Double-Strand Break Repair in Repetitive DNA Regions

Writing Assignment

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Layman’s summary

The human body is composed of many cells. In each one of these cells, there is DNA. DNA is the “instruction manual” for the human body. It tells each cell what to do, how to act, and what to look like. The DNA is two meters long. However, it needs to fit within this very small cell, which is a challenge. The DNA needs to be compacted in a very organized manner. To do so, the DNA wraps around small proteins which are called histones. Together this forms the chromatin. Imagine chromatin to be like a pearled necklace where the DNA is the string, and the pearls are the histones. Additionally, different chromatin structures exist. Just like a pearled necklace, your pearls can either be placed very tightly together or very distanced from each other. When the histones are spaced from each other, this is called euchromatin and when they are tightly packed, this is called heterochromatin.

Every day, your DNA is damaged. One of the significant damages that can occur is a double-strand break. Basically, your DNA is broken into two pieces. This process can occur due to outside factors (i.e., radiation) or factors from inside your body. The cell is well-trained to repair these breaks. It does this by putting back the two broken sides together through various processes. However, the repair of such breaks needs to be properly done otherwise your DNA can accumulate errors (mutations) which might impair the survival of your cell or give rise to cancerous cells.

The way your DNA will be repaired is specific to where in your DNA the break occurs. Some regions of your DNA are harder to repair and put back together than other regions. For example, it can be harder to repair such breaks when all the surrounding DNA looks the same. In these cases, the cell does not know what belongs where, and which pieces should go back together. This is the case in regions of the DNA that are repetitive. To deal with such complex areas, special repair “protocols” have been put in place by the cell to allow for safe and correct repair of the break. To help the cell in navigating which pieces of DNA belong together, these repair protocols can isolate the two ends of the broken piece of repetitive DNA far away from all the rest of the DNA. The isolation of the break allows the repair to be safely done. This reduces the risk of creating mutations and wrong recombinations. The cell has different repair “protocols” specifically for each region of the chromatin. In this report, we reviewed the different mechanisms that the cell has put into place to repair different regions of the chromatin after a double-strand break. We particularly looked into the repair of double-strand breaks in regions of the DNA that are repetitive.

Double-Strand Break Repair in Repetitive DNA Regions

Abstract

Every day, our cells are exposed to endogenous and exogenous sources of damage which cause double-strand breaks (DSBs). If left unrepaired or misrepaired, DBSs can be highly toxic for the cell resulting in cell death, mutations, or genomic instability, which is a hallmark of cancer development. To deal with such breaks, the cell activates the DNA damage response which will sense the DSB and activate the appropriate repair pathway. Two main repair pathways exist: non-homologous end-joining and homologous recombination. The choice of repair pathway is dependent on different factors such as the cell cycle phase and the chromatin context in which the break occurred. Moreover, the different chromatin organizations will recruit different repair proteins and lead to different repair mechanisms as a way of safeguarding genomic stability. However, in highly repetitive regions of the DNA, such as in constitutive heterochromatin, ribosomal DNA, and centromeres, there are higher risks of aberrant recombination and chromosomal rearrangement occurring during repair due to their repetitive nature. Even though these repetitive regions pose a particular danger to our genome integrity, repetitive elements cover half of our genome. It is therefore essential that the cell ensures proper repair in repetitive DNA regions to maintain genomic integrity. In this review, we give an overview of the current knowledge of the different ways repetitive DNA regions are repaired after DSBs specifically in heterochromatin, ribosomal DNA, and centromeres.

1. Introduction

In each cell, the DNA carries the genetic information that defines the cell's function and identity. However, DNA constantly undergoes damage. Every day, tens of thousands of DNA lesions occur in each cell of the human body (Lindahl & Barnes, 2000). Damage can arise due to exogenous or endogenous causes. Multiple types of DNA damage exist, but the most significant one is the double-strand break (DSB). Unrepaired or misrepaired DSBs are highly toxic for the cell and its survival (Khanna & Jackson, 2001). Therefore, this DNA damage must be detected and properly repaired to prevent cell death, accumulation of mutations, or genetic instability, which is one of the hallmarks of cancer development (Khanna & Jackson, 2001; Jackson & Bartek, 2009).

To deal with DSBs and repair them, the cell activates the DNA damage response (DDR). The DDR includes detecting the break, signaling its presence, and activating DNA repair pathways and cell cycle checkpoints (Jackson & Bartek, 2009). Altogether, the DDR safeguards the genome against improper repair. Different DSB repair pathways exist within eukaryotic cells. The two main ones are non-homologous end-joining (NHEJ) and homologous recombination (HR) (West, 2003). NHEJ allows for fast repair by ligating the two ends together without using a template, which may result in local mutation or deletion. On the other hand, HR relies on a DNA template for proper repair, resulting in a precise but time-consuming process (West, 2003).

DSBs can occur at any site in the DNA. However, the repair pathway choice depends on the chromatin context in which the DSB occurred (Clouaire & Legube, 2015). Chromatin is the structure that DNA packs itself into to fit in the nucleus (Felsenfeld & Groudine, 2003). Nucleosomes are the building blocks that enable the assembly of the chromatin. Each nucleosome is formed by a stretch of DNA wrapped around a histone octamer (Luger et al., 1997). Different factors such as histone modifications, DNA methylation, histone variants or histone-binding proteins influence chromatin organization (Clouaire & Legube, 2015). Throughout the genome, different chromatin structures and states are identified. As mentioned above, the DDR will be different between chromatin regions such as between the constitutive heterochromatin, ribosomal DNA (rDNA), and centromeric regions. Each one of these regions has a different chromatin -structure and -state influenced by the presence of different histone modifications and histone-binding proteins. Histone modifications and histone-binding proteins further impact the recruitment of specific repair proteins to each chromatin region, and consequently, the choice of repair pathway as well as additional mechanisms of repair (Clouaire & Legube, 2015). For example, after detection of a DSB in constitutive heterochromatin, relocalization is required before further repair through HR. However, this separation of events is not observed in euchromatin (Chiolo et al., 2011). Therefore, this example shows the presence of unique repair mechanisms in different chromatin states.

Heterochromatin, rDNA, and centromeres are all composed of a high number of tandem repeats (Lander et al., 2001). However, the repair of DSBs in repetitive sequences represents a true challenge. Due to their repetitive nature, they are highly prone to aberrant recombination and chromosomal rearrangement which results in genomic instability (Barra & Fachinetti, 2018; Kendek, Wensveen & Janssen, 2021; Ramsden & Nussenzweig, 2021). All three regions have essential roles in the cell such as gene expression regulation, production of the RNA elements of ribosomes, and chromosomal segregation which is important for maintaining genetic stability (Kendek et al., 2021; Barra & Fachinetti, 2018). Additionally, repetitive DNA represents a wide proportion of our genome as it makes up around 50% of our genome (Lander et al., 2001). It is therefore essential to properly repair DSBs in repetitive DNA to maintain genomic stability and cell viability (Khanna & Jackson, 2001; Jackson & Bartek, 2009). To deal with DSBs in such repetitive regions, the cell has developed specific strategies to avoid aberrant and/or ectopic recombination.

In this review, we give an overview of the current knowledge in the field of DSB repair in repetitive sequences. Here, we will describe the DSB repair mechanisms used in the three repetitive DNA regions (centromeres, heterochromatin, and rDNA) to safeguard their genetic information, mainly focusing on research from mammalian cells and *Drosophila melanogaster*. We will discuss the different repair pathways that are preferred depending on the chromatin factors present in each chromatin region (i.e., histone marks, histone binding proteins). Moreover, we will cover the mechanisms that the cell uses to avoid aberrant recombination. Finally, we will discuss the similarities and differences between DSB repair mechanisms in these different repetitive regions.

2. Chromatin Organization

Each cell contains two meters of DNA. The genetic information that the DNA encodes dictates cell properties and identity. However, all this DNA needs to fit within the cell's nucleus. To do so, DNA is compacted into a structure called chromatin (Felsenfeld & Groudine, 2003). Nucleosomes are the building blocks that enable the assembly of a chromatin structure. To form these nucleosomes, a stretch of 146 base pairs (bp) of DNA wraps around a histone octamer (H3, H4, H2A, and H2B) (Luger et al., 1997). Each histone contains an N-terminal histone tail which can sustain post-translational modifications (PTMs). These histone tails can undergo modifications such as acetylation, phosphorylation, ubiquitination, or methylation (Felsenfeld & Groudine, 2003). PTMs allow for the orchestration of changes in chromatin structures and epigenetic information (Quina, Buschbeck & Di Croce, 2006). The combination of different modifications and the presence of different chromatin binding proteins leads to different chromatin organizations, which as a consequence changes DNA accessibility (Quina et al., 2006). Classically, we distinguish two chromatin states: euchromatin and heterochromatin. Yet, additional chromatin organizations are present in the nucleus such as centromeres and rDNA. Each chromatin region is distinct from one another, as seen in the descriptions below and in Figure 1. Nonetheless, (pericentromeric) heterochromatin, centromeric DNA, and rDNA all contain repetitive DNA sequences.

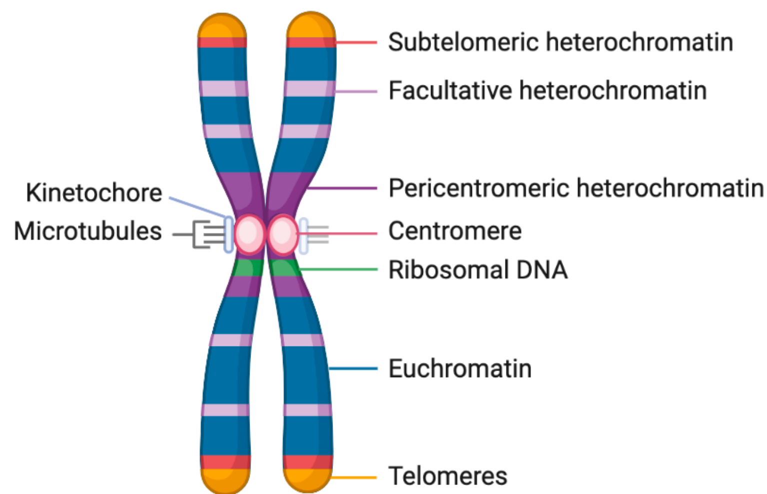

Figure 1: Mitotic chromosome of *D. melanogaster* and its different chromatin regions: telomeres, subtelomeric heterochromatin, euchromatin, facultative heterochromatin, pericentromeric heterochromatin, centromere with the kinetochore and its microtubules, and ribosomal DNA (if applicable) embedded in the pericentromeric heterochromatin. Figure created with BioRender.com

Euchromatin is defined by its characteristic open chromatin organization and transcriptionally active state (Quina et al., 2006). Euchromatin can contain many different "active" histone marks such as the trimethylation of the H3 histone at lysine 4 (H3K4me3) and histone acetylations. On the other hand, constitutive heterochromatin is typically associated with a condensed chromatin structure, resulting in inaccessibility and silenced transcription

(Huisinga, Brower-Toland & Elgin, 2006; Grewal & Jia, 2007). It is located at subtelomeric and pericentric regions of the chromosomes. Constitutive heterochromatin regions contain repetitive DNA sequences and are silenced throughout the cell cycle. This chromatin state is characterized by an enrichment in mainly di- and tri-methylation marks on the lysine 9 of H3 histones (H3K9me2 and H3K9me3). This enables the recruitment of the heterochromatin protein 1 (HP1) (Grewal & Jia, 2007; Allshire & Madhani, 2018). HP1 further dimerizes (Cowieson et al., 2000) and recruits histone methyltransferases (HMTase). In *D. melanogaster,* HP1 recruits the Su(var)3–9, an HMTase that methylates H3 at lysine position 9 (H3K9me2-3), further allowing for heterochromatin propagation and chromatin condensation (Rea et al., 2000; Schotta et al., 2002). All these factors are responsible for maintaining the stability of heterochromatin and its repetitive DNA (Peng & Karpen, 2008).

The centromere is a region of the DNA that is functionally and structurally distinct from either heterochromatin or euchromatin states (Sullivan & Karpen, 2004). The centromere is characterized by its repetitive nature and the presence of the H3 histone variant CENP-A (CID in *D. melanogaster*) (Yoda et al., 2000; Blower, Sullivan & Karpen, 2002; Sullivan & Karpen, 2004). It is embedded within pericentric heterochromatin, which for that reason, was often considered to be heterochromatic. However, the centromere has distinct chromatin features when compared to the flanking heterochromatin (Blower & Karpen, 2001). It does not contain di- or tri-methylation marks on H3K9 (Sullivan & Karpen, 2004). Yet, it does contain the active histone mark H3K4me2 and can be transcribed (Sullivan & Karpen, 2004). Centromeres play an essential role in chromosomal organization and stability. They serve as the foundation on which the kinetochore is built. The kinetochore interacts with the spindle microtubules during mitosis. The interaction between the kinetochore and the microtubules is crucial for chromosome segregation during mitosis, and thus, for proper cell division and chromosomal stability (Dobie et al., 1999; Cheeseman, 2014).

Finally, the last chromatin region with repetitive sequences is rDNA. rDNA specifically produces the RNA that makes up ribosomes. The tandem arrays of rDNA repeats are spread out over different genomic clusters, named nucleolar organizer regions (NORs). In interphase, the NORs cluster together within the cell's nucleolus (Lindström et al., 2018). Additionally, the number of NORs present in the genome is specific to each species. For humans, around 300 repeats are spread across the NORs and are found on chromosome 13, 14, 15, 21, and 22 (Henderson, Warburton & Atwood, 1972). Yet, the *D. melanogaster* genome has around 600 rDNA repeats present on the X and Y chromosomes (Ritossa et al., 1966). rDNA genes are highly transcribed by RNA polymerase I (Pol I). However, only half of the rDNA repeats are in an active state (McStay & Grummt, 2008). At the border of the nucleolus, inactive rDNA regions assemble into an intranucleolar heterochromatin region, which is in fact connected to the constitutive heterochromatin present around the nucleolus (Akhmanova et al., 2000).

Altogether, repetitive DNA sequences are found in constitutive heterochromatin, centromeres, and rDNA. These sequences are all functionally and structurally distinct from one another and each one of these regions has different chromatin organizations, post-translational modifications, histone-binding proteins, and locations within the nucleus.

3. DSB Repair Mechanisms

DNA is constantly exposed to damaging factors which can result in the production of DSBs. DNA is threatened by both exogenous and endogenous sources of damage. Exogenous damage results from environmental sources, either physical (e.g., UV, radiation) or chemical (e.g., cancer chemotherapy) (Ciccia & Elledge, 2010). Endogenous damage can be a result of metabolic processes or replication stress for example (Ciccia & Elledge, 2010). These double-strand breaks represent a threat to genome integrity. Unrepaired or misrepaired DSBs can lead to genetic instability, mutations, and chromosomal rearrangements. DSBs promote the activation of DSB repair machinery, activation of cell cycle checkpoint kinases, and apoptosis signaling. If the DSB is left unrepaired or misrepaired, cell apoptosis or senescence will be initiated. However, if the cell is unable to induce apoptosis, misrepaired DSBs could in the long run promote tumorigenesis by enhancing the level of genetic instability. In addition, if misrepaired DSBs occur in genes required to control proliferation, these mutations that inactivate these cell cycle genes could also lead to uncontrolled cell division and tumor growth (Khanna & Jackson, 2001; Jackson & Bartek, 2009; Roos, & Kaina, 2006). The DDR is responsible for the proper recognition and repair of such damage (Ciccia & Elledge, 2010). First, the breakage is sensed which leads to the activation of kinases. Three kinases of the phosphatidylinositol 3-kinase-related protein kinase (PIKK) family are of high importance, namely Ataxia Telangiectasia Mutated (ATM), ATM-related (ATR) and DNA-dependent protein kinase (DNA-PK). Once they are recruited to the DSB and activated, these kinases are responsible for the phosphorylation of a variety of substrates within the DDR, thus enabling complete repair (Blackford & Jackson, 2017). Next, repair pathways are activated. There are two main repair pathways to repair DSBs: NHEJ and HR, as illustrated in Figure 2. Other repair pathways include the Single Strand Annealing (SSA) and the Alternative End-Joining (Alt-EJ) pathways (Ciccia & Elledge, 2010). These repair pathways allow the DSB to be resolved.

Sensing the DSB

Once a DSB occurs, it is sensed by different protein complexes, namely poly (ADP-ribose) polymerase-1 (PARP1), the MRN complex (formed by MRE11, RAD50, and NBS1), the Ku70/80 complex, or SIRT6 (Ciccia & Elledge, 2010; Onn et al., 2020). These proteins lead to activation of ATM and DNA-PK. More specifically, the MRN complex leads to the recruitment and activation of ATM, while for DNA-PK, this is done by the Ku heterodimer Ku70/80 (Lee & Paull, 2005; Falck, Coates & Jackson, 2005). Their activation leads to the phosphorylation of the H2A.X histone variant on the Ser139 position. The phosphorylated H2A.X is named γH2A.X in mammals (referred to as γH2A.v in *D. melanogaster*) (Ward & Chen, 2001; Stiff et al., 2004). γH2A.X allows for the recruitment of MDC1 (Stewart et al. 2003). ATM and MDC1 will further spread γH2A.X across the neighboring chromatin up to 1-2 megabases around the DSB site (Rogakou et al., 1999; Stucki & Jackson, 2006). The phosphorylation of H2A.X further allows for the recruitment and stabilization of repair proteins at the DSB such as RNF8, BRCA1, and 53BP1, which enable repair through either of the repair pathways (Bekker-Jensen et al., 2006; Mailand et al., 2007).

The Homologous Recombination Pathway

HR is a repair pathway that relies on the use of a template to repair a DSB. This process can be slower than NHEJ but has a higher fidelity. HR relies on a homologous sequence which is often the sister chromatid. For this reason, HR is often limited to the S and G2 phases of the cell cycle (West, 2003). To allow for HR, the break first needs to be detected and signaled. When DSBs are sensed by PARP1, the MRN complex is recruited which in turn recruits ATM (Lee & Paull, 2005; Falck et al., 2005; Haince et al., 2008). As described before, this results in the phosphorylation of H2A.X and the recruitment of BRCA1, and later on, CtBP-interacting protein (CtIP) (Sartori et al. 2007). Together, CtIP and the MRN complex initiate short end resection of the DSB (Sartori et al. 2007). 5' to 3' end-resection is further elongated by Exonuclease 1 (EXO1), DNA replication helicase/nuclease 2 (DNA2) and Bloom syndrome protein (BLM) (Mimitou & Symington, 2008). The resulting 3' ssDNA is bound by replication protein A (RPA) (Wold, 1997; Sartori et al., 2007). Once RPA is loaded on the 3' ssDNA ends, ATR-interacting protein (ATRIP), followed by ATR, are recruited and activated (Zou & Elledge, 2003). RAD51 is then loaded onto the ssDNA by the BRCA1-PALB2-BRCA2 complex to replace RPA (Sy, Huen & Chen, 2009; Jensen, Carreira & Kowalczykowski, 2010). Subsequently, Rad51 initiates the search for a homologous sequence on the sister chromatid. Once found, the strand with the homologous sequence is invaded, which leads to the formation of a D-loop structure. The homologous sequence is then used by DNA polymerases as a template for the repair of the 3' ssDNA ends. Finally, the break is resolved by annealing and ligating the two ends of the DSB back together (West, 2003). This process is illustrated in Figure 2 (right).

The Non-Homologous End Joining pathway

Alternatively, DSBs can be repaired through the NHEJ pathway. NHEJ is a pathway that allows for fast repair of a DSB. NHEJ does this by religating the two ends of the DSB back together without the use of a template. However, this method can lead to the formation of small mutations or deletions (West, 2003). When DSBs arise, PARP1 and the Ku70/80 complex compete to sense the DSB (Wang et al., 2006). If the Ku70/80 complex senses the DSB, it binds to the break ends, recruits and activates the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Falck et al., 2005), which promotes NHEJ repair (Mahaney, Meek & Lees-Miller, 2009). If the ends are directly ligatable, X-ray repair cross-complementing protein 4 (XRCC4), ligase IV, paralogue of XRCC4 and XLF (PAXX), and XRCC4-like factor (XLF) will be recruited to the DNA-PK. These four proteins will align and ligate the ends back together (Ahnesorg, Smith & Jackson, 2006; Mahaney et al., 2009; Xing et al., 2015). However, if the ends of the breaks are non-ligatable, additional steps and factors will be needed before being able to ligate the ends of the DSB back together. In that case, DNA-PKcs autophosphorylates itself, causing conformational changes in the DNA-PKcs (Ding et al., 2003). This gives access to the nuclease Artemis to process the ends of the DSB (Ma et al., 2002). Additional factors such as polynucleotide kinase/phosphatase (PNKP), and aprataxin and polynucleotide kinase/phosphatase-like factor (APLF) also help to process the ends of the

DSB. Moreover, the polymerases λ and μ further reconstruct the DNA. Finally, PAXX, XRCC4, XLF and ligase IV rejoin the ends back together (Meek, Dang & Lees-Miller, 2008; Mahaney et al., 2009). This process is illustrated in Figure 2 (left).

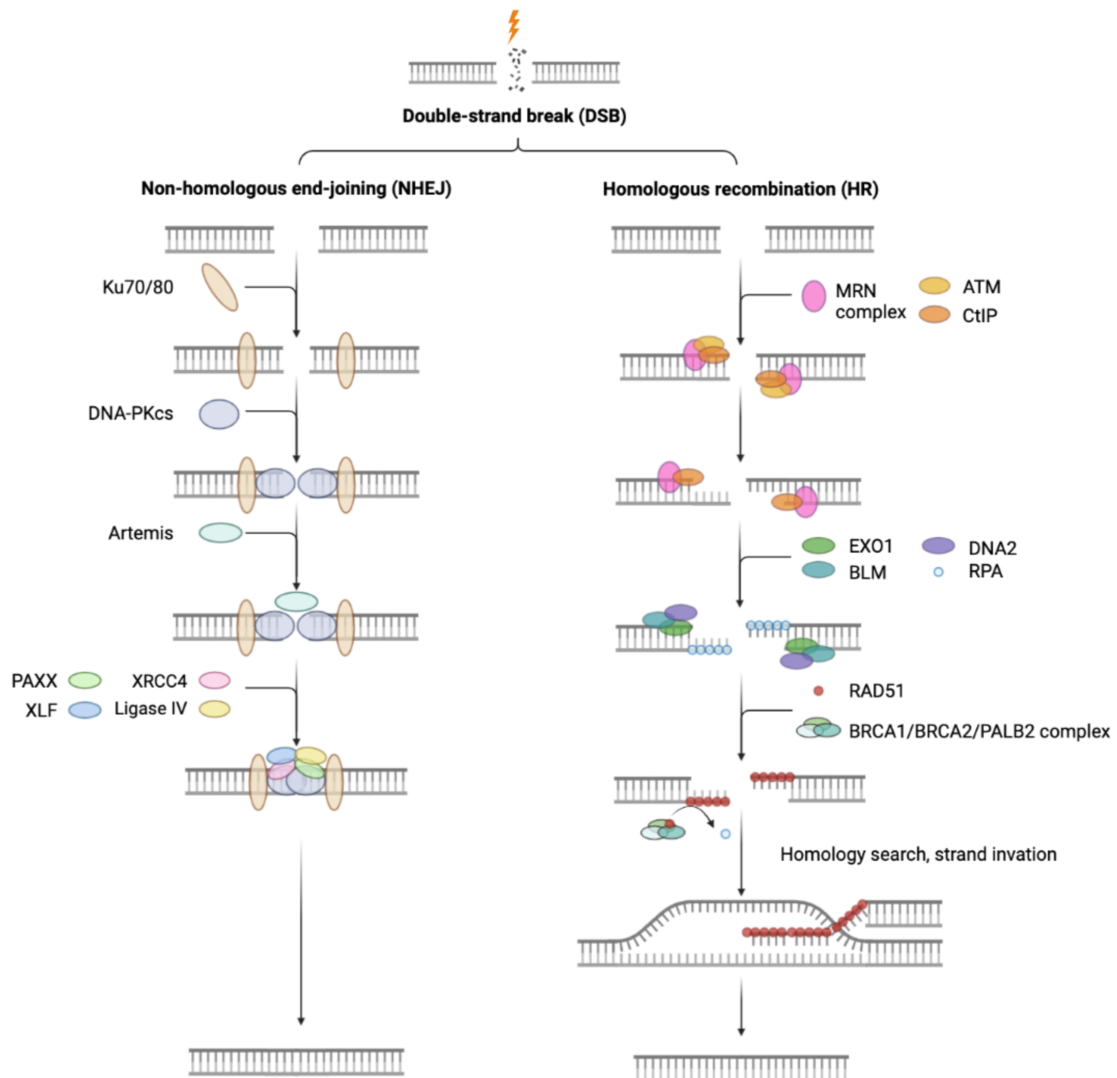

Figure 2: Repair of Double-Strand Breaks through the two main repair pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR). ***Left***. During NHEJ, the DSB is sensed by the Ku70/80 complex. The Ku70/80 complex will bind to the ends of the breaks and recruit the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Artemis will further be recruited to process the ends of the DSB. Finally, the ends can be aligned and ligated back together by the X-ray repair cross-complementing protein 4 (XRCC4), ligase IV, paralogue of XRCC4 and XLF (PAXX), and XRCC4-like factor (XLF). ***Right***. During HR, the MRN complex is recruited to the break, which in turn recruits ATM and CtIP. CtIP and MRN initiate short end resections. Further resection is performed by Exonuclease 1 (EXO1), DNA replication helicase/nuclease 2 (DNA2) and Bloom syndrome protein (BLM). Once the ends are resected, they are coated by RPA. Subsequently, RAD51 is loaded onto the ssDNA by the BRCA1-PALB2-BRCA2 complex to replace RPA. Once RAD51 is loaded, the homologous sequence can be invaded, forming the D-loop structure. The homologous sequence is used as a template for the repair of the 3’ ssDNA ends. Finally, the break is resolved by annealing and ligating the two ends of the DSB back together. Figure created with BioRender.com

4. Repetitive DNA repair

Previously, it was thought that the choice of DSB repair pathway was dictated by the cell cycle phases, cell type or the nature and complexity of the DSB (Symington & Gautier, 2011; Brandsma & van Gent, 2012). However, in the past decade, it has become clear that the process and fidelity of DSB repair is highly influenced by the chromatin and genomic context in which the break occurs. Recent work demonstrates that the chromatin context influences the choice of DSB repair pathway and the mechanisms behind the repair (Clouaire & Legube, 2015).

Repetitive DNA constitutes 50% of the human genome (Lander et al., 2001). These tandem repeats are found in three main chromatin regions: constitutive heterochromatin, rDNA, and centromeres (Lander et al., 2001). When it comes to the repair of DSBs in such repetitive DNA, there is the risk of aberrant recombination. The repetitive nature of the region results in the presence of many homologous sequences that can be used as templates for HR (Anand et al., 2014). Additionally, repeated homologous sequences can be found scattered on non-homologous chromosomes across the genome. Therefore, when HR occurs in repetitive regions, there is a risk of template switching between repeated sequences dispersed across the genome (Anand et al., 2014). Aberrant recombination can lead to chromosomal rearrangements such as translocations, duplications, deletions, dicentric or acentric chromosomes (Anand et al., 2014). These misrepaired DSBs are highly toxic for the cell and its survival (Khanna & Jackson, 2001). Thus, cell apoptosis or senescence will be initiated. However, if the cell is unable to induce apoptosis, aberrant recombinations that result in mutations in cell cycle genes can lead to tumor growth through uncontrolled cell division (Khanna & Jackson, 2001; Jackson & Bartek, 2009; Roos, & Kaina, 2006). Therefore, DSBs, especially when occurring in tandem repeats, need to be detected and properly repaired. To avoid aberrant recombination, the cell has developed unique repair mechanisms for each repetitive region. Here, we will discuss the repair of DSBs in each one of the three repetitive chromatin regions: constitutive heterochromatin, rDNA, and centromeres.

4.1. Repair of DSBs in Heterochromatin

Heterochromatin is a unique chromatin organization characterized by its histone marks (H3K9me2/3) and the recruitment of HP1. Pericentromeric heterochromatin represents roughly 30% of both the human and the *D. melanogaster* genomes (Lander et al., 2001; Hoskins et al., 2007; Caridi et al., 2017). Yet, in most species, pericentromeric heterochromatin is mainly formed by repetitive DNA sequences such as tandem repeats (Padeken, Zeller & Gasser, 2015). In *D. melanogaster*, roughly half of the heterochromatin is formed by 'satellite' repeats while the rest of the heterochromatin is composed of clusters of transposons, and around 250 isolated genes (Caridi et al., 2017). The high amount of repetitive DNA sequences in heterochromatin represents a challenge for the repair of DSBs (Caridi et al., 2017). Moreover, heterochromatin clusters itself in a 3D organization, forming heterochromatin domains or condensates within the nucleus (Strom et al., 2017). These heterochromatin clusters are hypothesized to also result in an increased risk of aberrant recombination as repeats found on different chromosomes are

present in the same cluster which could potentially promote chromosomal rearrangements (Mitrentsi et al., 2022).

The Repair of Heterochromatin is Controlled Through Space and Time

To repair DSBs, NHEJ is generally preferred in G1 while HR is mainly used in S and G2. During G1, repair through HR is blocked by ubiquitination of PALB2 which hinders the assembly of the BRCA1/PALB2/BRCA2 complex needed for HR, therefore leading to NHEJ (Orthwein et al., 2015). Moreover, CtIP is mainly activated in S and G2 through phosphorylation by CDK, promoting HR in those phases only (Huertas & Jackson, 2009). When DSBs occur in heterochromatin, it has often been thought that the main pathway of choice would be HR (Chiolo et al., 2011). Nonetheless, recent work demonstrates that heterochromatin actually relies on both HR and NHEJ for DSB repair, in both flies and mice (Janssen et al., 2016; Tsouroula et al., 2016). Moreover, in heterochromatin, HR is restricted to the S and G2 phases (Tsouroula et al., 2016).

The repair of DSBs in heterochromatin through HR is controlled through space and time to avoid ectopic recombination (Chiolo et al., 2011). To control the repair of DSBs in heterochromatin through space, one main mechanism has been found in different model systems: relocalization of the DSB to the heterochromatin periphery (Chiolo et al., 2011, Tsouroula et al., 2016). The movement of DSBs is thought to be mainly associated with HR (Chiolo et al., 2011; Tsouroula et al., 2016). However, whether NHEJ is also associated with relocalization remains to be determined. Therefore, the data discussed here will cover the role of HR in the relocalization of the DSB to the heterochromatin periphery. The process of relocalization is thought to allow the isolation and physical separation of the DSB from the rest of the homologous repetitive sequences, thus, avoiding aberrant recombination and chromosomal translocation during HR (Chiolo et al., 2011). To control the process of HR in heterochromatin in time, HR is divided into two distinct phases: an early phase occurring before DSB relocalization, and a late phase after relocalization (Chiolo et al., 2011). Here, we will cover the steps that allow for HR to repair DSBs in constitutive heterochromatin. This process is illustrated in Figure 3. It must be noted that a majority of research on the topic of DSB in heterochromatin has been performed in *D. melanogaster.* Therefore, here we will mainly focus on the work done in this organism except if stated otherwise.

In the early phase of HR repair in heterochromatin, the DSB is sensed and signaled. This leads to checkpoint activation, DNA end resection, RPA loading and ATR activation. These steps are required before DSB relocalization (Chiolo et al., 2011). It was found that the structural maintenance of chromosomes 5/6 (Smc5/6) complex is also recruited to the DSB (Chiolo et al., 2011). Smc5/6 further allows for the recruitment of three SUMO E3 ligases: protein inhibitor of activated STAT (dPIAS), as well as two Smc5/6 complex subunits, namely Quijote (Nse2/Qjt) and Cervantes (Nse2/Cerv) (Ryu et al., 2015; Ryu, Bonner & Chiolo., 2016). The Smc5/6 complex and the SUMO E3 ligases together block the recruitment of RAD51 and strand invasion while the DSB is still located in the heterochromatin domain (Chiolo et al.,

2011; Ryu et al., 2015; Ryu et al., 2016). RAD51 will only assemble after DSB relocation to the heterochromatin periphery (Chiolo et al., 2011). After the recruitment of the Smc5/6 complex and the different SUMO E3 ligase, SUMOylation of currently unknown targets occurs, helping promote DSB relocalization (Ryu et al., 2015). These SUMOylated proteins further enable the recruitment of a SUMO-targeted ubiquitin ligase (STUbL) protein, called Dgrn (Ryu et al., 2015). Altogether, in the early phase of HR, RAD51 binding is inhibited while still within the heterochromatin domain. The blockage of RAD51 assembly is controlled by the recruitment of specific SUMO-dependent proteins as seen in Figure 3.

Heterochromatic DSBs have often been demonstrated to relocate to the heterochromatin periphery to resume HR (Chiolo et al., 2011). Yet, recent work demonstrates that around 20% of heterochromatic DSBs can also relocate to the nuclear periphery for HR (Ryu et al., 2015; Caridi et al., 2018). In these studies, the relocalization of heterochromatic DSBs to the nuclear periphery was shown to be mediated by nuclear actin filaments (F-actin) and myosin (Caridi et al., 2018). The actin nucleator Arp2/3 was found to assemble nuclear F-actin from the DSB site to the nuclear periphery (Caridi et al., 2018). Moreover, the Smc5/6 complex was also found to recruit the myosin activator Unc45 which allows the activation of nuclear myosins. Myosin interacts with the Smc5/6 complex bound at heterochromatic DSBs and thereby enables the relocalization of the heterochromatic DSB along the nuclear F-actin fiber to the nuclear periphery (Caridi et al., 2018). There, the DSB is relocated to the inner nuclear membrane proteins (INMPs) or to the nuclear pores (Ryu et al., 2015). Once at the nuclear periphery, the DSB is anchored as Smc5/6 and STUbL interact with components of the nuclear pore/inner nuclear membrane through the STUbL/RENi complex (Ryu et al., 2015; Ryu et al., 2016). Additionally, once the heterochromatic DSB is stabilized at the nuclear periphery, STUbL is thought to ubiquitinate the proteins that were SUMOylated. This is hypothesized to promote the later steps of HR, which includes RAD51 recruitment and homology search on the homologous chromosome (Ryu et al., 2015). However, if the relocalization process fails, it has been identified in mouse cells that RAD52 is recruited to engage the alternative SSA repair pathway. NHEJ can also be used to repair DSB when relocalization fails (Tsouroula et al., 2016). Together, the relocalization of the heterochromatic DSB to the periphery is believed to be mediated by F-actin and myosin. Once the heterochromatic DSB has been relocated, RAD51 is recruited and HR can be resumed, as illustrated in Figure 3.

Together, the process of DSB relocalization reveals the specific spatial temporal control of the repair of DSBs within heterochromatin, therefore isolating the DSB and helping avoid aberrant recombination (Chiolo et al., 2011). The process of heterochromatic DSB movement to the heterochromatin periphery for HR has been observed both in *D. melanogaster* and mouse cells (Chiolo et al., 2011; Tsouroula et al., 2016). In both organisms, the repair through HR of heterochromatic DSBs occurs in two stages before and after relocalization. Before relocalization, the DSB needs to be sensed, the ends are resected, and further HR is blocked until relocalization. To allow for the heterochromatic DSB movement, DNA end-resection and RAD51 exclusion are required to occur in the heterochromatin domain. Once at the periphery, HR is resumed (Chiolo et al., 2011; Tsouroula et al., 2016). Unlike *D. melanogaster*, in mouse cells, heterochromatic DSB were not observed to relocate to the nuclear periphery for HR as

they only localized at the heterochromatin periphery (Tsouroula et al., 2016). This could be due to the fact that in mouse cells, there is the formation of the RAD51/BRCA2 complex that stabilizes the DSB at the heterochromatic periphery (Tsouroula et al., 2016). In *D. melanogaster*, however, heterochromatic DSBs can continue their relocalization to the nuclear periphery (Ryu et al., 2015; Caridi et al., 2018). Further research is needed to determine the extent of the conservation of the HR repair pathway in heterochromatic DSB between species.

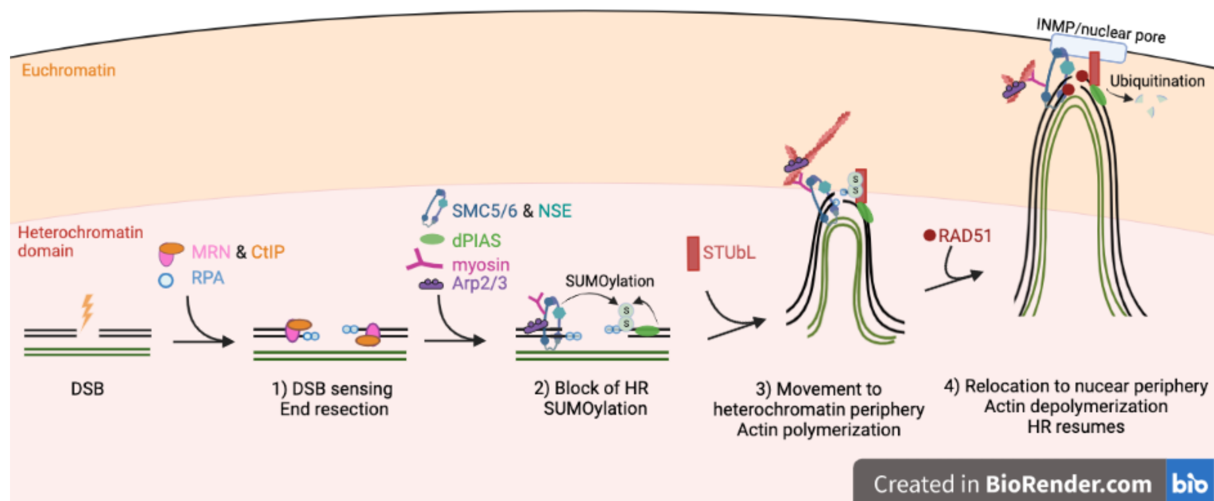

Figure 3: Model of homologous recombination repair of heterochromatic DSBs through space and time in *D. melanogaster*. *1)* To allow for HR, the heterochromatic DSB is sensed and the ends are resected by MRN and CtIP as well as EXO1, DNA2 and BLM (the last three are not shown). RPA coats the 3' ssDNA. ***2)*** Smc5/6 is recruited to the heterochromatic DSB, which recruits dPIAS, and two Smc5/6 subunits: Nse2/Qjt and Nse/Cerv. Together, they block RAD51 recruitment while in the heterochromatin domain. Arp2/3 and myosins are recruited to the DSB. SUMOylation of target proteins allows for localization to the heterochromatin domain periphery. ***3)*** At the heterochromatin periphery, the actin filament elongates, promoting relocalization of the break from the heterochromatin domain to the nuclear periphery. *4*) DSB is anchored as Smc5/6 and STUbl interacts with components of the nuclear pore or the INMPs. The anchoring allows for F-actin depolymerization. Now that the DSB is at the periphery, the blockage is removed, RAD51 is recruited, and HR is safely resumed. Figure created with BioRender.com

Changes in Heterochromatin Organization after DSBs

To facilitate the repair of DSB in heterochromatin, two main mechanisms are noted: chromatin expansion and removal of heterochromatin histone marks. In fact, DSB repair in heterochromatin is coupled with a global expansion of the heterochromatin domain surrounding the DSB (Chiolo et al., 2011). This is observed in both mouse and *D. melanogaster* cells (Ayoub et al., 2008; Chiolo et al., 2011). The expansion of heterochromatin is a result of the activity of DNA damage checkpoint kinases, namely ATM and ATR, and other resection elements (Chiolo et al., 2011). Yet, the expansion is not linked to a prominent eviction of HP1 components or a reduction of the total amount of H3K9me3 histone marks (Chiolo et al., 2011; Tsouroula et al., 2016).

Relaxation of the chromatin has been hypothesized to increase the speed of access of the DDR proteins to the break, thus promoting repair (Murga et al., 2007). However, other research observed that a compacted chromatin state does not necessarily hinder the recruitment of DDR proteins to heterochromatin (Tsouroula et al., 2016). Additionally, forced chromatin relaxation also did not increase RAD51's accessibility to the DSB (Tsouroula et al., 2016). The current model is that expansion of the heterochromatin is not necessarily the driver of DSB relocalization, yet it might facilitate the movement (Tsouroula et al., 2016; Ryu et al., 2016).

DSB repair in heterochromatin is also associated with local changes in the heterochromatin landscape around the DSB through loss of heterochromatin histone marks. In *D. melanogaster,* the process of demethylation of H3K9me2/3 is controlled by dKDM4A (drosophila KDM4A) (Colmenares et al., 2017; Janssen et al., 2019). dKDM4A is recruited to heterochromatin by HP1a (Lin et al., 2008). The removal of heterochromatin histone marks might facilitate the transient decompaction of heterochromatin and promote the accessibility of repair proteins to the DSB as well as relocalization (Colmenares et al., 2017; Janssen et al., 2019). Together, the change in heterochromatin organization through chromatin expansion and removal of heterochromatin histone marks is thought to facilitate repair protein accessibility and relocalization of heterochromatin DSBs.

4.2. Repair of DSBs in Centromeric DNA

Centromeres are characterized by their unique chromatin structure through the presence of the CENP-A histone H3 variant (Yoda et al., 2000; Blower, Sullivan & Karpen, 2002; Sullivan & Karpen, 2004). Centromeres are essential components for chromosomal organization and stability (Dobie et al., 1999; Cheeseman, 2014). The centromere is the foundation on which the kinetochore is built. The kinetochore interacts with the spindle microtubules during mitosis, which is essential for chromosome segregation during mitosis, and thus, for proper cell division and chromosomal stability (Dobie et al., 1999; Cheeseman, 2014). However, centromeres are highly susceptible to chromosomal rearrangements, highlighting the importance of proper repair to avoid disrupting the integrity of this region (Yilmaz et al., 2021). Yet, little is still known on how this specific chromatin region responds to DSBs and how it is repaired.

Homologous Recombination of DSBs also Occurs in G1 at the Centromeres

Similarly to heterochromatic DSB repair, the repair of DSBs in centromeres is also controlled through space and time (Tsouroula et al., 2016). The exclusion of RAD51 from DSBs is also identified in centromeres while surrounded by centromeric repeats. The centromeric DSBs relocate outside of the centromeric repeats to continue HR (Tsouroula et al., 2016). However, unlike pericentric heterochromatin, both the inhibition of RAD51 recruitment and relocalization of the DSBs occurs independently of the cell cycle phase, indicating that HR of centromeric DSBs can occur in both G1 and S/G2 (Tsouroula et al., 2016; Yilmaz et al., 2021). Additionally, most of the DSBs occurring in G1 in centromeric chromatin rely on the

recruitment of RAD51, RPA, and BRCA1, and thus, on HR (Tsouroula et al., 2016; Yilmaz et al., 2021). This allows centromeres to rely on both NHEJ and HR throughout the cell cycle (Tsouroula et al., 2016). Specific repair mechanisms, highlighted below, have been described to promote HR throughout all cell cycle phases at DSBs in centromeres.

One specific mechanism that has been shown for the repair of DSBs in centromeres is R-loop formation at DSBs (Racca et al., 2021; Yilmaz et al., 2021). R-loops are formed by a three stranded structure where the nascent RNA strand anneals to the DNA strand to which it is complementary to, forming an RNA:DNA hybrid (Racca et al., 2021). Although it has been described that removal of R-loops is necessary to prevent DSBs and genomic instability (Costantino & Koshland, 2018), the assembly of RNA:DNA hybrids during HR in fact promotes repair of centromeric DSBs (Yilmaz et al., 2021). When a centromeric DSB occurs in G1, HR is the preferred pathway even though there is no homologous sister chromatid present during that cell cycle phase (Tsouroula et al., 2016; Yilmaz et al., 2021). Recent work demonstrated that CENP-A with its corresponding histone chaperone HJURP, and the H3K4me2 histone mark are all essential components to make HR possible during G1 in centromeres (Yilmaz et al., 2021). It was revealed that the H3K4me2 histone mark increases transcription at the DSB site, which promotes the formation of RNA:DNA hybrids. In turn, these R-loops facilitate HR through promotion of DNA-end resection. Additionally, it was found that CENP-A and its histone chaperone HJURP interact with the deubiquitinase USP11 at DSBs (Yilmaz et al., 2021). USP11 is proposed to deubiquitinate HJURP allowing HJURP to chaperone and deposit new CENP-A. In turn, CENP-A further recruits USP11. Moreover, USP11 also promotes the assembly of the BRCA1-PALB2-BRCA2 complex by deubiquitinating PALB2 in G1, leading to RAD51 recruitment and licensing of HR while still in G1 (Orthwein et al., 2015; Yilmaz et al., 2021). Therefore, CENP-A, HJURP, and USP11 together grant HR in centromeres while still being in G1.

4.3. Repair of DSB in Ribosomal DNA

The nucleolus contains rDNA which specifically produces the RNA that makes up ribosomes. To produce these RNAs, rDNA genes are highly transcribed by Pol I. The high Pol I occupancy and frequent replication fork stalling make rDNA especially prone to DSBs (Lindström et al., 2018). Moreover, its repetitive nature makes the repair of DSBs in the rDNA complex at risk of chromosomal rearrangements (Lindström et al., 2018). Also, the localization of rDNA in the nucleolus represents an additional challenge for repair as repair proteins need to access this phase-separated area of the nucleus (Korsholm et al., 2020). To face the challenges of DSBs in rDNA, the cell has developed certain mechanisms to repair DSBs in the nucleolus, thus allowing it to maintain genomic stability (Korsholm et al., 2020). Below, we will discuss the mechanisms of structural reorganization, nucleolar cap formation, transcription repression, and repair pathway choice after rDNA DSBs.

rDNA DSBs Relocate and Form Nucleolar Cap

Nucleolar caps in the context of rDNA DSBs were first observed to form upon irradiation as the upstream binding factor (UBF1) was identified to segregate to these nucleolar caps (Kruhlak et al., 2007). In this initial work, after irradiation, the authors identified that DSBs in rDNA resulted in Pol I transcriptional inhibition. Subsequently, the transcriptional inhibition led to nucleolar segregation and nucleolar cap formation (Kruhlak et al., 2007). Transcriptional inhibition was a process already identified earlier to result in nucleolar cap formation (Shav-Tal et al., 2005). Moreover, later work demonstrated that the process of rDNA DSB relocalization to form nucleolar caps allows for HR at the nucleolar caps (van Sluis & McStay, 2015). To do so, a cluster of DSBs is formed in the nucleolar interior and relocates to its periphery at the nucleolar caps (Korsholm et al., 2019). The nucleolus reorganization to form nucleolar caps allows the rDNA DSBs to become attainable by repair factors (van Sluis & McStay, 2015). Diverse HR repair proteins, notably RAD51, RAD52, BRCA1 colocalize to the nucleolar cap for repair (Harding et al., 2015; van Sluis & McStay, 2015). The process of relocation of rDNA DSBs to the nucleolar caps could allow for safe repair through HR and therefore avoid aberrant recombination within the nucleolus. Additionally, the relocalization process of rDNA DSBs for repair through HR was also observed in *Saccharomyces cerevisiae* (Torres-Rosell et al., 2007). In short, when Pol I transcriptional inhibition occurs upon rDNA DSBs, this leads to nucleolar segregation. The rDNA DSBs relocate to form nucleolar caps at the nucleolar periphery where HR factors colocalize, enabling repair, as seen in Figure 4.

Other factors in addition to Pol I transcriptional repression have been identified to control rDNA DSB relocalization and nucleolar cap formation. In fact, the linker of the nucleoskeleton and cytoskeleton (LINC) complex was found to control the restructuring of the nucleolus to promote repair (Marnef et al., 2019). With the help of actin filaments, the LINC complex enables the invagination of the nuclear envelope to form contact with the nucleolus (Marnef et al., 2019). When depleting one of the subunits of the LINC complex, as well as actin and myosin factors needed to assemble actin filaments, there is a decrease in the number of nucleolar caps formed after DSBs without affecting transcriptional inhibition (Marnef et al., 2019). Therefore, the action of the LINC complex and the actin pathway are needed to promote the relocalization of rDNA DSBs to the nucleolar periphery (Marnef et al., 2019). In addition, the histone arginine demethylase JMJD6, which is recruited to rDNA DSBs and interacts with the nucleolar protein Treacle, has been suggested to play a role in nucleolar cap formation (Fages et al., 2020). In fact, depletion of JMJD6 results in decreased nucleolar cap formation, without affecting transcription inhibition (Fages et al., 2020). Together, both work done on the LINC complex and JMJD6 suggest that nucleolar cap formation can be uncoupled from transcription inhibition and that nucleolar cap formation is also dependent on factors such as the LINC complex and JMJD6 (Marnef et al., 2019; Fages et al., 2020).

Persistent rDNA DSBs Cause Transcriptional Repression

As mentioned above, Pol I transcriptional inhibition is one of the mechanisms required for nucleolar cap formation, and thus for HR (Kruhlak et al., 2007; Harding et al., 2015; van Sluis & McStay, 2015). Transcription is only repressed around the rDNA DSB (Kruhlak et al., 2007). The current prevailing hypothesis is that rDNA transcription is only inhibited in the presence of persistent DSBs in the nucleolus (Harding et al., 2015). To do so, different DNA repair factors are required for the transcriptional blockage (Kruhlak et al., 2007).

Persistent DSBs in the nucleolus lead to the recruitment of ATM by the MRN complex, which initiates the inhibition of rDNA transcription and the phosphorylation of Treacle (Kruhlak et al., 2007; Harding et al., 2015; Mooser et al., 2020). Once phosphorylated, Treacle enables further accumulation of NBS1, allowing for a positive feedback loop on ATM recruitment and accumulation at the DSB site (Larsen et al., 2014). Treacle along with ATM and NBS1 recruit DNA Topoisomerase II Binding Protein 1 (TOPBP1), further leading to end processing, enhancement of rDNA transcription inhibition, and ATR activation (Larsen et al., 2014; Mooser et al., 2020). The activation of ATR plays a central role in the inhibition of transcription while also stimulating nucleolar reorganization through nucleolar cap formation (Korsholm et al., 2019).

The cohesin and the Human Silencing Hub (HUSH) complexes were also identified to be important in repressing the RNA Pol I transcription activity at DSBs (Marnef et al., 2019). To this end, cohesin recruits the HUSH complex which introduces H3K9me3 methylation marks at rDNA repeats, further driving silencing (Marnef et al., 2019). Together, the transcriptional repression is controlled by the DNA repair factors ATM, NBS1, TOPBP1, ATR, as well as the HUSH complex. Transcriptional repression further enables nucleolar segregation, cap formation, and thus HR. This process is illustrated in Figure 4. In line with this, transcription repression is an essential step to avoid chromosomal rearrangements during the repair of persistent rDNA DSBs (Meisenberg et al., 2019).

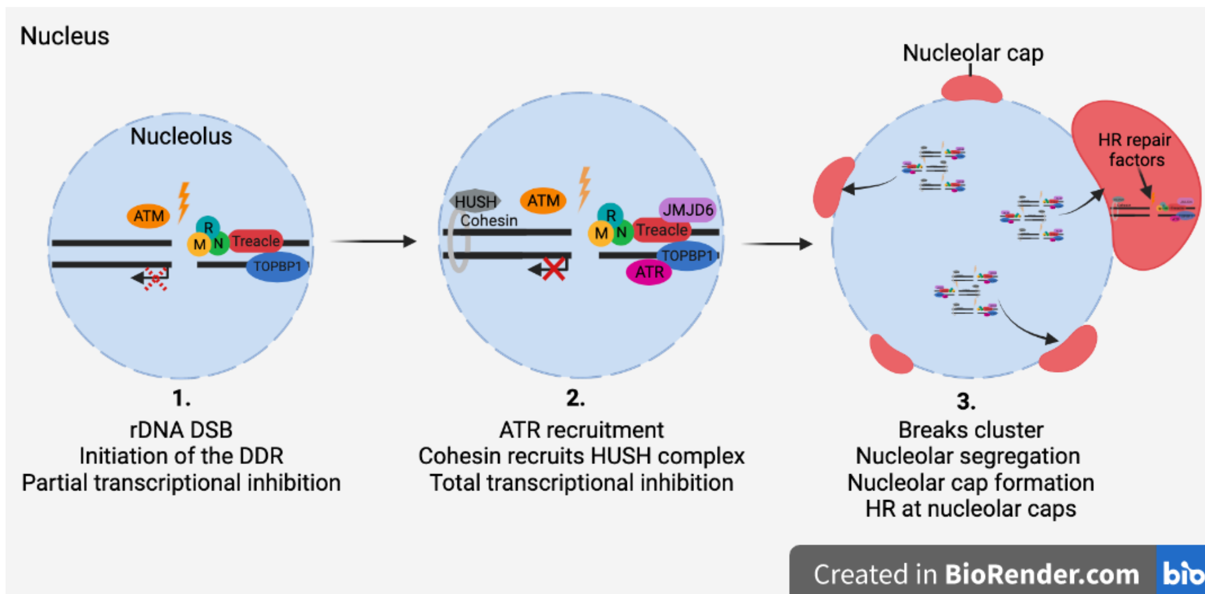

Figure 4: Model of repair of persistent rDNA DSBs in the nucleolus. ***1.*** Once a rDNA DSB occurs, ATM is recruited to the DSB by the MRN complex. Treacle further allows to accumulate NBS1 which increases ATM recruitment. This leads to an initial transcriptional inhibition. TOPBP1 is recruited to the DSB by Treacle along with NBS1 and ATM. ***2.*** This will lead to end processing and ATR activation. ATR and HUSH complex further repress the transcriptional activity of Pol I. ***3.*** Transcriptional inhibition enables DSBs to cluster, nucleolar segregation, and nucleolar cap formation. JMJD6 and the LINC complex (not shown) also play essential roles for nucleolar cap formation. Nucleolar caps allow the rDNA DSBs to be attainable by HR repair factors that colocalize to the nucleolar caps. Figure created with BioRender.com

Repair of rDNA DSBs through HR and NHEJ

To repair DSBs in rDNA, both NHEJ and HR can be used. When NHEJ repair is being used, this does not lead to ATM activation, or any major changes in transcription or nucleolar reorganization (Harding et al., 2015). It has been proposed that when there are persistent DSBs in rDNA, there could be a shift from NHEJ to HR. In that case, ATM does get activated which in turn leads to rDNA silencing, nucleolar reorganization, and formation of nucleolar caps (Harding et al., 2015). HR components and transcription factors are recruited at the nucleolar caps, where HR is suggested to occur (van Sluis & McStay, 2015). Also, HR factors are present at the nucleolar caps throughout each cell cycle phase (Harding et al., 2015; van Sluis & McStay, 2015). Nonetheless, it is still debated whether all the mechanisms behind the repair through HR are conserved in G1 compared to S/G2 in the nucleolus (Korsholm et al., 2020).

5. Discussion: Three Types of Repetitive DNA, Three Different DSB Repair Mechanisms

Proper repair of DSBs is essential to prevent cell death and the accumulation of mutations as well as to maintain the genomic stability of a cell, which is essential to prevent cancer formation (Khanna & Jackson, 2001; Jackson & Bartek, 2009). The repair of DSBs occurs in the context of chromatin organization and is tightly controlled both spatially and temporally (Clouaire & Legube, 2015; Tsouroula et al., 2016). In this review, we demonstrate that different chromatin regions will opt for different repair mechanisms depending on their chromatin organization, their location within the nucleus, or the cell cycle phase the cell resides in. We saw that the three repetitive regions, namely constitutive heterochromatin, rDNA, and centromeres, all rely on spatial temporal control for DSB repair. Yet, these three regions respond differently to DSBs in terms of movement of the DSBs and choice of repair pathway.

DSBs in Repetitive Regions Undergo Spatial and Temporal Control for Repair

Constitutive heterochromatin, rDNA, and centromeres are the three regions composed of highly repetitive DNA sequences. Yet, repairing DSBs in repetitive DNA makes them prone to aberrant recombination, chromosomal rearrangements, and therefore genomic instability (Barra & Fachinetti, 2018; Kendek et al., 2021; Ramsden & Nussenzweig, 2021). This is because in repetitive regions many homologous sequences can be used as templates for HR, leading to potential template switching (Anand et al., 2014). To overcome this challenge, the three regions mentioned here all rely on movement of DSBs. Heterochromatic DSBs relocate to the heterochromatin domain or nuclear periphery (Chiolo et al., 2011; Ryu et al., 2015). Centromeric DSBs relocate outside of the centromeric repeats (Tsouroula et al., 2016). rDNA DSBs move to the nucleolar periphery where nucleolar caps are formed (Kruhlak et al., 2007; van Sluis & McStay, 2015). The movement of DSBs to the domain's periphery is thought to isolate the DSBs from the rest of the repetitive sequences for safe repair. Therefore, relocalization is thought to reduce the risks of template switching, and thus, avoid aberrant recombination.

The process of relocalization of DSBs has been associated with HR (Chiolo et al., 2011; Tsouroula et al., 2016; van Sluis & McStay, 2015). However, whether NHEJ is also associated with relocalization remains to be determined. The repair of DSBs through relocalization occurs in a two-step process, before and after DSB relocalization (Chiolo et al., 2011). Before relocalization, the DSBs are recognized, and end resection is initiated. At this stage, HR is blocked while the DSB is still located in the domain of origin. Upon DSB movement to their respective periphery, the HR block is lifted, which allows for the finalization of repair (Chiolo et al., 2011). However, it remains unclear what process allows the HR blockage to be lifted so that HR can resume. In addition to this process, centromeres specifically rely on R-loop formation to facilitate DNA-end resection in G1 (Yilmaz et al., 2021). On the other hand, rDNA DSB relocalization in the presence of persistent DSBs relies on Pol I transcriptional inhibition as it is a required step for nucleolar segregation and cap formation, and thus for HR (Kruhlak

et al., 2007; Harding et al., 2015; van Sluis & McStay, 2015). However, it is still unclear how the clusters of rDNA DSBs exactly relocate to the nucleolar periphery. Therefore, it would be of interest to research whether proteins such as Smc5/6 and SUMOylation could drive rDNA DSBs relocation similarly to heterochromatin DSBs. Together, the process of DSB repair through HR in constitutive heterochromatin, rDNA, and centromeres is controlled through space and time and relies on movement of the DSBs. Yet, it remains unclear whether all mechanisms of repair and relocation are conserved between species. It would especially be of interest to investigate whether this process is conserved in human cells. Each region has developed specific mechanisms for its repair and the exact processes that coordinate the repair of each one of these regions need to be further investigated. Nonetheless, using the findings within one region and applying them to one of the other regions can be used to better understand the similarities and differences in repair in these different regions.

Recent research also points to the role of phase separation in repair. Heterochromatin domains and the nucleoli are both formed under the biophysical properties of phase separation (Feric et al., 2016; Larson et al., 2017; Strom et al., 2017). Phase separation is hypothesized to affect the accessibility of repair proteins by either retaining or excluding certain factors from the phase separated domain (Janssen, Colmenares & Karpen, 2018). The exclusion or retention of certain repair proteins could also influence the repair pathway of choice or the kinetics of repair. Thus, while factors responsible for resection could initially be recruited in the domain for HR, other proteins such as for NHEJ or RAD51 could be actively excluded. It has also been hypothesized that certain changes in the biophysical properties (i.e., heterochromatin expansion), would initiate later HR events such as DSB movement (Rawal, Caridi & Chiolo, 2019). Therefore, phase separation could be an important factor in DSB repair and would require further research to understand how changes in the biophysical properties affect stages of repair and protein accessibility. Phase separation could promote spatial and temporal control on the accessibility of repair factors to the break site. This could facilitate correct repair and avoid aberrant recombination.

A limitation of certain research when investigating the mechanisms behind the repair of DSBs is the method they use to induce DSBs. In certain cases, there is no knowledge of how many DSBs were induced and where they occurred. This makes it hard to really understand the repair of DSBs in each specific chromatin region individually as DSBs could also be induced in other regions at the same time. To overcome this problem, future research should concentrate on using a diploid model system where single targeted DSBs can be induced. For example, such a system has already been developed to study DSBs in heterochromatin and euchromatin in *D. melanogaster* (Janssen et al., 2016). The use of systems that induce DSBs at specific loci would allow further investigation of the temporal and spatial dynamics occurring during DSB repair in a genomic region of interest. Moreover, these systems could be used to further elucidate which proteins are recruited at the DSBs and facilitate the relocalization of DSBs through Chromatin Immunoprecipitation for example. Together, the use of systems that induce single DSBs at specific loci would improve the understanding of the DDR and how the DDR varies between chromatin regions.

Repair Pathway Choice

The choice of DSB repair pathway and the mechanisms behind the repair have been shown to be influenced by the chromatin context in which the DSB occurred (Clouaire & Legube, 2015). In constitutive heterochromatin, rDNA, and centromeres, both HR and NHEJ are used. Moreover, the choice of repair pathway can also be influenced by the cell cycle phase (Symington & Gautier, 2011). In general, for DSBs occurring in G1, it has been demonstrated that HR will be blocked and NHEJ will be preferred (Orthwein et al., 2015). This is notably the case for the repair of heterochromatic DSBs but not for rDNA or centromeric DSBs (Tsouroula et al., 2016; van Sluis & McStay, 2015). To repair heterochromatic DSBs in S/G2, it has often been thought that the main pathway of choice would be HR. However, recent work demonstrates that heterochromatin relies both on HR and NHEJ for DSB repair (Janssen et al., 2016; Tsouroula et al., 2016). On the other hand, pathways to repair centromeric and rDNA DSBs are not restricted by the cell cycle phases. For both of these repetitive regions, HR repair factors are able to be recruited even in G1 (Harding et al., 2015; van Sluis & McStay, 2015; Tsouroula et al., 2016). The process of end resection inhibition in G1 must not be active in both of these regions as end resection has been observed both at rDNA and centromeres DSBs in G1 (Tsouroula et al., 2016; Mooser et al., 2020). However, in G1, the sister chromatid cannot be used as a template. Thus, for HR to occur in G1, the DSB repair is hypothesized to be templated in *cis* or it could rely on repeats from the same NOR as template for repair (van Sluis & McStay, 2015). Therefore, to allow the use of HR to repair centromeric and rDNA DSBs throughout each one of the cell cycle phases in contrast to heterochromatic DSBs, this would be a result of their individual chromatin contexts. Future research would be needed to investigate how different components of those chromatin regions (e.g., CENP-A presence) influence the ability to perform HR in G1 and whether those components are essential for HR to occur in G1. The reliance on HR in G1 could be a necessity to maintain centromeric integrity and NOR genomic stability (van Sluis & McStay, 2015; Yilmaz et al., 2021).

Altogether, in this report, we gave an overview of the current knowledge in the field of DSB repair in the context of repetitive DNA. We demonstrate that chromatin context influences repair mechanisms. Each chromatin region has a unique built-in system to respond to DSBs. These responses safeguard the genome and avoid aberrant recombination, especially in the context of repetitive DNA regions. Nonetheless, a better insight of how each chromatin context is repaired will allow us to get a clearer understanding of the processes that occur to maintain genomic stability. Genomic instability is one of the hallmarks of cancer. Therefore, having in-depth knowledge of repair mechanisms in different chromatin contexts will give us a better overview of the mechanisms that potentially drive cancer development. This could in the long term enable the development of new anti-cancer therapies.

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