinson & Loparo, 2021). The DNA-PK complex can phosphorylate itself and other factors involved in the repair process. In the case of blunt end breaks in the DSB, the cell quickly responds by promoting the binding of XRCC4-LIG4 to the Ku70/80 complex. The C-terminus of LIG4 has two BRCA1 C-Terminus (BRCT) domains that allow the binding of two Ku complexes on each side of the DSB. Between the BRCT and ligase 4 domain of LIG4, an interaction domain is present that binds a homodimer of XRCC4 (Pannunzio et al., 2018). LIG4 in complex with XRCC4 performs the DNA-end ligation. This step is promoted by PAXX which increases the stability of DNA-PK by 20-fold and helps stabilize the DNA ends, while XLF helps to maintain the DSB ends close together (Groelly et al., 2023)

Although blunt-ended DSBs can be relatively easily resolved, direct ligation is often prevented by incompatible ends caused by mismatched overhangs or chemical modifications. To maintain the dedication to the NHEJ pathway, the mismatched or modified ends must be prepared for ligation (Cannan & Pederson, 2016). Therefore, Ku70/80 recruits DNA-PKcs to the DSB site, which in turn recruits phosphorylates, and forms a complex with Artemis. This complex has endonuclease activity and can remove 5' and 3' DNA overhangs to create small regions of microhomology (<4bp) to facilitate the end joining by the XRRC4-LIG4 complex (Cannan & Pederson, 2016; Pannunzio et al., 2018). Ku70/80 plays a critical role in this process by preventing extensive end resectioning (>20bp) which could initiate the HR or SSA pathways (Pannunzio et al., 2018). Besides Artemis, many other factors participate in the processing of incompatible ends such as Aprataxin, Flap Endonuclease 1 (FEN1), and EXO nuclease 1 (EXO1) (Chang et al., 2017; Pannunzio et al., 2018).

NHEJ is a single pathway, but due to the variety of enzymes at its disposal, NHEJ can repair DSBs with different DNA end configurations. The flexibility of NHEJ makes it a useful tool for repairing DSBs but it does not mean it is a precise tool. Because NHEJ contains multiple tools for the editing of a DSB end, multiple processes can be active at the same time which could result in multiple rounds of editing needed before the ends can be ligated together (Pannunzio et al., 2018). Multiple rounds could result in small insertions or deletions. Because NHEJ occurs in the absence of a DNA template or homologous region, these mutations will not be restored and thereby make NHEJ a mutagenic pathway. However, NHEJ is still remarkably efficient and accurate and in human cells is the major pathway to repair DSBs (Blackford & Jackson, 2017).

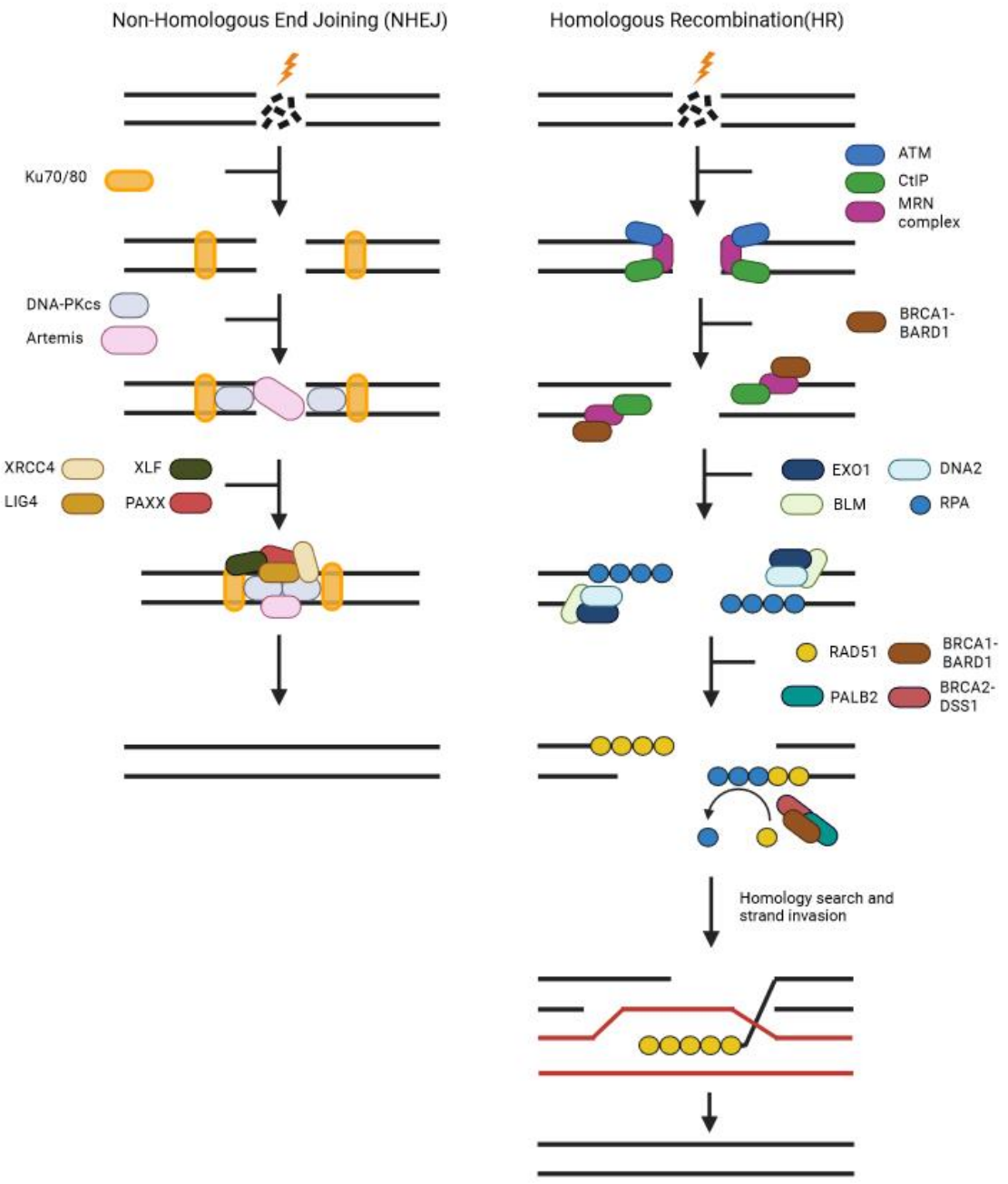



**Figure 2: Non-homologous End Joining and Homologous Recombination of double-strand breaks. Left**: Non-homologous End Joining is initiated by the binding of Ku70/80 to the ends of the break. Ku70/80 recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Artemis can be recruited to the DSB to process incompatible DNA ends. LIG4 with XRCC4 facilitates the ligation of the DSB while XLF and PAXX are responsible for the stability during ligation. **Right:** During HR, the MRN complex is recruited to the DSB, which recruits ATM and CtIP. ATM triggers the recruitment of BRCA1-BARD1 and together with CtIP and MRN 5' end resection occurs. BLM and EXO1 perform further end resection. RPA rapidly coats the 3' ssDNA. RPA is replaced by RAD51 which is mediated by a complex of BRCA1/BARD1, BRCA2/DSS1, and PALB2. RAD51 facilitates homology search and strand invasion and in the process forms a D-loop. By copying the complementary strand, the break is resolved. This figure was created using BioRender.com.

***Homologous recombination***

The other canonical pathway for the repair of DSBs is HR (Figure 2 right panel). Unlike NHEJ, HR repairs DNA using an identical DNA sequence as a template for repair. NHEJ can operate throughout the eukaryotic cell cycle, while HR is often restricted to the S and G2 phases of the cell cycle as HR involves recombination between sister chromatids, post-replicative copies of chromosomal DNA (Scully et al., 2019). HR is also restricted to S/G2 because the DSB end resection machinery that is used in HR repair is regulated by cell cycle-specific proteins such as cyclins and cyclin-dependent kinases (CDKs) which are S and G2-specific (Vu et al., 2022). HR starts with the recruitment of the MRN complex and CtBP (C-terminus-binding protein of adenovirus E1A )-interacting protein (CtIP) to the DSB. The MRN complex also recruits and activates ATM which phosphorylates several substrates of the DDR including H2AX (Hartlerode & Scully, 2009). The formation of γH2AX leads to the remodeling of the chromatin resulting in the recruitment of BRCA1-BARD1 (Hartlerode & Scully, 2009). In association with BRCA1-BARD1 and CtIP, MRN creates 3' hydroxyl ssDNA overhangs by first nicking up to 300bp internal to the DNA end on the free 5' terminus, and then using the 3'-5' exonuclease activity of MRE11 to promote 'short-range' resection. During short-range end-resection, Ku70/80 is removed which initiates HR and ensures inhibition of the NHEJ pathway (Scully et al., 2019).

The short-range resection creates a starting point for a bigger resection performed by EXO1, exonuclease DNA2, and the helicase Bloom syndrome protein (BLM) (Scully et al., 2019). The free ssDNA is rapidly coated by the heterotrimeric complex Replication Protein A (RPA), which prevents the pairing of the ssDNA with other ssDNA sequences (Hartlerode & Scully, 2009). Because the binding of RPA to ssDNA is extremely abundant, several mediators are required to replace RPA for the recombinase RAD51. The most critical mediators include BRCA1/BARD1 and BRCA2/DSS1 which is most likely bridged by Partner and Localizer of BRCA2 (PALB2) (Hartlerode & Scully, 2009). Although all mediators are required, BRCA2 directly interacts with RAD51. RAD51 is a DNA-dependent ATPase that forms nucleoprotein filaments with DNA, it facilitates strand invasion and strand pairing by scanning the genome to locate a homologous region within the dsDNA (usually in the form of a sister chromatid) (Haber, 2018). The RAD51- 3' ssDNA filament invades and anneals to the complementary sequence in the sister chromatid, thereby displacing the opposing strand in the sister chromatid and resulting in the formation of the Displacement loop (D-loop) (Cannan & Pederson, 2016).

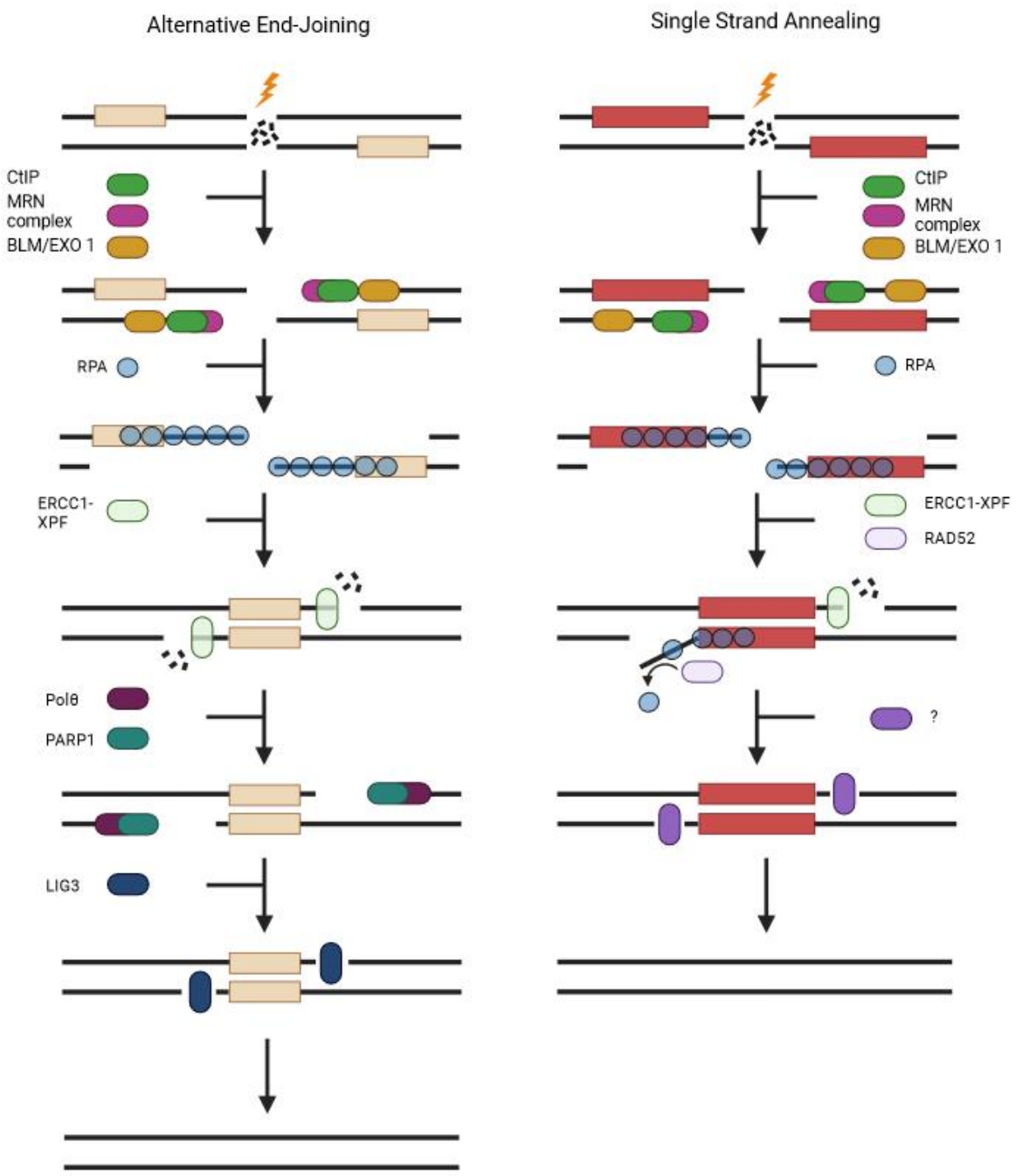
***Alternative pathways***

In addition to NHEJ and HR, two alternative DNA repair pathways are used to repair DSBs, termed a-EJ and SSA (Figure 3). Overall, these alternative pathways have lots of similarities, and both are mutagenic events because large pieces of DNA are often deleted during the repair process (Bhargava et al., 2016). Both pathways are also implicated in the formation of chromosomal translocation and rearrangements which are often associated with tumorigenesis but could also explain why many breakpoint junctions feature microhomology (Deng et al., 2014; Sfeir & Symington, 2015). Both a-EJ and SSA use similar mechanisms as HR and depend on the resection of 5' DNA ends. Both pathways mainly operate during the S-phase of the cell cycle and do not require Ku70/80 or DNA-PK but do require the MRN complex (Cannan & Pederson, 2016; Deng et al., 2014; Vu et al., 2022). The main difference between a-EJ (Also referred to as microhomology-mediated end joining (MMEJ)) and SSA is the amount of resection, the size of the homologous region used, and the specific role of PARP1 and DNA polymerase theta in a-EJ (Bhargava et al., 2016).

As discussed above, NHEJ predominantly repairs DSBs in the G1 phase, and HR repairs DSBs in S and G2. Interestingly, while in mitosis HR and NHEJ are repressed, a-EJ was recently found to be the only DSB repair pathway active in the M phase. This repair is driven by the accumulation of RHINO which promotes the recruitment of polθ to the damaged sites (Brambati et al., 2023).

Similar to HR, the first step in a-EJ and SSA is the resection of 5' DNA ends using the MRN complex together with CtIP to create a 3' ssDNA overhang. In both pathways, a further 5'-3'resection is performed by BLM and EXO1. However, during a-EJ, this extensive resection is only needed if microhomology regions are >2kb apart, while in SSA more extensive resection is always required (Sfeir

& Symington, 2015; Vu et al., 2022). The 3' ssDNA tails are protected and stabilized by the RPA complex to prevent the annealing of the ssDNA. In SSA, Rad52 plays a key role in removing RPA in a RAD51-independent manner and in the annealing of homologous regions with the 3' ssDNA (Figure 3 right panel), while in a-EJ Rad52 is not required (Figure 3 left panel) (Sfeir & Symington, 2015). After annealing, the remaining non-homologous DNA at the 3' end of the overhangs are removed by the nuclease activity of the ERCC1/XPF complex (Bhargava et al., 2016). In a-EJ, the produced 3' -hydroxyl termini are extended by DNA polymerase theta and PARP1 (Figure 3 left panel) (Deng et al., 2014; Sfeir & Symington, 2015). In SSA the polymerase involved in this gap-filling is not exactly known but it might be similar to the second strand synthesis process in HR which requires DNA polymerase delta or zeta (Vu et al., 2022). For both pathways, the exact ligation pathway is not yet known. In a-EJ, there are still discrepancies but ligase 3 acts as the primary ligase (Figure 3 left panel) (Sfeir & Symington, 2015).




**Alternative DNA repair pathways Alternative End-Joining (A-EJ) and Single Strand Annealing (SSA). Left.** In A-EJ the 5' DSB end resection is performed by the MRN complex and CtIP thereby creating 3' ssDNA overhangs. BLM and EXO1 can perform further end resection. RPA protects the 3' ssDNA from annealing. Nuclease activity of the ERCC1/XPF removes the 3' ssDNA non-homologous DNA. The gap that is created in this process is filled by polθ and PARP1. Ligation of the dsDNA gap is performed by ligase 3. **Right.** Similar to A-EJ 5' end resection is performed by MRN and CtIP but in SSA further end resection by EXO1 and BLM is required. RPA protects the 3' ssDNA and removal of RPA is mediated by RAD52. The polymerase involved in gap filling and the pathway of ligation is not exactly known yet. This figure was created on Biorender.com.

# 4. The influence of chromatin state on the DSB repair pathway

HR and NHEJ can co-exist in the same cell and are often both used in a cell to repair DSBs. In most cells, other DSB repair pathways can compensate if one of these mechanisms fails (Clouaire & Legube, 2015). This shows that DNA repair pathways compete to some extent for the repair of a DSB. As described in the previous chapter every DNA repair mechanism has unique features, and some of these could also be harmful on sequence and chromosomal level. The choice of pathway is therefore a critical aspect in the repair of a DSB (Clouaire & Legube, 2015). Cells have developed a complex mechanism to ensure that the DSB repair pathway matches the cellular context in which the DSB is present. Crucial factors in this mechanism are cell type, age, cell cycle phase, location, nature, and complexity of a break (Scully et al., 2019). Recently more effort has been put into the role of chromatin context in DSB repair. Chromatin is affected by multiple factors such as histone PTMs, histone variants, and the local nucleosome density. These chromatin signatures direct gene expression. However, these pre-established chromatin structures could also play a role in the process of DNA repair pathway choices, depending on the chromatin structure at the DSB location (Clouaire & Legube, 2015).

***DSB repair in euchromatin***

Euchromatin is characterized by a more open structure, active genes, and 'active' histone modifications such as H3K4me3, H3K27ac, H3K36me, and histone variants such as H2A.Z (Morrison & Thakur, 2021). Similar to heterochromatin and centromeric chromatin, the combination of euchromatic histone marks, histone variants, and chromatin-interacting proteins can also impact DSB repair.

The accurate repair of DSBs in this region is important to maintain the integrity of crucial genes and their regulatory elements. It has been found that RAD51-bound DSBs are more enriched at transcription-associated histone marks such as H3K9ac and H3K36me3 (Aymard et al., 2014). This suggests that active transcription is associated with an increase in HR repair (Aymard et al., 2014). Genome-wide mapping of H3K36me3 revealed that genes closer to RAD51-bound DSBs have higher H3K36me3 levels than those located near RAD51-unbound DSBs (Aymard et al., 2014). These findings indicate that RAD51 is preferentially recruited to transcriptionally active loci. However, at these euchromatin sites, HR is still only active in G2 and S of the cell cycle. Indeed, other pathways such as NHEJ have also been shown to repair DSBs in euchromatin (Aymard et al., 2014).

As described earlier, γH2AX plays a significant role in the DDR. Interestingly, the dynamics of γH2AX formation are slightly different across the genome. γH2AX is more efficiently formed in euchromatin than in heterochromatin which could increase DDR efficiency (Van & Santos, 2018). Phosphorylation of H2AX initiates the accumulation of repair factors such as MRN, BRCA1, and 53BP1. However, it is also important for the recruitment of chromatin remodelers to the DSB such as histone acetyltransferase TIP60 (Van & Santos, 2018). TIP60 plays a role in acetylation of H4K16. Acetylated H4K16 promotes HR by counteracting the binding of 53BP1 to demethylated H4K20 while deacetylated H4K16 facilitates NHEJ (Tang et al., 2013).

A major step specific to DNA repair in euchromatin is also the silencing of transcription to create a chromatin state that can facilitate DNA repair. The demethylation of H4K4me3 is an example of a chromatin modification in euchromatin to facilitate repair (Gong et al., 2017). The histone demethylase KDM5A is recruited to DNA damage where it demethylates H3K4me3. This promotes the interaction between the ZMYND8 and nucleosome remodeling and histone deacetylation (NuRD) complex and the damaged chromatin which represses transcription and facilitates repair by HR (Gong et al., 2017). Besides the removal of euchromatin-specific modifications, silencing histone modifications such as H3K27me3 and H3K9me2/3 also a play role in silencing the local chromatin structure to facilitate the repair of euchromatin (Ayrapetov et al., 2014; Chou et al., 2010).

In conclusion, DSB repair in euchromatin relies on silencing transcription and creating a chromatin structure that can facilitate DSB repair. Euchromatin-specific pre-existing and damaged chromatin marks help to facilitate the transformation into this state.

Although much research has been performed on DSB repair in euchromatin, repair in heterochromatin is relatively understudied. Nevertheless, emerging research in the past decade has highlighted the unique repair dynamics associated with these silent domains. I will therefore focus the remaining part of this report on the repair and relocation of DSBs in constitutive heterochromatin, and the role of facultative heterochromatin as well as centromeric chromatin on DSB repair.

***Clustering of euchromatin DSBs***

Besides changes in histone modifications in euchromatin, clustering of DSBs has also been found to play a role in DNA repair. Smaller chromosomes contain more clustered DSBs but there is no correlation between the position of the DSB in the three-dimensional nucleus and the ability to cluster. (Aymard et al., 2017). Clustering can occur between DSBs on different chromosomes; however, clustering is favored between loci in proximity (Aymard et al., 2017). Moreover, DSBs with higher γH2AX levels had a higher ability to cluster (Aymard et al., 2017). Interestingly, clustering also depends on the repair pathway, since DSBs with higher levels of RAD51 (HR prone) have an increased tendency to cluster (Aymard et al., 2017). DSBs induced in active genes are more HR-prone than silenced genes as DSBs in silenced regions are unable to recruit RAD51 (Aymard et al., 2014). This indicates that actively transcribed which are more HR-prone also tend to cluster more than DSBs in silenced regions (Aymard et al., 2017). Counterintuitively, DSB clustering is enhanced in the G1 phase of the cell cycle in which HR is usually strongly inhibited (Aymard et al., 2017). The authors propose that DSBs induced in euchromatin during the G1 phase are not completely ready for repair and are therefore more prone to cluster (Aymard et al., 2017). The MRN complex was found to be involved in clustering together with the microtubule-and actin-related networks (Aymard et al., 2017).

The data discussed above demonstrates that DSB repair in euchromatin relies on many factors such as histone marks and transcriptional silencing. Further research is focused on unraveling the exact mechanisms underlying DSB repair and getting a better understanding of the clustering of euchromatic DSBs in the genome.

***Repair in constitutive heterochromatin and movement of DSBs***

cHC is characterized by the histone marks H3K9me2/3, H3K56me2/3, and the H3K9me2/me3 binding protein HP1. The biggest part of cHC is located at peri-centromeres and sub-telomeres and contains high levels of repetitive DNA such as TEs and satellite sequences (Morrison & Thakur, 2021). The large number of repeated sequences, and their proximity, form a risk for genome rearrangement as these repetitive sequences can engage in ectopic recombination and generate chromosome rearrangements when DSBs occur (Mitrentsi et al., 2022; Ryu et al., 2015). Besides that, these regions exhibit greater vulnerability to replication stress due to the high concentration of repetitive DNA. These repetitive areas can form Common Fragile Sites (CFSs), regions in the genome more prone to DNA breakage and rearrangements due to replication stress (Janssen et al., 2018). Chromatin surrounding CFSs is often hypoacetylated indicating that they are associated with a more compact chromatin state such as heterochromatin (Janssen et al., 2018). The compact state of heterochromatin does not result in blocking the accessibility or exchange of molecules and DNA repair factors, but it does reduce it (Fortuny & Polo, 2018). However, in general, it is thought that DSB detection and signaling is not delayed in these domains (Caridi et al., 2017). In heterochromatin, the repair of DSBs relies on both HR and NHEJ. However, in both *Drosophila* and mouse cells repair in the S and G2 phases of the cell cycle is preferably by HR (Caridi et al., 2017; Tsouroula et al., 2016). *Drosophila* and mouse cells both form heterochromatic chromocenters in which satellite repeats are clustered and have adopted a unique strategy to repair DSBs within heterochromatin (Mitrentsi et al., 2022). By relocating DSBs away from the pericentromeric heterochromatin core, aberrant HR between repetitive sequences is suppressed, which thereby prevents the formation of genome rearrangements (Figure 4 right panel) (Chiolo et al., 2011; Mitrentsi et al., 2022; Tsouroula et al., 2016).

Important proteins involved in the relocation of DSBs are ATR, SMC5/6, HP1, and regulator proteins of early HR (Chiolo et al., 2011). Interestingly, the formation of DSBs results in an expansion

of the heterochromatin domain (Chiolo et al., 2011; Ryu et al., 2016; Tsouroula et al., 2016), which, in *Drosophila,* starts within minutes following the formation of the IR-induced DSBs. Peak expansion occurs during the relocation of the repair sites, leading to an increase of approximately 50% in the heterochromatin volume occupied within the nucleus (Chiolo et al., 2011; Ryu et al., 2016). Interestingly in *Drosophila* the factors involved in expansion such as ATR and resection components are also involved in DSB relocation (Caridi et al., 2017). While expansion might facilitate relocation it is not required for relocation (Caridi et al., 2017). In both *Drosophila* and mouse cells, the initial steps of HR repair such as the recruitment of the MRN complex, activation of ATM/ATR which phosphorylates H2AX, and the 5'-3' end resection of the DSB occur inside the heterochromatin domain (Chiolo et al., 2011; Janssen et al., 2016). After the initial steps of HR are completed, the repair is stopped temporarily and the DSB is relocated to the heterochromatin periphery to resume HR (Chiolo et al., 2011). In *Drosophila* the exclusion of RAD51 in the initial stages of HR relies on HP1a. HP1a recruits and associates with the Smc5/6 complex which recruits SUMO E3 ligase subunits Nse2/Qjt and Nse2/Cerv but also SUMO-E3 ligase Protein Inhibitor of Activated STAT (dPIAS) that together block the recruitment of RAD51. (Caridi et al., 2017; Chiolo et al., 2011; Ryu et al., 2015). The exclusion of RAD51 from the DSB in the repetitive heterochromatin area is thought to be an extra barrier to prevent premature recombination between repeats. The factors involved in the exclusion of RAD51 are also involved in the relocation of the DSB to the heterochromatin periphery via SUMOlyation of unknown targets that help to promote relocation (Ryu et al., 2015). Interestingly, the loss of Smc5/6 leads to abnormal formation of RAD51 in *Drosophila* while it does not in mammalian cells, suggesting that alternative mechanisms of RAD51 exclusion exist in mammalian cells (Caridi et al., 2017).

In *Drosophila* cells, 20% of the heterochromatic DSBs can also relocate to the nuclear periphery (Caridi et al., 2018; Ryu et al., 2015). Relocation to the nuclear periphery is mediated by nuclear actin filaments (F-actin) and myosin (Caridi et al., 2018). Two important actin nucleators in this process are Arp2/3 which can assemble F-actin between the DSB and nuclear periphery (Caridi et al., 2018). Via HP1a and Mre11, Arp2/3 is recruited to DSBs, and together with Smc5/6, mediates the relocation of DSBs (Caridi et al., 2018). Besides Arp2/3, relocation also relies on nuclear myosin which promotes DSB movement along actin filaments (Caridi et al., 2018). The myosin activator Unc45 also plays a significant role in this nuclear actin-mediated DSB movement, as it is required for myosin stability and function (Caridi et al., 2018). Before relocalization, myosin and Unc45 are enriched at the DSB site, similar to Arp2/3. Myosin recruitment relies on Mre11 and HP1 while the recruitment of Unc45 relies on Smc5/6 (Caridi et al., 2018). Unc45 is suggested to function as a trigger for the activation of myosin and relocalization of the DSB along the actin filaments (Caridi et al., 2018). After relocation, the later HR is continued and events such as the recruitment of BRCA2, and binding of RAD51 are initiated (Chiolo et al., 2011). Although the movement of DSBs to the heterochromatin periphery has also been found in mouse cells, the relocation of DSBs to the nuclear periphery during HR is not conserved in mouse cells. It is possible that the formation of the RAD51/BRCA-2 complex in mammalian cells stabilizes the DSB in the heterochromatic periphery and further relocation is not required to continue HR repair (Tsouroula et al., 2016). In cells that fail to relocate the DSB the SSA repair pathway factor RAD52 is recruited. This suggests cells can repair a DSB using SSA if relocation fails (Tsouroula et al., 2016). DSB movements in the nucleus do not only occur at DSBs in repeated sequences but also in other areas where relocation is required to prevent aberrant recombination with ectopic repeated sequences such as collapsed forks, sub-telomeric regions, and eroded telomeres (Caridi et al., 2017).

In both *Drosophila* and mouse cells, it was demonstrated that DSBs increase the levels of H3K9me3 and HP1 isoforms (Janssen et al., 2019; Tsouroula et al., 2016). However, in *Drosophila* additional chromatin modifiers are thought to play a role in heterochromatin repair. A crucial factor in the heterochromatin of *Drosophila* is histone demethylase dKDM4A, which was identified as a protein enriched in heterochromatin required for the structure and function of heterochromatin (Colmenares et al., 2017). dKDM4A can directly bind to HP1a and demethylate H3K9me3 and H3K56me3 specifically at DSB sites in heterochromatin (Janssen et al., 2019). The demethylation of H3K9me2/3 to H3K9me1 and H3K56me2/3 to H3K56me1 plays a significant role in the proper repair of DSBs in heterochromatin.

dKDM4A not only inhibits the recruitment of early HR proteins, thereby promoting NHEJ but the demethylation of H3K9me2/3 and H3K56me2/3 could also result in a local loss of heterochromatin structure (Janssen et al., 2019). The loss of heterochromatin structure could increase the accessibility of DNA repair factors to the DSB and help in the relocation of the DSB to the heterochromatin (Janssen et al., 2019).

In conclusion, DSB repair in constitutive heterochromatin relies on a specialized pathway of decompaction of heterochromatin, timely regulation and recruitment of repair factors, and movement of the DSB away from the repetitive area to enable repair.

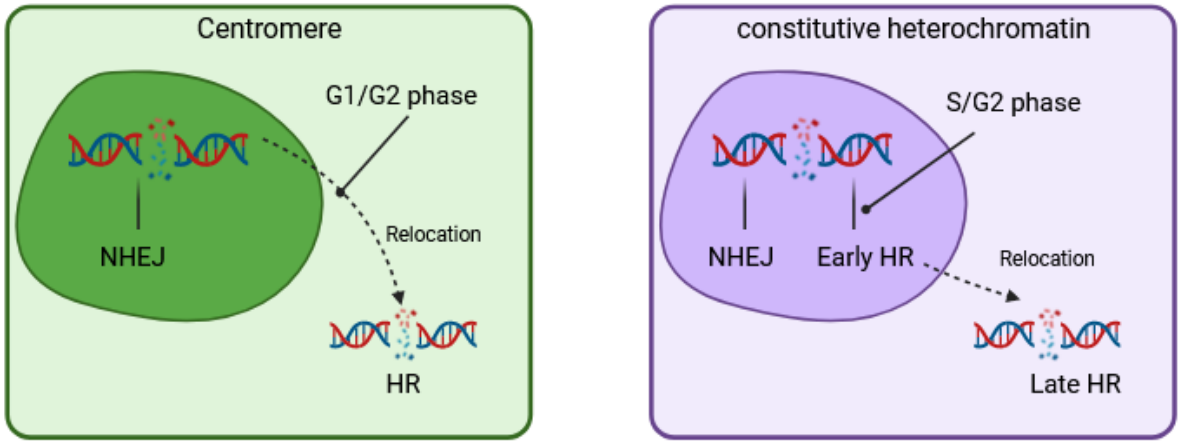



**Figure 4 Relocation of DSB in different chromatin domains**. DSB can relocate from their chromatin domain for DSB repair. In centromeres (green) DSBs can relocate in both the G1 and G2 phase of the cell cycle to be repaired by HR. In constitutive heterochromatin (purple) DSB repair in the S and G2 phase starts with early HR within the domain which is followed by relocation to the periphery to continue late HR. This figure was created on BioRender.com.

***DSB repair in pericentromeric heterochromatin in human cells***

To assess if these mechanisms are conserved in human cells an experimental system using CRISPR-Cas9 was set up to specifically induce DSBs in human pericentromeric SatIII domains (Mitrentsi et al., 2022). This revealed that, unlike mouse cells, the Cas9-induced DSBs in human heterochromatin do not relocate, revealing a fundamental difference in heterochromatin DSBs between mouse and human cells (Mitrentsi et al., 2022). To determine if this is caused by a difference in the DSB repair mechanism, the investigators measured the RAD51 distribution. This showed that RAD51 was recruited to the core of the SatIII domain while in mouse cells RAD51 is located at the heterochromatic periphery to promote DSB relocation (Mitrentsi et al., 2022). In addition, it was observed that in most S/G2 cells, repair pathway factors exhibit a spatial separation, HR factors are recruited to the SatIII core domain while NHEJ factors are confined to the periphery (Mitrentsi et al., 2022).

The extent of clustering of repetitive elements in pericentromeric heterochromatin differs between mice, *Drosophila,* and humans. Mouse and *Drosophila* cells cluster satellite repeats in chromocenters which could lead to aberrant recombination while human cells do not cluster their repeats (Mitrentsi et al., 2022). Indeed, when repeat clustering was depleted in mouse cells, RAD51 was no longer excluded indicating that clustering itself plays a significant role in the DNA repair pathway (Mitrentsi et al., 2022). Unlike *Drosophila* and mouse cells, DSBs in the SatIII domain did not show an increase in HP1α/β but instead resulted in the removal of HP1γ leading to higher accessibility to the domain (Mitrentsi et al., 2022). Overall, this shows that the DNA repair pathway dynamics in pericentromeric heterochromatin are not conserved between human (osteosarcoma) cells and mouse or *Drosophila*, and DSBs in human SatIII are positionally stable throughout the cell cycle and can be repaired within the heterochromatin domain by HR (Mitrentsi et al., 2022).

Collectively, this study suggests that there is a fundamental difference in heterochromatic DSB repair between mouse, *Drosophila,* and human cells. Future work will likely give more information about the exact differences and conserved elements in pericentromeric DSB repair.

***Repair in facultative heterochromatin***

Although fHC covers large parts of the genome, its reaction to DSBs has received limited research attention. Because fHC participates in many biological processes such as X-chromosome inactivation, stem cell maintenance, cell cycle progression, genomic imprinting, and cell differentiation, misregulation of polycomb chromatin can have severe consequences (Parreno et al., 2022). A good understanding of DSB repair and maintenance of the silent state in DSB repair in fHC is therefore essential.

fHC is characterized by an abundance of H3K27me3 and polycomb group (PcG) proteins. The PcG proteins form multiprotein complexes, also known as polycomb bodies, which can compact chromatin thereby suppressing gene expression (Cheutin & Cavalli, 2012). In both *Drosophila* and mammalian cells, PRC1 has been observed to form polycomb bodies and is thought to bring multiple PcG-repressed genes together (Cheutin & Cavalli, 2012). To study the repair of DSBs in fHC the Janssen group created a set of inducible single DSBs in the model system *Drosophila* (see Wensveen et al., 2023 for full report). Using this system, it was identified that ~20% of the DSBs are repaired by HR and ~80% by NHEJ (Wensveen et al., 2023), which was similar for DSBs in fHC and euchromatin. Depleting the end-resection protein CtIP resulted in a decrease in HR-repaired DSBs, confirming that DSBs in fHC are indeed repaired using end-resection-dependent HR (Wensveen et al., 2023). To investigate if the specific molecular and biophysical properties of polycomb bodies could influence the dynamics or movement of DSBs, live imaging of the DSBs was performed. This revealed that the majority of the DSBs that appeared inside the polycomb bodies were moved outside the domain within 10 minutes to continue repair (Wensveen et al., 2023). To investigate if local chromatin changes coincide with DSB movement in polycomb bodies, levels of the fHC histone modification H3K27me3 were assessed. Indeed, at two of the three fHC DSB sites, a decrease in H3K27me3 was observed, while this is not seen in euchromatin (Wensveen et al., 2023). This DSB-induced reduction in H3K27me3 was found to be mediated by the histone demethylase dUtx, specifically in fHC (Wensveen et al., 2023). To assess if this local loss of H3K27me3 is required for the movement of DSBs outside polycomb bodies, DSB movement was imaged in cultured cells in the presence or absence of dUtx. In the absence of dUtx, the movement of DSBs is reduced, which coincides with an accumulation of DSBs within polycomb bodies (Wensveen et al., 2023). Together, these findings indicate that the initial stages of HR take place within the polycomb bodies and are not influenced by the methylation status of H3K27me3 at the DSB site. However, for the movement of DSBs outside the polycomb bodies, H3K27me3 demethylation by dUtx is required (Wensveen et al., 2023). In addition, the movement of DSBs is also required for repair of the DSBs using HR as loss of dUtx resulted in a relative reduction of HR repair in fHC specifically (Wensveen et al., 2023).

Although in Drosophila, no differences in DSB repair pathway usage were identified between facultative heterochromatin and euchromatic DSBs sites, a sequencing-based reporter screen in human tumor cells using CRISPR-Cas9-induced DSBs revealed a difference in repair pathway usage in H3K27me3 regions (Schep et al., 2021). This reporter creates >1000 DSBs in random locations in the genome of human cancer cells of which the repair products are subsequently sequenced. This setup revealed a relative increase in a-EJ and a decrease in NHEJ repair products in H3K27me3-rich regions when compared to euchromatin regions (Kendek et al., 2021; Schep et al., 2021). Inhibition of the H3K27me3 methyltransferase EZH2 resulted in a global loss of H3K27me3 and shifted the a-EJ: NHEJ balance towards NHEJ to a similar level as in euchromatin.

***DSB dynamics in centromeric chromatin***

The centromere is a unique chromatin structure characterized by the presence of the histone variant CENP-A (Sullivan & Karpen, 2004). Centromeres are essential for kinetochore formation and therefore segregation of chromosomes. Similar to pericentromeric heterochromatin, the DNA at the centromeres is characterized by tandem repeats (Schalch & Steiner, 2017). However, there are differences in DSB repair between these two types of chromatin (Tsouroula et al., 2016). For example, in mouse cells and human cells (U2OS, HeLa, and RPE1), DSBs at centromeres recruit proteins from the HR machinery throughout the cell cycle (Yilmaz et al., 2021), which is in contrast to the S/G2-specific HR usage in euchromatin and heterochromatin. Centromeres are often actively transcribed and contain active chromatin marks such as H3K4me2 (Yilmaz et al., 2021). To determine if H3K4me2 participates in recruiting HR proteins in G1, DSBs were induced in mouse cells with artificially reduced H3K4me2 levels at the centromere. Surprisingly, this resulted in a substantial decrease in the recruitment of RAD51, BRCA1, and RPA (Yilmaz et al., 2021), indicating that H3K4me2 plays a role in the recruitment of HR factors to centromeres in the G1 phase.

The effect of H3K4me2 loss on HR in G2 was only marginal indicating that the mechanism is not essential in centromeric DSB repair in S/G2 (Yilmaz et al., 2021). Ku80 is recruited at the same level in G1 and G2 similar to what was found in pericentromeric heterochromatin. In addition to the role of H3K4me2 in promoting HR, it was also found that de novo transcription at DNA breaks can promote DNA-RNA hybrid formation which in turn facilitates HR (Yilmaz et al., 2021). Finally, albeit counterintuitive, HR at centromeric DSBs in G1 also prevents chromosomal rearrangements and therefore promotes centromeric integrity (Yilmaz et al., 2021).

The authors conclude that H3K4me2-dependent transcription at DSBs within centromeres increases the formation of DNA-RNA hybrids which facilitates DNA-end resection and initiates HR in G1 (Yilmaz et al., 2021). Because HR is usually largely suppressed in G1, it is unlikely that H3K4me2 alone is sufficient for HR activation. However, the unique features of centromeres, such as CENP-A and its chaperone HJURP could play a role. Indeed, both CENP-A and HJURP were found to play a role in RAD51 nucleation specifically in G1 (Yilmaz et al., 2021). Additionally, it was found that DSBs at centromeres trigger the deposition of CENP-A which results in the specific recruitment of USP11. The recruitment of USP11 results in the recruitment of RAD51 to the DSB resulting in licensing of HR (Yilmaz et al., 2021). These results indicate that, unlike pericentromeric heterochromatin, DSBs in centromeric chromatin can recruit both HR and NHEJ throughout the cell cycle (figure 4 left panel) (Tsouroula et al., 2016).

In conclusion, the unique features of centromeres such as the presence of CENP-A but also the active chromatin marks play a role in the choice of DSB repair pathway and promote HR in G1. To better understand DSB repair in the centromere more understanding of the role of CENP-A and other factors in the centromeres and DSB repair pathway is required.

# 5. Discussion and future perspectives

In this review, I focused on the influence of chromatin context on the repair of DSBs in euchromatin and heterochromatin. This revealed that histone marks, histone variants, and chromatin-associated proteins all contribute to the regulation of the DDR. The pre-existing and damage-induced chromatin marks especially play an important role in the initiation of the DDR, and the reorganization of the chromatin environment to facilitate DNA repair and recruitment of repair factors. These factors also play an important role in the repair pathway choice as was seen with dKDM4A in the relocation of DSBs in constitutive heterochromatin and CENP-A in the centromere-repair response. However, there are still many unanswered questions regarding the influence of chromatin context on DSB repair.

***Important factors in experimental setup DSB research***

There are many important factors to consider when researching DSB repair in the context of chromatin. For example, the choice of a model organism is essential when addressing specific questions. Budding yeast is a much-used model and has greatly contributed to our understanding of chromatin dynamics at DSBs (van Bueren & Janssen, 2024). However, it is less complex than *Drosophila,* mouse, or human cells which are more often used in studies on DSB repair in the context of chromatin domains (van Bueren & Janssen, 2024). As was highlighted in the relocation of DSBs in pericentromeric heterochromatin, not all mechanisms are conserved. It is therefore interesting to see if the mechanisms observed in model organisms are indeed conserved in humans.

Another important factor to consider when studying DSB repair is the method used to induce the formation of DSBs. DSBs can be randomly induced by, for example, ionizing radiation (IR). An advantage of this technique is that both the induction of DSBs as well as the subsequent analyses can be precisely timed (van Bueren & Janssen, 2024). However, when using IR, DSBs are randomly induced in the genome, and there can be variation in the induction of DSBs due to the chromatin context (van Bueren & Janssen, 2024). Moreover, because multiple DSBs are induced it is harder to determine the exact effect of one DSB in a specific chromatin context. It is therefore important that future research focuses more on single DSB models such as those described by (Janssen et al., 2016) to ensure that the analyzed DSB is induced in the chromatin context of choice. In the future, it is also important to not underestimate the importance of visualization techniques. New visualization techniques could give us more insight into DSB dynamics, and many other DDR processes (van Bueren & Janssen, 2024).

Heterochromatin can be divided into two types, constitutive and facultative heterochromatin. Both heterochromatin domains are characterized by specific histone marks and chromatin proteins. Although fHC covers large parts of the genome, much of the research on the role of heterochromatin on DSB repair has been performed in cHC. Although a recent study has given more insight into the role of the fHC-specific histone mark H3K27me3 and PcG proteins, much is still unknown. This study revealed that DSBs in fHC can be repaired by both HR and NHEJ. 60% of the DSBs in fHC were relocated outside the polycomb bodies for HR, which is mediated via local H3K27me3 removal (Wensveen et al., 2023). While this shows that H3K27me3 is involved in DSB relocation and HR repair, H3K27me3 also plays a role in the a-EJ: NHEJ balance in DDR repair in fHC (Schep et al., 2021). A global loss of H3K27me3 shifted the balance towards NHEJ (Schep et al., 2021). This shows that a loss of H3K27me3 can promote HR but on the other hand, can also shift the balance towards NHEJ repair. However, the results by Schep et al., 2021 were obtained in unsynchronized cells and have a limitation in the detection method as perfect repair cannot be detected. Therefore, HR is not measurable. Given that repair and cutting occur at the same time and considered that HR is not measurable it would be interesting to see if the same results can be achieved using different methods for inducing DSBs, or different detection methods.

***The relocation of DSBs in mouse cells and Drosophila***

An important discovery in the DSB repair process in heterochromatin is the relocation of DSBs. During the G2 phase of the cell cycle, DSBs in *Drosophila* and mouse cells can be relocated from the heterochromatic environment to the heterochromatic periphery and in *Drosophila* even to the nuclear periphery (Chiolo et al., 2011; Tsouroula et al., 2016). Cells relocate DSBs to separate the repetitive sequences from each other and to reduce the risk of aberrant recombination. In *Drosophila* and mouse cells, relocation of DSBs has been associated with HR (Chiolo et al., 2011; Tsouroula et al., 2016). The initial steps of HR such as recognition of the DSB and end resection occur within the heterochromatin domain (Chiolo et al., 2011). After the initial steps of HR repair are halted, the DSB is relocated to the periphery, the HR block is lifted and the repair is finished (Chiolo et al., 2011). At the centromeres, DSB relocation, HR repair and RAD51 exclusion occur throughout the cell cycle (Tsouroula et al., 2016).

The difference in cell cycle phase restrictions between DSB relocation in centromeres and pericentromeric heterochromatin might be caused by the fact that centromeric chromatin contains active

genes and active chromatin histone marks (Yilmaz et al., 2021). These histone marks most likely influence the DSB repair pathway choice and can promote HR protein binding already in G1. To investigate one could add transcription inhibitors to the experiment and determine whether active transcription plays a role in DSB repair in centromeres.

Unlike *Drosophila*, the relaxation of heterochromatin does not increase access of RAD51 to the core domain in mouse cells and is also not the cause of DSB relocation (Tsouroula et al., 2016). Recent work also demonstrates that this mechanism of DSB relocation is most likely not conserved in humans (Mitrentsi et al., 2022). DSBs in human SatIII are repaired within the heterochromatin domain and the DSBs remain positionally stable throughout the cell cycle (Mitrentsi et al., 2022). This difference might be caused by the fact that human pericentromeric repeats cluster much less than mice (Mitrentsi et al., 2022). Clustering could build a refractory environment for HR and result in the relocation of DSBs. The absence of relocation in human cells could therefore be caused by a lack of clustering.

However, the human cells used in many of the heterochromatin repair experiments are tumor cells (Mitrentsi et al., 2022; Schep et al., 2021), which are often genetically unstable. As a researcher, I understand that immortalized cell lines such as U2OS, Hela, or HEK293T are easier to handle, more cost-effective, and give more reproducible results than primary- or stem cells. However, one could argue that the results obtained in these cells are biased because the cells are genetically modified to be immortal. Although it might not always be possible, it could be very interesting to do research into DDR and the context of chromatin in DSB repair using (embryonic) stem cells or primary cells. Despite significant progress in the field of relocation of DSBs, there are still many open questions. For example, what are the targets of SUMOylation in heterochromatin repair, what is the exact role of SUMOylation in HR at the nuclear periphery, what mechanisms drive relocation to the nuclear periphery, and is relocation also involved in NHEJ repair of DSBs? Finally, the mechanisms responsible for lifting the block on the HR pathway following the relocation of the DSB remain unknown. Future research must clarify these questions and give us a better understanding of the relocation of DSBs, SUMOylation, and repair pathway choice in the chromatin context.

***The role of condensate formation in DSB repair in heterochromatin***

H3K9me2/3 and HP1 characterize constitutive heterochromatin. Recent studies have proposed a role for LLPS of HP1a in the compartmentalization of heterochromatin. Compaction via various factors leads to the exclusion of RNA polymerase from the DNA thereby preventing transcription (Strom et al., 2017). However, the exact role of LLPS in heterochromatin structure and heterochromatin dynamics remains largely unresolved. LLPS has also been proposed to play a role in a variety of processes such as enrichment of factors required for DNA repair, replication, and translation (Strom et al., 2017; Zhang et al., 2023). Recent advances show that LLPS is also involved in DSB repair. First LLPS plays a role in sensing the DSB and triggering DDR factors to form DNA repair centers (Chen et al., 2023). In this stage, LLPS is important for the stability of the DSB and the recruitment of sensor proteins such as Ku70/80 and the MRN complex (Chen et al., 2023). It was also shown that 53BP1 LLPS acts as a scaffold for P53 and its activating proteins (Kilic et al., 2019). 53BP1 could therefore play a role in transmitting signals from the DSB to P53 thereby coordinating the cell cycle checkpoint activation (Kilic et al., 2019). Multiple mechanisms such as damage-induced long non-coding RNA, PARylation, and PTMs drive LLPS (Chen et al., 2023). However, there are still many questions regarding the role of LLPS in DNA repair, such as the role of LLPS alternative pathways. what is the underlying mechanism of LLPS? And is there an interplay between the LLPS DSB repair factors and the biophysical properties of heterochromatin? The answers to these questions would help us further understand DSB repair mechanisms (Chen et al., 2023).

***The role of clustering in DSB repair***

DSBs in active genes are sometimes refractory to repair in G1 and undergo clustering to subsequently be repaired by HR during and after the S phase (Aymard et al., 2017). It remains unknown why the clustered DSBs in active genes in the G1 phase are not repaired. Clustering was decreased by

transcription inhibition with the compound DRB but not with other transcription inhibitors, suggesting that transcription itself is not necessarily responsible for clustering (Aymard et al., 2017). It is striking to me that DSBs cluster, given the fact that inhibition of clustering reduces translocation (Aymard et al., 2017). This suggests that clustering could promote aberrant repair and therefore cause more genomic rearrangements. From an evolutionary perspective, it is therefore striking that this potentially dangerous system is selected throughout evolution. In euchromatin, clustering of DSBs was suggested to play a role in creating "repair centers" in which repair machinery can be concentrated (Chen et al., 2023; Zhang et al., 2023). Data by Aymard et al., 2017 would argue against this because they observe delayed repair in these clusters. It has also been suggested that clustering is used to prepare DSBs for correct repair and to prevent these DSBs in active genes from being repaired by the mutagenic alternative pathways such as a-EJ or SSA (Aymard et al., 2017). Further research must clarify the mechanisms behind DSB clustering, the potential role of LLPS in clustering, and the role of clustering in DSB repair.

***Chromatin restoration following DNA damage***

As described in Chapter Two, the organization of chromatin is defined by the combination of histone modifications, incorporation of histone variants, and presence of chromatin remodelers and chromatin binding proteins (Martire & Banaszynski, 2020). Synthesis of many histones is coupled to replication except for histone variants (Martire & Banaszynski, 2020). Many processes are required to maintain the correct chromatin organization in the context of both euchromatin and heterochromatin. In euchromatin "active" modifications promote gene expression, in heterochromatin "silencing" modifications promote the silencing of the DNA. I highlighted some of the specific factors in these processes. Most importantly I discussed the fact that in both heterochromatin and euchromatin, the chromatin organization is modified to facilitate DNA repair. Although we have learned a lot about chromatin changes occurring upon DSB induction, our understanding of how the chromatin structure is restored after DNA repair is still limited. In response to DNA damage, local expansion of chromatin is observed (Chiolo et al., 2011). Because chromatin reorganization is only examined close to the damaged area it is often not known how far this reorganization spreads and if and how spreading is involved in chromatin restoration (Polo, 2015). It has been shown that core histones are lost over a region of 3kb surrounding the DSB (Goldstein et al., 2013). However, it is yet to be determined if this is caused by disruption or damage to the histone or a result of remodeling (Polo, 2015). Histone chaperones play an important role in restoring the chromatin organization by navigating and depositing newly synthesized histones to the DNA after damage (Polo, 2015). However, it is most likely a combination of histone chaperones, histone modifications, and remodeling factors that facilitate the restoration of the initial chromatin organization.

Overall, this report gave an overview of the current knowledge in the field of DSB repair in the context of chromatin organization. Chromatin organization plays an important role in the response to DSBs. This response not only protects the genome from aberrant recombination but helps to facilitate the activation of the most effective repair tool in that specific organization. A better understanding of DSB repair in the context of chromatin allows us to get a better understanding of how genomic stability is maintained in different chromatin organizations. As genomic instability is a hallmark of cancer, resolving fundamental processes in DNA repair in a chromatin context can help us to get a better understanding of what processes potentially drive cancer development. Answers to these fundamental questions can help us in the future in the development of anti-cancer therapies.

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