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Journal:	<i>Biochemistry and Cell Biology</i>
Manuscript ID	bcb-2016-0085.R2
Manuscript Type:	Invited Review
Date Submitted by the Author:	14-Jul-2016
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Keyword:	Nuclear Organization, DNA repair, Genome Stability

A decade of understanding spatio-temporal regulation of DNA repair by the nuclear architecture

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Keywords: Nuclear periphery, DNA damage, Chromatin, Nuclear bodies

Running title: DNA repair in the context of Nuclear organization

Abstract

The nucleus is a hub for gene expression and is a highly organized entity. The nucleoplasm is heterogeneous due to the preferential localization of specific metabolic factors, which led to the definition of nuclear compartments or bodies. The genome is organized into chromosome territories, heterochromatin and euchromatin domains. Recent observations indicate that nuclear organization is important for maintaining genomic stability. For example, nuclear organization has been implicated in stabilizing damaged DNA, repair pathway choice and in preventing chromosomal rearrangements. Over the past decade, several studies have revealed that dynamic changes in the nuclear architecture are important during double-strand break repair. Stemming from work in yeast, relocation of a damaged site prior to repair appears to be at least partially conserved in multicellular eukaryotes. In this review, we will discuss genome and nucleoplasm architecture, particularly the importance of the nuclear periphery in genome stability. We will also discuss how the site of relocation regulates repair pathway choice.

Introduction

The nucleus is heterogeneous and its organization involves the dynamic three-dimensional arrangement of the genome and nuclear bodies. The genome has all the information necessary to build and maintain life, thus preserving genomic integrity is essential for the continuation of all species. The cell nucleus, where the genome resides, is a complex organelle with dynamic internal architecture involving numerous nuclear bodies that compartmentalizes the nuclear space, creating distinct environments within the nucleus called nuclear bodies (Dundr and Misteli 2001; Misteli 2007). The underlying mechanism(s) by which nuclear bodies function are highly diverse. In some cases, a nuclear body may be host to a particular activity such as transcription; in other cases, it acts indirectly by regulating the local concentration of components in the nucleoplasm. However, in contrast to conventional cytoplasmic organelles, nuclear bodies are not delineated by a lipid membrane as their structural integrity appears to be entirely mediated by protein–protein and possibly protein–RNA interactions.

Efforts to understand how the genome is regulated initially emerged from investigations on supposedly distinct processes within the nucleus including transcription, replication, DNA repair and mutagenesis. Currently, a more encompassing view of how these nuclear events are integrated has emerged as collectively a body of work suggests that genome organization not only regulates functionality, but unites and has an integral role in each of these processes. Regarding DNA repair, two observations coming from work in *Saccharomyces cerevisiae* in the last decade identified that damaged genomic loci are relocated to permissive repair compartments (Torres-Rosell et al. 2007; Nagai et al.

2008). Indeed, the spatial organization of the cell nucleus plays a primary role in genome maintenance where microenvironments impact how DNA damage is detected and which pathway is utilized in repair. Here we describe some of the recent advances that have provided understanding to DNA repair in the context of nuclear organization.

Genome organization and DNA repair

Traditional approaches for studying genome organization were dominated by electron and light microscopy to describe, at increasingly higher resolution, the arrangement of genes and chromosomes within the nucleus. These efforts led to the realization that genomes are, in fact, spatially arranged at several, hierarchical levels in three dimensional space. Genome organization initiates from the folding of chromatin fibers into higher-order structures, that involve the formation of loops and chromosome domains, which culminate in chromosome territories (Schardin et al. 1985; Cremer et al. 1993; Bystricky et al. 2004; Bystricky et al. 2005). Additionally, it has been recognized that chromosomes are non-randomly arranged in the nucleus, with many genes occupying preferred positions relative to other regions in the genome or to nuclear structures/compartments (Fig. 1) (Zimmer and Fabre 2011; Lanctôt et al. 2007; Misteli 2007; Rajapakse and Groudine 2011).

Several studies on the yeast *Saccharomyces cerevisiae* permitted the establishment of model-maps of the chromosome organization inside the nucleus and the corresponding described nuclear compartments (Bystricky et al. 2005; Lassadi and Bystricky 2011; Berger et al. 2008; Duan et al. 2010; Zimmer and Fabre 2011). More recent work, where the authors labelled single specific loci on the yeast's chromosome XII, permitted the definition of gene territories and revealed a dynamic aspect for the

chromosome organization (Albert et al. 2013). The 16 centromeres of the haploid yeast chromosomes cluster at the spindle pole body (Q. -w. Jin 1998; Q. W. Jin, Fuchs, and Loidl 2000), and the 32 telomeres localize at the nuclear periphery in five to eight clusters (Gotta et al. 1996; Laroche et al. 1998), whereas the ribosomal DNA (rDNA) localizes opposite the centromeres in the yeast's single crescent-shaped nucleolus (Fig. 2) (Gotta et al. 1997; Albert et al. 2013).

The topology of the genome is dynamic and can change depending on cell cycle phase, transcriptional states, and the persistence of DNA damage (Bernardi 2015; Dekker and Mirny 2016). For example, large blocks of active chromatin (3-5 Megabases on average) associate with other active chromatin domains, supposedly for more efficient transcription, while inactive chromatin regions associate with other inactive regions (Lieberman-Aiden et al. 2009; Zhang et al. 2012). Also, it was seen in yeast that the activation of some genes led to their relocalization to the nuclear pores (Taddei et al. 2006). This association can be considered as a way to facilitate the expression of a gene by combining transcription, processing and export at the nuclear pores, which supports the initial hypothesis of gene gating at the nuclear pores (Blobel 1985).

Genomic organization influences DNA damage detection and repair (Soutoglou and Misteli 2007; Mekhail and Moazed 2010; Nagai, Heun, and Gasser 2010; Nagai, Davoodi, and Gasser 2011). Damaged DNA, such as a double-strand break (DSB), is repaired to avoid chromosomal rearrangements and genomic instability. In contrast to non-homologous end joining (NHEJ), homology mediated repair involves resection of the 5' ends of DNA at the break site through enzymatic degradation, resulting in 3' ends

of single stranded DNA (ssDNA) (for review see (Symington 2016)). These 3' ssDNA tails then recruit and bind the homologous recombination (HR) protein, Rad51. Once bound, Rad51-coated ssDNA starts scanning in search of a homologous donor to repair from. It remains to be determined how the homology search proceeds *in vivo* and what controls if the search is confined to a particular area or if/when it can sample a larger nuclear space (review see (Renkawitz, Lademann, and Jentsch 2014)) .

Repair following resection involves a physical contact between broken ends and homologous regions. In yeast, repair of the DSB via homology search is predominately intra-chromosomal (Agmon et al. 2009). The linear distance of a homologous donor contributes to the site of D-loop formation. However, the organization of the chromosome within the nucleus also influences repair efficiency for two main reasons: the search for homology is driven by proximity and each chromosome occupies a specific territory in the nucleus. It is also known that intra-chromosomal interactions are more frequent than inter-chromosomal events (Duan et al. 2010), and if the latter occurs, they are most common between chromosomes in close spatial proximity to one another. Therefore, a central determinant of repair is the proximity of a homologous donor or the vicinity of a partner site in the case of a translocation (Sachs et al. 1997). Similarly there is lower efficiency repair between spatially distant regions of the genome (Reviewed in Parada and Misteli 2002)(Roukos et al. 2013).

The Nuclear Periphery: silencing chromatin and mediating repair

The nuclear periphery of higher eukaryotes is characterized by the presence of a meshwork of intermediate filament proteins called lamins. The lamins enclose the genome of higher eukaryotes and provide anchor points for chromatin at the nuclear

periphery (for details see (Burke and Stewart 2012). The lamins interact with the chromatin through large genomic areas, termed lamina-associated domains (LADs) (Fig. 1). LADs are known for low gene-expression levels, thus representing a repressive chromatin environment. The borders of LADs are demarcated by the insulator protein CTCF, by promoters oriented away from LADs, or by CpG islands (Guelen et al. 2008). Lamins A and C interact with the telomere-repeat binding factor 2 (TRF2) (Wood et al. 2015) and defects in *LMNA*, the gene encoding Lamins A and C, result in accelerated telomere shortening (Allsopp et al. 1992; Decker et al. 2009). Moreover, lamin defects result in aberrant DNA repair, such as delays in recruiting 53BP1 and RAD51 (Liu et al. 2005; for review see Gonzalo and Kreienkamp 2015).

Although lamins are not present in plants or lower eukaryotes like *S. cerevisiae*, many proteins of the nuclear membrane are present, suggesting evolutionary conservation of the periphery in nuclear organization. Nuclear membrane proteins are generally linked to transcriptional silencing, however the nuclear pore complexes (NPC) has been linked to both transcriptionally inactive and active loci in different organisms (for details see (Mekhail and Moazed 2010). Also, it was seen in yeast that the activation of some genes led to their relocalization to the nuclear pores (Taddei et al. 2006) (Fig. 2).

A connection has been established between chromosome positioning at the nuclear periphery and genome stability (Therizols et al. 2006; Nagai et al. 2008; Kalocsay, Hiller, and Jentsch 2009; Oza et al. 2009; Schober et al. 2009).

In yeast cells, telomeres gather at the nuclear periphery in 5-8 foci (M Gotta et al. 1996). Their distribution is not static as they are not anchored, but they show less subnuclear mobility than bulk chromatin (Nagai, Heun, and Gasser 2010). Organization

of telomeres at the periphery is driven by partially redundant pathways involving Sir4 binding to membrane bound Esc1 and both Yku70/80 and Sir4 interacting with Mps3 in S phase (Hediger et al. 2002; Taddei et al. 2004; Horigome et al. 2011) (Fig. 2). The organization of telomeres impacts various aspects of telomere homeostasis, including length regulation and prevention of inappropriate recombination (Schober et al. 2009; Taddei et al. 2009). Telomere length is maintained by telomerase, which offsets the continual loss of telomere sequence during DNA replication. Mammalian cells respond to short telomeres by activating Ataxia Telangiectasia Mutated (ATM, Tel1 in yeast) and initiating replicative senescence (Herbig et al. 2004). The rescue of short telomeres by a HR –dependent mechanism signifies that a short or uncapped telomere is recognized by the repair machinery as a DSB. The repair of this DSB initiates with resection and the formation of Rad51-coated ssDNA followed by searching for a homologous donor. In order to prevent genomic rearrangements and telomere fusions, these eroded telomeres remain at the nuclear periphery, but move to the NPCs (Khadaroo et al. 2009). Relocation plays an important role in preventing chromosome fusions and maintaining genomic stability by separating the naked telomeric DNA from the highly compacted yeast genome.

Repair at the Nuclear Periphery

The nuclear pore complexes (NPC) enable the exchange of molecules between the cytoplasm and the nucleus across the nuclear envelope (NE). Each NPC consists of ~30 different proteins termed nucleoporins (Nups) that are highly conserved from yeast to humans (Rout et al. 2000; Cronshaw et al. 2002). The structure of the NPC has been recently visualized using three-dimensional cryo-electron microscopy (von Appen and

Beck 2016). The NPC consists in one central structure, known as the scaffold ring complex localized in the plane of the nuclear envelope and two peripheral extensions: the cytoplasmic filaments and the nuclear basket. The nuclear basket extends the NPC domains into the nucleus (Niño et al. 2016).

In addition to its role in nucleo-cytoplasmic exchange, the nuclear basket substructure of the NPC is emerging as an important coordinator of diverse nuclear functions. It is implicated in genome architecture, gene expression, mRNA surveillance and the DNA-damage response (DDR) and repair (Bermejo et al. 2011; Guet et al. 2015) (for further understanding see (Ptak, Aitchison, and Wozniak 2014))

The nuclear basket in *S. cerevisiae* consists of five proteins including Nup60, Nup1, Nup2, and two myosin-like proteins, Mlp1 and Mlp2, whereas the basket in vertebrates is comprised of Nup153 (which is likely the combined functional equivalent of Nup1 and Nup60), Nup50 (Nup2 homolog), and Tpr (homolog of the Mlps) (Strambio-de-Castillia, Blobel, and Rout 1999; Dilworth et al. 2001). Recently, it was shown that DNA damage promotes the monoubiquitylation of Nup60, which stabilizes its interactions with the NPC and functions in the DNA damage response (Niño et al. 2016). The human homologue, Nup153, also plays an important role in activating the DNA damage checkpoints (C Lemaître et al. 2012), and in regulating the nuclear import of 53BP1 in response to damage (Moudry et al. 2012)..

Studies in *S. cerevisiae* indicate that damage is targeted to at least two sites at the periphery, the NPC and the SUN domain containing protein, Mps3 (Schober et al. 2009; Oza et al. 2009; Lemaître and Soutoglou 2015; Su et al. 2015), and the site of relocation influences the pathway of repair (Fig. 2). For example, nuclear pores mediate

repair through pathways distinct from canonical HR. Support for this comes from monitoring Rad52 foci formation, which suggested sites of active HR are in the nuclear interior, and specifically excluded from the nuclear envelope, and the nucleolus (Bystricky et al. 2009; Dion et al. 2013). Moreover recently, the NPC was identified as a site of repair by break induced replication (BIR) and relocation of damage to the pore is mediated by the kinesin-14 motor protein complex (Cik1-Kar3) and chromatin remodelers (discussed further below) (Chung et al. 2015). Relocation of damage to the NPC also requires the Nup84 complex, which includes Nup84, Nup120, Nup133 and the associated Nup60 protein (Nagai et al. 2008; Khadaroo et al. 2009), and occurs in G1, without the need of extensive resection (Horigome et al. 2014). Tracking the localization of induced DSBs lacking an intact homologous donor or resulting from repeated cleavage/ligation cycles, revealed a shift of the unrepaired loci to the NPC within 2 hours of induction (Nagai et al. 2008). The loss of the Nup84 complex or its associated Slx5/Slx8 SUMO targeted ubiquitin ligase (STUbL), compromised survival after replication fork collapse and enhanced ectopic recombination (Nagai et al. 2008; Oza et al. 2009; Horigome et al. 2014). Slx5 harbors two small ubiquitin-like modifier (SUMO) recognition motifs and Slx8 harbors a RING domain and ubiquitin ligase activity (Mullen et al. 2001; Nagai et al. 2008).

Mps3, which is a member of the Sad1-Unc-84 (SUN) domain family of proteins, is also located at the nuclear envelope, but at a location different from the NPC (Fig. 2). Mps3 sites appear to sequester Rad51-coated ssDNA filaments and reduces promiscuous recombination events during S and G2 phases of the cell cycle (Kalocsay, Hiller, and Jentsch 2009; Oza et al. 2009; Ferreira et al. 2011). In contrast to the NPC, which not

only favors BIR, but repair by imprecise end joining, repair at the Mps3 location appears to be mediated by HR (Horigome et al. 2016). Factors known to regulate which site a break shifts to include the cell cycle phase and a differential requirement for the INO80 and the SWR1 complex (SWR-C) chromatin remodelers (Horigome et al. 2014). INO80 and the SWR-C are sequentially recruited to breaks (reviewed in Peterson and Almouzni 2013). SWR-C is required for binding to the NPC and Mps3, however INO80 assists binding to only Mps3. Regarding the cell cycle phase, DSBs bind to pores in G1, S, and G2, but associate with Mps3 only in S and G2 (Horigome et al. 2014). The absence of homology also is a major determinant for the destination of damage relocation at the periphery which is consistent with G1 events at the NPC and SWR-C regulating repair by NHEJ (van Attikum, Fritsch, and Gasser 2007).

It has also been observed, in what is more likely a physiologically relevant system in proliferating cells, that collapsed or stalled replication forks in regions of expanded triplet repeats also shift to the nuclear periphery as repair at these loci are favored by mechanisms other than precise end-joining or canonical HR (Su et al. 2015). In contrast to induced-DSB, stalled forks in triplet repeats associate transiently with the nuclear pores in late S/G2 phase but are not associated with Mps3. Highlighting the functional significance of nuclear pore association, the frequency of triplet repeat expansion/contraction events increase when Slx5 or Slx8 at the NPC is disrupted (Su et al. 2015).

Relocation of an irreparable DSB to the periphery depends on SUMOylation events that are mediated by the E3 ligases Siz2 (PIAS in humans) and the SMC5/6-associated NSe2/Mms21 (Nagai et al. 2008; Ryu et al. 2015 Horigome et al. 2016). Relocation of

damage to the NPC in G1 is regulated by polySUMOylation and Slx5. However, in S phase, monoSUMOylation mediated by Mms21 is sufficient to drive DSBs to Mps3, independently of Slx5. From the above observations, it would be predicted that polySUMOylation events at sites of damage preserve triplet repeat regions via BIR or imprecise end joining at the NPC.

Repair in Heterochromatin links with the nuclear periphery

In *Saccharomyces cerevisiae*, heterochromatin is limited to the *HM* silent mating-type loci (*HML* & *HMR*), regions within the ribosomal DNA (rDNA), and some telomeres. The silencing complex composition and mechanisms are different for the three regions. Telomeres and the *HM* loci are similar to each other, whereas the rDNA locus has distinct features (Wierman and Smith 2014).

Pericentromeric heterochromatin occupies about 30% of the human and fly genomes and is characterized by contiguous stretches of repeated sequences, the transposons and satellite repeats. Epigenetically, heterochromatin is characterized by hypoacetylation of the histone tails and the presence of trimethylation on lysine 92 and 27 of histone H3 (H3K9me3, H2K27me3) and the presence of heterochromatin protein 1 (HP1). HP1 binds to H3K9me3 and helps in higher order chromatin packing (Campos and Reinberg 2009; Luijsterburg et al. 2009; Riddle et al. 2011; Ho et al. 2014). Pericentromeric heterochromatin is absent in budding yeast, however in multicellular eukaryotes it represents chromatin that is predominantly transcriptionally inactive and is a major threat to genome stability (Chiolo et al. 2013).

The sensitivity of DNA to damage and the kinetics of repair are believed to be affected by the level of chromatin compaction. This compaction presents an obstacle for various

DNA template processes, including DNA repair (Araki et al. 2000; Ura and Kaneda 2001). One possible mode of protection is that chromatin proteins may act as radical scavengers, reacting with unstable radical species produced by radiation (Lee et al. 2012). These proteins also push water molecules away from tightly packaged DNA, perhaps reducing damage from ionized hydroxyl radicals. These observations may have interesting implications for the mechanisms leading to DNA damage in embryonic and adult stem cells, which have comparatively more open chromatin structures than their differentiated counterparts (Meshorer and Misteli 2006; Bártová et al. 2008).

Heterochromatin represents a physical barrier. For repair to proceed, Kap-1, the heterochromatin formation protein, must be phosphorylated by ATM. Kap-1 phosphorylation mediates the release of CHD3, a chromatin remodeling factor, resulting in chromatin relaxation in the vicinity of the lesion to aid repair (Goodarzi et al. 2008; Goodarzi, Kurka, and Jeggo 2011; Goodarzi and Jeggo 2012).

Very interesting insights about the repair kinetics of DSBs in heterochromatin have come from studies in *Drosophila* cells. Early HR steps, involving the recruitment of *Mus304/Mus101* (homologues of the human ATRIP/TopBP1) and resection, occur quickly within heterochromatin. However, subsequent steps, including DmRad51 loading, occur only after the damaged site is relocated to outside the heterochromatic domain (Chiolo et al. 2011; Dronamraju and Mason 2011) (Fig. 1). Relocation has also been observed in mouse cells where a DSB within heterochromatin first leads to rapid γ H2AX formation, then relocation of the damage to the periphery of the heterochromatic domain. The authors also demonstrated that relocalization is independent of ATM kinase activity (Jakob et al. 2011) (Fig. 1). The functional significance of this movement

is to prevent aberrant recombination, by separating damaged DNA from similar repeats on non-homologous chromosomes, and promote safe exchanges with the sister chromatid or homologous chromosome (Chiolo et al. 2013; Chiolo et al. 2011). As relocation is documented in both mouse and *Drosophila* cells it is likely a conserved mechanism in repair.

The Smc5/6 complex, which is a member of the structural maintenance of chromosome (SMC) protein complex family is present at heterochromatin and is required to exclude Rad51 from the heterochromatic regions (Chiolo et al. 2011). This confers a role for the Smc5/6 complex in regulating the important step of strand invasion, as it prevents Rad51 filament formation and abnormal recombination within the heterochromatic domain.

There are many parallels between moving damage outside the heterochromatin domain prior to HR mediated repair in multicellular eukaryotes and the relocation of damage in yeast. HR is the preferred pathway of repair in *Saccharomyces cerevisiae* and SUMOylation mediates the relocalization of DSBs in rDNA to outside the nucleolus and the movement of persistent DSBs to the nuclear periphery (Torres-Rosell et al. 2007, Kalocsay, Hiller, and Jentsch 2009; Horigome et al. 2016). In the Smc5/6 complex, non-structural maintenance of chromosomes element 2 (Nse2, NSMCE2 in higher eukaryotes), is a SUMO E3 ligase and required for the DNA damage response (Andrews et al. 2005; Potts and Yu 2005; Zhao and Blobel 2005). In *Drosophila*, it is enriched in heterochromatic domains in the absence of damage (Chiolo et al. 2011). Interestingly, Nse2 is recruited in a similar manner to other early repair components, to DSBs foci inside the heterochromatin, as seen by its colocalization with the DSB

markers γ H2Av and TopBP1 (Ryu et al. 2015). Nse2 focus formation depends on the core subunits Smc5 and Smc6. Yeast Nse2/Mms21 shares numerous targets with the SUMO E3 ligases Siz1 and Siz2, homologues of *Drosophila* dPIAS (Albuquerque et al. 2013; Hari, Cook, and Karpen 2001). dPIAS is also recruited to heterochromatic repair foci with kinetics similar to ATRIP/TopBP1 and this recruitment seems to precede the spreading of γ H2Ax (Ryu et al. 2015). This suggests important and early functions of the SUMO ligases Nse2 and dPIAS at heterochromatic DSBs. Similar to yeast, the nuclear periphery appears to be a specialized site for repairing heterochromatic DSBs in *Drosophila* (Ryu et al. 2015). Binding of damaged sites to the nuclear periphery is mediated by interactions of Smc5/6 and with the nuclear periphery components, STUbL/RENi (Ryu et al. 2015). These findings identify the nuclear periphery as an important repair environment for heterochromatic DSBs in *Drosophila* and highlight the similarities between anchoring heterochromatic DSBs in *Drosophila* and irreparable-persistent DSBs in yeast at the nuclear periphery (Ryu et al. 2015; Horigome et al. 2016). Moreover, the importance of SUMOylation and SMC5/6 in preventing HR in a vulnerable environment and in relocating damage to a preferred site appear to be a conserved mechanism for repair, which underscores their role in maintaining genomic stability by preventing illegitimate rearrangements.

Repair in the nucleolus and connections with genome stability and disease

The nucleolus, a major hallmark of nuclear organization, is a non-membrane-bound nuclear organelle found in all eukaryotes. Its structure is functionally compartmentalized into three components defined by electron and light microscopy: The fibrillar centers (FCs) surrounded by dense fibrillar components (DFCs) embedded in a granular

component (GC). The FC contains pools of unengaged RNA Polymerase I (RNA Pol I) transcription factors such as the upstream binding factor (UBF), whereas the DFC contains early pre-RNA processing factors. Transcription is believed to occur at the border of these two regions (Raska, Shaw, and Cmarko 2006).

Saccharomyces cerevisiae relies on one crescent-shaped nucleolus formed by approximately 150 tandem copies of a 9.1 kb rDNA that occupies approximately 1.5 Mb region of chromosome XII. This cluster covers about 60% of chromosome XII and about 10% of the whole yeast genome (T Kobayashi et al. 1998), and the nucleolus itself occupies nearly one third of the nuclear volume. In human cells, there are 10 rDNA clusters or nucleolar organizing regions (NOR), located on the 5 acrocentric chromosomes 13, 14, 15, 21 and 22. Each cluster contains ~70 copies, bringing the total number of rDNA gene copies to ~700 (~30Mbps), which represents less than 0.5% of the diploid genome (Sakai et al. 1995).

The rDNA repeats within the nucleolus are found in two forms: an open, highly transcribed conformation and in a silenced heterochromatin state (Drygin et al. 2011; Grummt and Längst 2013). The transcription of the active rDNA copies is mediated by RNA Pol I and a large number of co-factors in the nucleolus (Grummt 2013). This led to the description of the nucleolus as a ribosome factory as it is the site of transcription and maturation of ribosomal RNAs (rRNAs), and pre-ribosome assembly prior to export in the cytoplasm. Nucleolar proteins and non-coding RNAs (ncRNAs) with non-ribosomal roles have also been localized to the nucleolus (Vitali et al. 2005; Hutten et al. 2011), however their functional role remains to be clearly identified.

The stability of the rDNA and nucleolar function are important for cell viability and to prevent cancer. When the stability is reduced, some cellular functions are affected (Takehiko Kobayashi 2008), and one of its prominent effects can be cellular senescence. It has been shown in yeast that mutants with unstable rDNA are known to have a short lifespan. In humans, over half of the adult solid tumors show detectable rDNA rearrangements relative to either surrounding non-tumor tissue or normal peripheral blood (Sakai et al. 1995). This makes rDNA instability one of the most common chromosomal alterations in adult solid tumors. In addition, increased mitotic instability of rDNA has been found in at least 50% of human colon or lung cancer samples (Stults et al. 2009). Finally, genomic instability disorders including ataxia telangiectasia and Bloom syndrome have been linked to very high rates of mitotic rDNA cluster length instability (Killen et al. 2009).

Due to the repetitive structure and high transcription level, rDNA repeats are some of the most fragile sites in the genome. As mentioned, DSBs in the rDNA are transiently relocated outside of the nucleolus for repair as HR is actively excluded from this nuclear body (Torres-Rosell et al. 2007). In mammals, Bloom syndrome patients, which lack functional BLM, display increased rates of sister chromatid exchange and have highly unstable rDNA arrays with significant genomic rearrangements (Killen et al. 2009).

Silenced nucleolar heterochromatin presents an obstacle to DSB repair. Silenced rDNA repeats share epigenetic features similar to that of constitutive heterochromatin, and are highly condensed (Guettg and Santoro 2012). Thus characterizing pathways of heterochromatin repair are directly relevant to understanding repair in the nucleolus.

There are however, some unique outcomes that are specific to nucleoli. For example, DSB induction directly inside the nucleoli of primary mouse embryonic fibroblasts (MEFs) using laser micro-irradiation inhibits rRNA synthesis. Inhibition does not appear to spread into surrounding nucleoli suggesting down-regulation of transcription by RNA Pol I transcription is in *cis*, and within close proximity to the breaks. Cells irradiated with 10 Gy also displayed segregation of UBF, a transcription factor that is important in maintaining euchromatin, into nucleolar caps, a hallmark of transcriptionally inactive cells (Kruhlak et al. 2007; Shav-Tal et al. 2005). The Pol I transcription is not blocked directly by damage, but is controlled by ATM that is present in the nucleoli prior to damage, but which localizes to nucleolar caps when activated (van Sluis and McStay 2015). Data collected after γ -irradiation of repair-deficient knock-out MEFs showed that two DNA damage response (DDR) adaptor proteins, NBS1 and MDC1, were also required for transcription inhibition, whereas KU, BRCA1, 53BP1 and H2AX were dispensable (Kruhlak et al. 2007). Important information extracted from mathematical modeling of fluorescence recovery after photobleaching (FRAP) experiments suggest that the molecular mechanisms leading to RNA Pol I inhibition under local damage involve inhibition of initiation complex assembly as well as progressive displacement of elongating Pol I holoenzyme from DNA. In a similar manner, Chromatin immunoprecipitation also showed loss of RNA Pol I association with actively transcribed rDNA regions upon γ -irradiation (Kruhlak et al. 2007). This model was also supported by results obtained using mass spectrometry after IR (Moore et al. 2011).

DSBs induced at non-nucleolar sequences also appear to impact rRNA synthesis inside the nucleoli.

Damage from laser micro-irradiation at any sub-nuclear region of the nucleus, revealed a pan-nuclear signaling mechanism that resulted in rRNA transcriptional silencing (Larsen et al. 2014). This silencing is associated with transient recruitment of Nijmegen breakage syndrome protein 1 (NBS1) into the nucleoli, which interacts with Treacle (referred to as TCOF1) (Larsen et al. 2014; Ciccio et al. 2014). Recent study showed that NBS1 accumulation in the nucleoli occurs in an MRE11 independent manner (Lafrance-Vanasse, Williams, and Tainer 2015), and indicates that one function of NBS1 might be in regulating RNA Pol II transcribed genes upon damage. Previous to this, NBS1 functions in response to DNA damage were only described in association with MRE11 and RAD50.

A very important question remained which was how DSBs that are generated between the rDNA repeats are most commonly repaired. A recent report, based on inducing DSBs inside the rDNA using I-Ppol, which has a 15 base-pair recognition site in the 28S coding sequence of each rDNA, suggested that non-homologous end-joining (NHEJ) is the privileged repair pathway of DSBs in rDNA (Harding, Boiarsky, and Greenberg 2015). The number of DSBs significantly increased, as well as exacerbated ATM dependent signaling, after usage of chemical inhibitors against DNA-PK, transient depletion of NHEJ factors by siRNA transfection and genetically defined mouse fibroblasts (DNA-PK^{-/-}).

No significant increase was seen after depletion or knock-out of homology directed repair (HDR) factors. Indeed, the high rate of transcription would benefit from faster repair via NHEJ and if this for some reason fails, perhaps the break is relocated

to the periphery of heterochromatin for repair.

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Figure 1. The nuclear architecture in mammalian cells and the relocation of heterochromatic DSBs.

The nucleoplasm is organized into nuclear compartments. This organization is important for maintaining a smooth cellular metabolism and division. The organization is dynamic and is known to change during metabolic processes. Several studies have and are revealing the functional importance of the nuclear organization. The nuclear envelope and the nuclear pore complexes are implicated in the repair of heterochromatic regions. The lamina is known to be a gene poor compartment with a limited number of Lamina associated domains (LADs). The nucleoli, which appear as dark spheres using DAPI staining are the sites transcription of the rDNA genes into rRNA and are known to be non-permissive for recombination events. Other nuclear compartments such as the Cajal bodies, nuclear speckles and paraspeckles are implicated in metabolic processes. The chromosomes are organized into territories, with limited inter-chromosomal interactions and favored intra-chromosomal. Silent regions of the chromosome can be organized as the stiff heterochromatin (red oval), DSBs induced inside the heterochromatin are repaired by homologous recombination which initiates inside the heterochromatic domain by the recruitment of ATRIP/TopBP1, Nse2, SMC5/6 and dPIAS. Repair by HR is blocked once the single stranded Rad51 filament is exposed. In *Drosophila*, the product is relocated to the nuclear periphery where the HR proceeds. This relocation of the highly “recombinable” Rad51 filament is to prevent unlawful recombination inside the heterochromatin and prevent genomic instability. In mouse

cells, the damage site relocates to the periphery of the heterochromatic domain. DSBs inside euchromatic domains (green oval) are most commonly repaired by NHEJ.

Figure 2. The relocation of some types of DSBs (and genes) to the NPC and Mps3 in *S. cerevisiae*

DSBs in the *S. cerevisiae* genome are preferentially repaired by HR. DSBs inside the nucleolus, unique and crescent shaped, are moved outside of the nucleolus in order to be repaired by the recombination machinery. This is supposed to be a self protection of the nucleolus from recombination between the rDNA copies that are present in over 100 copies. The telomeres are known to form 5-8 clusters that are mostly at the nuclear periphery. The telomeres are anchored to the inner nuclear membrane by Mps3. The telomere clusters are known to be repressive for transcription. DSBs that occur at sub-telomeric regions are repaired at the NPC. Also, the activation of some genes in yeast led to their relocation to the nuclear pores. Persistent irreparable DSBs are relocated to the nuclear periphery for repair. In G1/S phases of the cell cycle and by the activity of the chromatin remodeling complex SWR-C, the repair foci of the DSBs are relocated to the NPCs supposedly for repair by NHEJ. However, DSB that occur during S/G2 phases are recruited to Mps3 by SWR-C and INO80 chromatin remodeling complexes, for repair by HR. Stalled replication forks at tri-nucleotide repeats (TNR) are also relocated to the NPCs for repair.