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Review

Nucleolar DNA Double-Strand Break Responses Underpinning rDNA Genomic Stability

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Nucleoli, the sites of ribosome biogenesis, form around ribosomal gene (rDNA) arrays termed nucleolar organiser regions (NORs). These are the most transcriptionally active regions of the human genome and specialised responses have evolved to ensure their genomic stability. This review focuses on nucleolar responses to DNA double-strand breaks (DSBs) introduced into rDNA arrays using sequence-specific endonucleases, including CRISPR/Cas9. Repair of rDNA DSBs is predominantly carried out by the homology-directed repair (HDR) pathway that is facilitated by inhibition of transcription by RNA polymerase-I (Pol-I) and ensuing dramatic nucleolar reorganisation. Additionally, we review evidence that nucleoli can sense and respond to DSBs elsewhere in the genome.

Highlights

DNA DSBs within rDNA arrays illicit a specialised nucleolar DNA damage response.

Persistent rDNA DSBs result in Pol-I transcriptional inhibition and nucleolar reorganisation, resulting in the formation of nucleolar caps that contain rDNA arrays from individual nucleolar organiser regions (NORs).

Accessibility of damaged rDNA in nucleolar caps promotes HDR. HDR occurs even in G1 cells and it has been argued that it can be templated in *cis* by undamaged rDNA repeats present in the same NOR.

At subthreshold levels of DSBs repair is carried out by the nonhomologous end joining (NHEJ) pathway, without transcriptional inhibition or nucleolar reorganisation.

The role of nucleoli as major cellular stress sensors is revealed by their response to nonnucleolar DSBs.

DNA Damage Responses Are Nonuniform across the Nucleus

Maintaining genome integrity is critical for cell survival. The genome is constantly threatened by endogenous sources of damage, such as collapsed replication forks, collisions between replication and transcription machineries, incomplete separation of sister chromatids during mitosis, as well as exogenous sources, such as irradiation and **xenobiotics** (see Glossary) [1–3]. DNA double strand breaks (DSBs) are the most toxic form of DNA damage. To prevent damage accumulating, mammalian cells have evolved a complex network, collectively referred to as the DNA damage response (DDR) to sense and repair DSBs (Box 1). Additionally, the DDR can also activate checkpoints that pause cell cycle progression to facilitate repair. If levels of damage exceed the cells repair capacity, the outcome is either **senescence** or **apoptosis** [4].

Recent investigations have addressed whether DSBs located anywhere in the genome are sensed and repaired uniformly or whether DSBs at certain loci elicit specialised responses. In particular, DSBs in repetitive sequences such as telomeres, centromeres, or ribosomal gene (rDNA) arrays might pose additional challenges due to their repetitive nature and high potential for causing genomic instability [4–6]. In this review, we focus on recent progress on understanding the nucleolar response to DSBs within rDNA arrays and how nucleoli respond to DSBs elsewhere in the nucleus. We focus on mammals (primarily humans), where rDNA arrays have been identified as a hotspot for DSBs [7]. Moreover, instability in rDNA has been observed in cancer, premature aging syndromes, and neurodegenerative diseases [8–10]. Nucleolar size and morphology facilitates an exploration of how they deal with targeted DSBs using cell biology approaches. Furthermore, as nucleoli are major stress sensors within mammalian cells, how they deal with their own internal stress can have major downstream consequences on cell growth and proliferation [11]. Before describing the emerging strategies for targeting DSBs to rDNA arrays, we review relevant nucleolar biology. The nucleolar DNA DSB response is then reviewed from both cell biology and biochemical perspectives. Finally, we discuss the interplay between nuclear and nucleolar DSB responses.

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Box 1. Response to DNA DSBs

Cells have evolved elaborate pathways to respond to DNA damage. The Phosphatidylinositol 3-kinase-related (PIK) kinases DNA-PK, ATM, and ATR all respond to different kinds of DNA damage [75]. ATR is activated by ssDNA, which is exposed after replication stress. Recruitment of the Ku70/80 complex to DBSs activates DNA-PK, while binding of the Mre11–Rad50–Nbs1 (MRN) complex activates ATM. These kinases phosphorylate a large number of proteins initiating DSB repair and cell cycle arrest. Histone variant H2AX gets phosphorylated around the break site, referred to as γ-H2AX, providing a platform for recruitment of repair factors.

There are two main DSBs repair pathways; NHEJ and HDR [4,68]. In NHEJ, the ends of the break are processed and ligated. This pathway, involving DNA-PK and XRCC4, can be error prone resulting in small insertion or deletions (indels).

HDR requires 5′-end resection exposing a long stretch of ssDNA, which will invade in a homologous sequence serving as a template for repair. For most loci in the genome, the repair template is the sister chromatid and therefore HDR is most active in S and G2 phase of the cell cycle. NHEJ is the predominant repair pathway and can operate throughout the cell cycle.

Pathway choice seems to be influenced by chromatin environment and the factors 53BP1 and BRCA1 [67]. 53BP1 protects the ends against resection and promotes NHEJ, while BRCA1 promotes resection and HDR. Inhibitory phosphorylation of resection endonucleases provides an additional layer of regulation [75]. Resected DNA is initially coated by the single-strand binding protein RPA2 and subsequently replaced by recombinase Rad51 [4,68,75]. Coated ssDNA searches and invades into a homologous double strand DNA (dsDNA) forming D loops. The ssDNA strand is extended by unscheduled DNA synthesis.

To complete HDR, D loops must be resolved [67,68]. Strand invasion of a homologous sequence by a single resected strand can be resolved when DNA is synthesized across the break site, which is referred to as SDSA. This pathway does not lead to crossovers. When resected ends from both sides of a DSB invade into a homologous sequence, a double Holliday junction is formed. This can be resolved by branch migration, which moves branch points towards each other and does not result in crossovers [68,71]. Holliday junctions, single and double, can also be resolved by nicking the DNA either side of the branch-point and the strands are religated. Depending on how the strands are religated, this can result both in crossovers and noncrossovers.

Nucleoli, Sites of Ribosome Biogenesis

Human **nucleolar organiser regions (NORs)**, containing rDNA arrays, are located on the short p arms of the acrocentric chromosomes HSA13, HSA14, HSA15, HSA21, and HSA22 (Figure 1A) [12]. The length of the human rDNA repeat is 43 kb, with a 30-kb intergenic spacer, and 13-kb transcribed region containing the 18S, 5.8S, and 28S rRNA [13]. A haploid genome contains on average 300 repeats, distributed over the five chromosomes, but there is large variability in the number of repeats in each person [14,15]. A number of methods have been used to characterise rDNA repeat copy number variation in human cells. One approach is to digest high-molecular-weight genomic DNA in agarose plugs with a restriction enzyme that does not cut within the rDNA repeat. The sizes of released rDNA arrays/NORs are then determined by Southern blotting, performed after pulsed-field gel electrophoresis. The cluster lengths exhibit striking variability between and within human individuals, ranging from 50 kb to >6 Mb. They exhibit heterozygosity and provide each person with their own unique rDNA karyotype [14]. Analysis of these karyotypes in multigenerational human families demonstrates that the rDNA clusters are subject to meiotic rearrangement at a frequency of >10% per cluster per meiosis. More recently, a computational approach to determine rDNA copy number across human genomes by using whole-genome DNA sequencing data has been described. This approach establishes a maximum of 410 repeats per genome and an improbable minimum of 14 [15]. In the last few years, **digital PCR** has been increasingly used to measure rDNA copy number in both normal and cancer cells [16]. These studies combine to reveal NORs as highly variable and recombinogenic. A view that is reinforced by molecular combing studies, where it has been reported that ~30% of rDNA repeats in the cell lines tested were organised in a noncanonical fashion, forming large palindromic structures [17]. In **Werner syndrome** cell lines this could rise to ~60%. More recently, a sustained effort at sequencing an rDNA array from a human chromosome 21 has provided some sequence-based evidence for the existence of these structures [18]. This sequencing effort involved transformation-associated recombination (TAR) cloning in yeast. As palindromic

Glossary

Actinomycin D (AMD): an antibiotic and chemotherapeutic that intercalates into DNA. AMD preferentially binds to GC-rich DNA at low concentrations. The rDNA transcribed region is GC rich and therefore AMD blocks RNA Pol-I elongation.
Apoptosis: pathway for programmed cell death that can be activated in response to cellular stress.
Bloom syndrome: rare, autosomal recessive disorder characterised by short stature, photosensitivity of the skin, and predisposition to cancer. Blooms syndrome is caused by mutations in the Bloom (BLM) RecQ helicase. Cells from patients have highly elevated levels of sister chromatid exchanges between homologous chromosomes due to excessive crossovers.
Box C/D snoRNPs: ribonucleoprotein (RNP) complex containing Box C/D small nucleolar RNAs (snoRNAs) and proteins fibrillarin, Nop56, Nop58, and 15.5kD protein. snoRNAs base pairing with pre-rRNA guide methylation of rRNA.
Branch migration: the process of moving Holliday junctions along the DNA sequence. Double Holliday junctions can be resolved by branch migration of crossovers towards each other.
Chromocenters: densely staining aggregation of heterochromatic regions, including centromeres, from a number of chromosomes
Digital PCR: quantitative PCR method that provides an accurate measure of the amount of target DNA sequence in a sample or copy number within a genome.
Distal junction (DJ): sequences immediately on the telomeric side of rDNA arrays present on the short arms of all five human acrocentric chromosomes. These sequences are thought to anchor the rDNA in PNH. Nucleolar caps form adjacent to DJ sequences.
Double Holliday junctions: intermediates of DNA break repair. Each Holliday junction forms when homologous DNA duplexes are joined by the interchange of a pair of identical single strands.
Indels: insertions or deletions that result after DSB repair by NHEJ.
Neuronal crest cells: cells derived from the embryonic ectoderm that migrate to generate a variety of differentiated cell types, including

sequences have not been confidently recovered from long-read whole genome sequence data, their prevalence in uncloned DNA may not be as high as previously suggested.

The chromosomal context of human rDNA arrays has been somewhat elusive as the p arms of the acrocentric chromosomes are not included in the reference human genome. Despite this, ~400 kb of sequence on the distal (telomeric) side of the rDNA array has been identified and shown to be shared between all five acrocentric p arms [19,20]. Indeed, these sequences are now included in the most recent version of the human genome reference, GRCh38.p13. These **distal junction (DJ)** sequences appear to play a role in aspects of nucleolar function and are embedded in **perinucleolar heterochromatin (PNH)** [19]. Isolation of NORs on human acrocentric p arms, and sharing of distal sequences between them, points to a role for chromosomal context on NOR function, possibly including the genome stability of rDNA arrays [20]. It is worth pointing out that less is known regarding the chromosomal context of rDNA arrays in other mammals, including mouse, where even the chromosomal location of rDNA arrays varies between laboratory strains [21].

In active NORs, rDNA is bound extensively across the repeat by a nucleolar transcription factor, upstream binding factor (UBF) [22]. This enables rDNA repeats to remain undercondensed on metaphase chromosomes, which is visible as **secondary constrictions** [23]. Silent NORs are not bound by UBF and therefore fully condensed. Such NORs remain transcriptionally silent in daughter cells [24,25]. At the end of mitosis, transcription resumes and nucleoli form around each of the active NORs, which fuse in early G1 to form fewer larger mature nucleoli containing multiple NORs.

The rDNA repeats are transcribed by RNA Pol-I yielding a 45S pre-rRNA transcript [20]. Within nucleoli, 45S pre-rRNAs are rapidly processed into the mature 18S, 5.8S, and 28S rRNAs. The internal structure of the nucleolus is tripartite, reflecting the various stages of ribosome biogenesis (Figure 1B). Unengaged transcription factors and RNA Pol-I are located in the fibrillar centres (FCs) [26]. Transcription takes place at the interface between the FC and the surrounding dense fibrillar component (DFC). Nascent pre-rRNA transcripts are modified and early processing occurs in the DFC. Late processing of released pre-rRNA species and ribosome assembly occurs in the granular component (GC).

Nucleolar structure is dynamic, and upon transcription inhibition by **actinomycin D (AMD)**, which at low concentration preferentially inhibits RNA Pol-I, results in large-scale nucleolar reorganisation, including the formation of **nucleolar caps**. Proteins in the FC and DFC move together with the rDNA to the nucleolar periphery. Each cap comprises a single NOR and forms adjacent to its linked DJ, which appears to anchor rDNA arrays [19].

Subnuclear bodies such as nucleoli, P bodies, and Cajal bodies are not membrane bound and have recently been described as displaying liquid-like properties [27]. Initial experiments were performed with isolated nuclei, also called germinal vesicles (GVs), from *Xenopus* oocytes. These contain many extrachromosomally amplified nucleoli [28]. When these nucleoli are brought into close proximity by physical manipulation, they fuse in a manner that resembles the behaviour of a viscous liquid. Further mechanistic insight came from purified recombinant fluorescently tagged proteins fibrillarin and nucleophosmin (NPM), which form liquid droplets in the presence of RNA with fibrillarin droplets enveloped by NPM [29]. How this process, termed liquid–liquid phase separation (LLPS), applies to chromosomally tethered nucleoli in human cells remains unclear. However, upon transcriptional inhibition, nucleoli in human cells change shape from somewhat irregular structures (seemingly incompatible with the LLPS) to more spherical

craniofacial cartilage and bone, smooth muscle, and neurons.

Nucleolar caps: structures at the nucleolar periphery that form upon transcription inhibition. Caps contain proteins from the FC and DFC as well as the rDNA.

Nucleolar organizer regions (NORs): these contain rDNA arrays that, when transcribed by Pol-I, direct nucleolar formation.

Perinucleolar heterochromatin (PNH): heterochromatin surrounding nucleoli. In human cells the PNH is derived from acrocentric p-arms as well as heterochromatic regions from metacentric chromosomes.

Secondary constrictions: achromatic gaps in metaphase chromosomes. All chromosomes have a primary constriction at the centromere, while chromosomes with active NORs contain a secondary constriction.

Senescence: irreversible cell cycle arrest, which is characterised by changes in gene expression and genome organisation.

Treacher Collins syndrome: rare, autosomal dominant disorder, involving neural crest cells, that is characterised by cranial facial abnormalities. Affected individuals have deformities of the ears, eyes, cheekbones, and chin. Treacher Collins syndrome is caused primarily by mutations in *TCOF1*.

Werner syndrome: rare, autosomal-recessive disorder characterized by the appearance of premature ageing. Affected individuals develop and grow normally until puberty, but in young adulthood, symptoms of accelerated aging develop. It is caused by mutations in the Werner (WRN) RecQ helicase.

Xenobiotics: foreign chemicals, including carcinogens, not normally present in an organism.

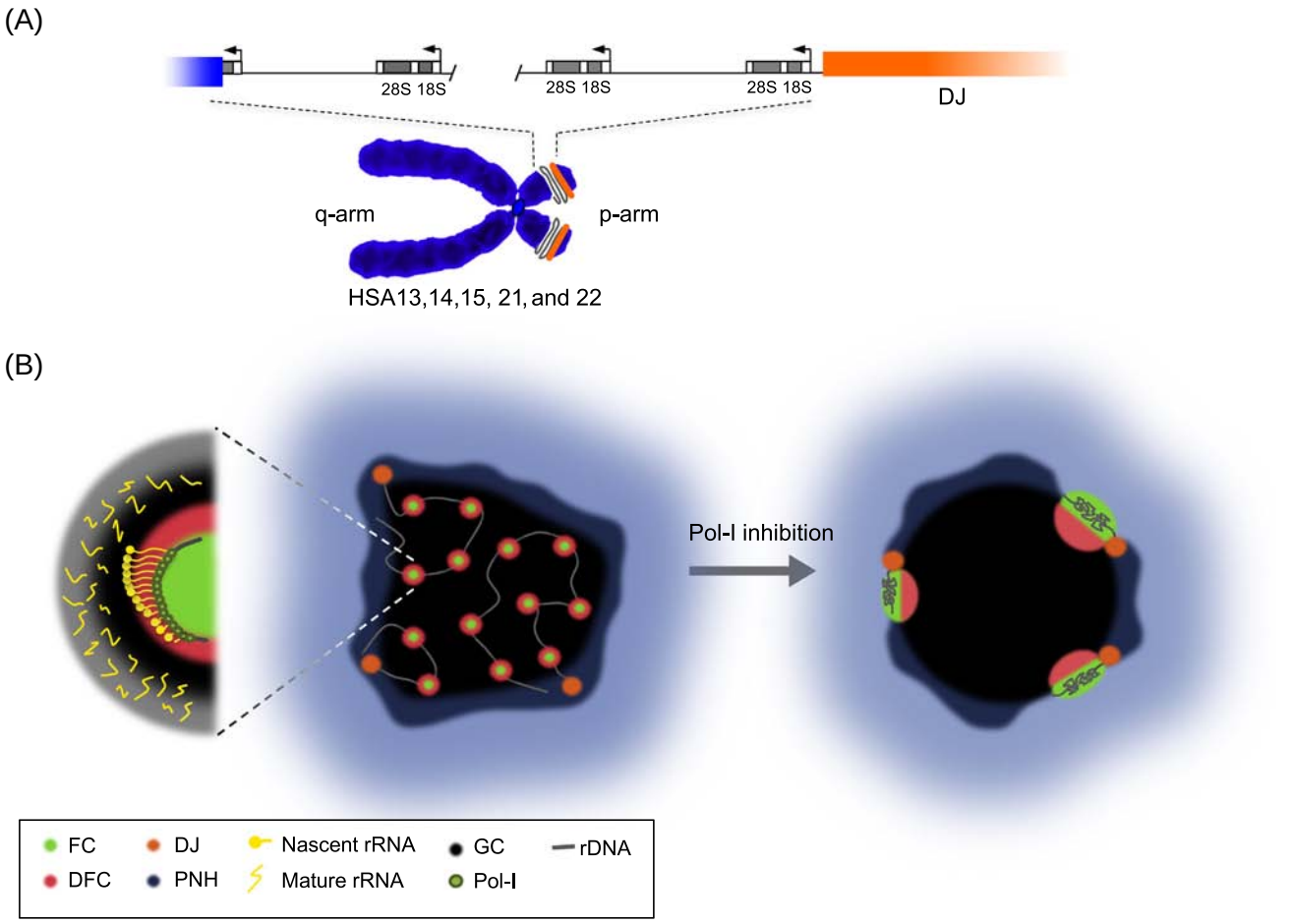

Figure 1. Organisation of Nucleolar Organiser Regions (NORs) and Nucleoli. (A) The genomic architecture of NORs containing the ribosomal gene arrays (rDNA), which are located on the short p arms of the human acrocentric chromosomes HSA13, 14, 15, 21, and 22. Transcription of rDNA arrays occurs in a telomere to centromere direction. The distal junction (DJ) (orange) is located adjacent to the ribosomal gene (rDNA) array and shared amongst all acrocentrics. (B) The internal organisation of nucleoli. In a mature interphase nucleolus, multiple NORs come together. The fibrillar centres (FCs) (green) are surrounded by the dense fibrillar component (DFC) (red). At the interphase between FC and DFC, RNA polymerase I (Pol-I) (green circles) transcription is initiated. Nascent pre-ribosomal RNA (pre-rRNA) transcripts (yellow) are bound by the small subunit processome (sphere at end of nascent transcript) and cotranscriptionally modified and processed. Late pre-rRNA processing intermediates are released into the granular component (black) for maturation and assembly into ribosomes. When RNA Pol-I transcription is inhibited, for example, by actinomycin D or rDNA double-strand breaks, rDNA arrays with associated proteins in the FC and DFC migrate to the periphery of nucleoli. These so-called nucleolar caps form adjacent to the DJ sequences, which anchor the linked rDNA in the perinucleolar heterochromatin (PNH) (blue). Abbreviations: GC, granular component.

(LLPS compatible) objects. Nucleolar caps are bipartite in structure, with the FC facing the nucleoplasm and the DFC facing the nucleolar interior and the GC [30].

Targeting DSBs to Nucleoli

Initial studies, aimed at studying DSBs within nucleoli, induced damage with γ irradiation or UV irradiation [31,32]. As the concentration of rDNA in nucleoli is low, it would be expected that rDNA DSBs would be under-represented compared with the rest of the genome. Targeted irradiation such as laser microirradiation and focussed ion beams [31,33] provide localised DSBs but still have the disadvantage that the number and exact location of the DSBs in the rDNA repeat cannot be controlled. Furthermore, the potential for collateral damage in the surrounding, more concentrated, DNA remains high.

Introducing DSBs using sequence-specific endonucleases provides a way to target DSBs to rDNA with minimal or no DSBs elsewhere in the genome. The homing endonuclease I-PpoI from *Physarum* has a single 15-bp recognition sequence, in each rDNA repeat and in ~13 other sites (mostly 28S rRNA pseudogenes) elsewhere in the genome [34–36]. Recently, targeting DSBs to specific sequences by CRISPR/Cas9 has significantly expanded the possibilities for studying DSB response at repetitive loci [37,38]. The Cas9 nuclease can be targeted to specific sequences by a 19- or 20-nucleotide guide RNA (gRNA), creating a DSB at the binding site. The only limitation is the presence of a protospacer adjacent motif (PAM, NGG for Cas9). With CRISPR/Cas9, the consequences of DSBs in the transcribed region or nontranscribed intergenic spacer of the rDNA repeat, as well as sequences adjacent to the rDNA repeat, can be studied [39]. Apart from the rDNA, this approach has been used to study the DNA damage response at other repetitive loci, such as the 5S rDNA arrays, centromeres, and pericentromeric heterochromatin [40,41]. However, DSBs generated by CRISPR/Cas9 have highly variable and relatively slow kinetics of repair (half-times up to 10 h) [42]. In contrast, DSBs generated by ionising radiation are usually repaired within 1 h [43]. Repair of DSBs generated by CRISPR/Cas9 have nonrandom repair outcomes, which is dependent on the target sequence and the cell line [44].

Targeted DSBs can also be induced by transcription activator-like effector nucleases (TALENs). These engineered nucleases recognise specific DNA sequences through 30–35-amino-acid repeats, which each recognise a specific single nucleotide [45]. These DNA recognition domains are fused to the nonsequence-specific FOK-I nuclease domain. The future use of TALENs for DDR studies has been simplified with the availability of a modular construction kit [46].

DNA Damage-Induced RNA Pol-I Inhibition Causes Nucleolar Cap Formation

The first indications of a specific nucleolar DNA damage response came from experiments in which DNA DSBs were introduced into mouse cells by ionizing radiation. Such damage resulted in ataxia–telangiectasia mutated (ATM) dependent inhibition of RNA Pol-I transcription and nucleolar reorganisation [31]. Furthermore, laser microirradiation targeting individual nucleoli resulted in localised silencing of RNA Pol-I transcription. Other nucleoli within the same nucleus were unaffected. Similar results have not yet been reported in human cells.

In human cells, generation of rDNA DSBs by expression of I-PpoI or CRISPR/Cas9 with an appropriate gRNA, results in dramatic alteration of nucleolar morphology. Nucleoli change from irregularly shaped objects to spherical structures with nucleolar caps [39,41,47]. This reorganisation is identical to that observed during AMD treatment, and is induced because rDNA DSBs lead to inhibition of RNA Pol-I transcription. Transcriptional inhibition is dependent on ATM [39,47].

Currently, it is unclear whether RNA Pol-I transcription is inhibited directly through phosphorylation of the transcription machinery or indirectly through modification of chromatin structure. DNA-dependent protein kinase (DNA-PK) has been shown to phosphorylate RNA Pol-II directly in the vicinity of the break site [36]. There are also a number of reports that RNA Pol-I can be directly inhibited by DNA-PK in response to DNA damage [48,49]. However, in response to targeted DSBs, DNA-PK does not seem to contribute to transcriptional silencing [39,41,47]. Ataxia telangiectasia and Rad3-related protein (ATR), a kinase related to ATM and DNA-PK, can inhibit ribosomal transcription in response to single-stranded DNA exposed by R loops forming after

hypo-osmotic stress [50]. However, in response to rDNA DSBs, transcriptional inhibition is not dependent on ATR [50].

Large-scale phospho-proteomic studies provide a list of potential DSB-induced phosphorylation targets, which include Treacle, UBF, and TAF_IC [51,52]. The only phosphorylation that has been independently confirmed is on the protein Treacle, encoded by the gene *TCOF1*, which is mutated in **Treacher Collins syndrome** [53]. Treacle interacts both with UBF and Nop56, a component of **Box C/D snoRNPs** that are responsible for methylation of rRNAs [54,55]. The consequences of phosphorylation on Treacle function are unknown. Among the other potential phospho-targets are UBF on S23 and TAF_IC, a component of RNA Pol-I-specific TBP containing complex, on S848 and S858 [51,52]. Phosphorylation of neighbouring T852 on TAF_IC silences nucleolar transcription during mitosis [56].

Alternatively, transcriptional inhibition could be indirect, through modification of the local chromatin environment. For example, ATM indirectly inhibits RNA Pol-II transcription in the immediate vicinity of DSBs through histone ubiquitylation [57,58]. A word of caution, however, it has been controversial for many years as to whether histones and/or nucleosomes are present on the pre-rRNA coding sequences on actively transcribed repeats [59]. The most reasonable conclusion is that nucleosomes are at least highly depleted over transcribed rDNA sequences. Despite these concerns, a recent study has shown phosphorylation of H2B is involved in the inhibition of RNA Pol-I transcription in response to rDNA DSBs [60]. Further work is clearly needed to elucidate the mechanism of RNA Pol-I transcriptional inhibition. Additionally, it will be critical to determine whether inhibition is restricted to damaged repeats, NORs that contain damaged repeats, all the NORs present in a mature fused nucleolus, or systemically throughout the nucleus.

Movement of DSBs Is Not Unique to the rDNA

A striking feature of the nucleolar response to DSBs is the formation of nucleolar caps with the associated movement of rDNA arrays including damaged repeats to the nucleolar periphery. Movement in response to DSBs is not unique to nucleoli and rDNA. DSBs in repetitive sequences present in heterochromatin and pericentromeric heterochromatin have also been shown to move to the periphery of **chromocenters** in *Drosophila* and mouse cells [40,61]. Also, DSBs in actively transcribed genes cluster together in a manner that is dependent on ongoing RNA Pol-II transcription [62].

Nucleolar reorganisation and the associated movement of rDNA DSBs is most likely a downstream consequence of ATM-dependent inhibition of transcription [39]. But how this reorganisation is generated is not yet known. For DSBs in the nucleoplasm, nuclear actin has been shown to form filaments, which induces movement of these breaks [62–65]. This movement has been suggested to facilitate HDR [65]. Filaments forming in and around nucleoli have been observed, when cells are treated with the DNA alkylating agent, methyl methanesulfonate [63]. The role of such filaments in nucleolar cap formation has not yet been explored.

An alternative explanation for capping involves LLPS [30,66]. One might imagine that individual small FC and DFC domains in transcriptionally active nucleoli are prevented from fusing as they are tethered to individual or small local clusters of rDNA repeats. However, upon inhibition of transcription, by AMD or ATM, these tethers are broken and LLPS may take over. Cap formation is likely important as each cap contains a single NOR and forms adjacent to the DJ sequence, which anchors rDNA in PNH. The formation of caps enables the recruitment of repair factors

normally excluded from nucleoli. Currently, it is unclear how these proteins are excluded from nucleoli. Perhaps they lack appropriate unstructured domains and are incompatible with nucleolar liquid phases [29]. Caps also concentrate all the repeats on a damaged NOR in close proximity to each other. Undamaged repeats on the same chromosome could serve as repair templates [39]. The formation of nucleolar caps containing a single NOR may limit interchromosomal recombination and translocations.

Repair Pathway Selection

The presentation of rDNA DSBs in nucleolar caps has profound consequences on repair pathway selection (Box 1). It would appear that following nucleolar reorganisation, HDR is the default pathway. Binding of p53 binding protein 1 (53BP1) around DSBs favours nonhomologous end joining (NHEJ), while additional binding of BRCA1 promotes HDR [67,68]. Additionally, 53BP1 can limit hyper-resection and thus ensure HDR fidelity [69]. Both 53BP1 and BRCA1 are recruited to the same nucleolar caps [39]. During HDR, end-resected DNA is initially coated by the single-strand binding protein RPA2 and subsequently replaced by recombinase Rad51 [4]. Coated single-strand DNA (ssDNA) then searches for a homology template forming a displacement loop (D loop). Subsequently, ssDNA is extended by unscheduled DNA synthesis. RPA2, Rad51, and unscheduled DNA synthesis, are all observed at nucleolar caps [39]. Surprisingly, these are observed even in G1 cells, where no sister chromatid is present. The most likely interpretation is that undamaged rDNA repeats in *cis* may provide a template for repair in G1 cells. In G2 cells repair of rDNA DSBs could be templated in *cis* or *trans* by the sister chromatid.

It seems likely that when rDNA DSBs fail to reach a threshold level they can be repaired by the NHEJ pathway rapidly before transcription is silenced. When breaks persist, and cross a threshold level, transcription is inhibited, and nucleolar caps form to facilitate HDR [47]. It should also be pointed out that the 'clean ends' produced by endonucleases such as I-PpoI may aid rapid accurate repair by NHEJ without introducing characteristic **indels** [4]. In this case, I-PpoI can recleave the repeat, leading to cycles of break and repair. Such a scenario may influence checkpoint activation and repair pathway selection. Future development of inducible and tunable expression systems for site-specific endonucleases could alleviate this problem. TALENs that generate more frayed ends could also provide an alternative.

NORs are not the only repeat containing loci that recruit the HDR machinery throughout the cell cycle. In mouse cells, DSBs in centromeric repeats likewise recruit Rad51 in all stages of the cell cycle at the periphery of chromocenters [40]. In contrast, DSBs in the pericentromeric heterochromatin only recruit HDR factors in S/G2 phase.

HDR of DSBs in the rDNA has been suggested to result in loss of a large number, up to 90%, of repeats [41]. This loss of repeats was inferred indirectly by loss of UBF staining intensity and by endpoint PCR. This result needs to be confirmed by quantitative direct methods such as fluorescence *in situ* hybridisation and digital PCR. Nevertheless, significant changes in rDNA repeat copy number are reminiscent of the instability seen in cancer, premature ageing syndromes, and neurodegenerative diseases [8–10]. In yeast, the nontranscribed repeats are essential for repairing DNA damage and without those, yeast cells are more sensitive to DNA-damaging agents [70]. Possibly inactive rDNA repeats in humans have a similar function. Importantly, HDR in *cis* does not necessarily result in the loss of repeats. HDR via synthesis dependent strand annealing (SDSA) or dissolution of **double Holliday junctions** by **branch migration** can mediate noncrossover repair [68,71]. Bloom RecQ DNA helicase (BLM) is involved in branch migration. Mutation of the gene encoding BLM, as observed in **Bloom syndrome**, results in hyper-recombination within rDNA arrays [8].

DNA Damage Outside the Nucleolus Can Transiently Inhibit Nucleolar Transcription

Many stress responses including whole-cell γ irradiation disrupts nucleolar transcription [11,31]. This inhibition is not necessarily due to DSBs within the rDNA. DSBs outside nucleoli can also transiently inhibit transcription [72]. This transcription inhibition is dependent on the DNA damage protein Nijmegen breakage syndrome protein 1 (NBS1) and Treacle. When nonnucleolar DSBs

Key Figure

Model of rDNA Double-Strand Break (DSB) Repair

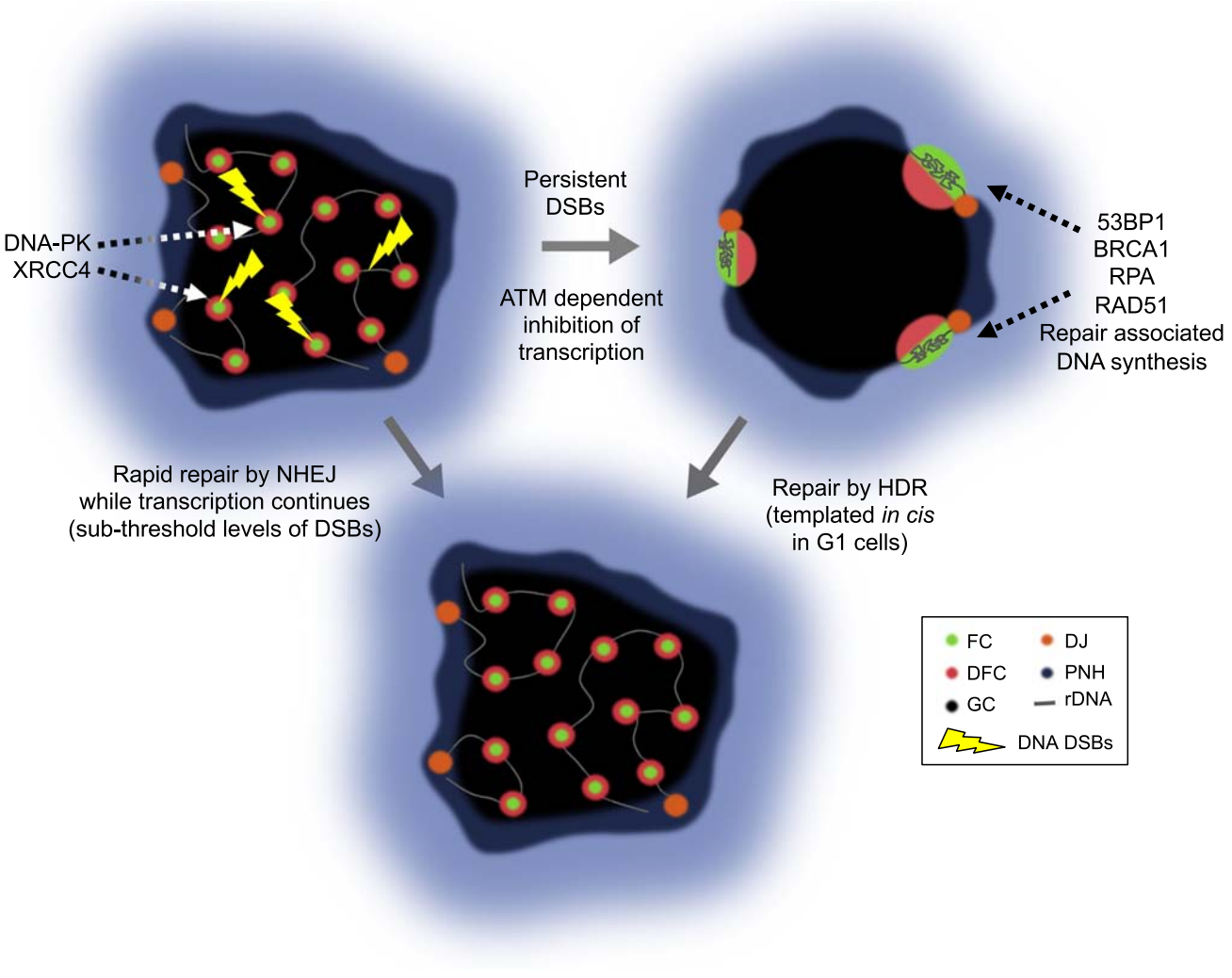

Figure 2. At subthreshold levels, DSBs in the rDNA will be rapidly repaired by nonhomologous end joining (NHEJ), involving DNA-dependent protein kinase (DNA-PK) and the ligase XRCC4, while RNA polymerase I (Pol-I) transcription continues. However, when the breaks persist, the kinase ataxia–telangiectasia mutated (ATM) is activated, which silences Pol-I transcription. Transcriptional inhibition initiates nucleolar cap formation at the nucleolar periphery. Note that each cap represents a single nucleolar organiser region (NOR). Cap formation facilitates recruitment of factors shown, required for homology-directed repair (HDR) and normally excluded from nucleoli. Ribosomal gene DSBs are repaired by HDR throughout the cell cycle. In G1 phase of the cell cycle at least, HDR is templated by other repeats in *cis*, that is, within the same NOR. Abbreviations: 53BP1, p53 binding protein 1; DFC, dense fibrillar component; DJ, distal junction; FC, fibrillar centre; GC, granular component; PNH, perinucleolar heterochromatin.

are induced by γ irradiation in both human and mouse cells, a nucleoplasmic pool of Treacle is phosphorylated by ATM, which enables the Treacle–NBS1 complex to localise to nucleoli where it inhibits nucleolar transcription. While full-length Treacle is highly enriched in nucleoli, shorter isoforms appear to be nucleoplasmic and largely excluded from nucleoli [53,72]. It is uncertain at the moment which isoform is responsible for targeting NBS1 to nucleoli, and whether this isoform is present in all cell types. Unlike rDNA DSBs, the transient inhibition induced by non-rDNA DSBs affects all nucleoli within the nucleus and does not result in the formation of nucleolar caps. The exact function of this transcriptional inhibition is not known and requires further investigation.

Concluding Remarks and Future Perspectives

The essential features of a specialised nucleolar response to DSBs within rDNA can be summarised as follows (Figure 2, Key Figure). ATM is activated and RNA Pol-I transcription is inhibited [39,41,47]. This inhibition induces nucleolar cap formation, which facilitates HDR throughout the cell cycle [39]. The formation of the nucleolar caps offers a number of advantages. It concentrates all the repeats from a single NOR in close proximity to each other possibly stimulating HDR in *cis*. Cap formation also exposes rDNA DSBs to repair factors, which are normally excluded from nucleoli. At subthreshold levels of damage, rapid repair by the NHEJ pathway may circumvent the requirement for transcriptional silencing and nucleolar reorganisation.

Most studies to date have used high levels of DSBs and use established cell lines; in many cases, cancer cell lines. In the future it will be important to study the response with more complex cellular models, with biologically relevant levels of damage and determine the long-term consequences (see Outstanding Questions). An interesting recent study found that injection of low amounts of I-PpoI mRNA into *Xenopus* embryos resulted in apoptosis selectively within **neuronal crest cells**, leading to craniofacial abnormalities in the embryos [73]. This result mirrors Treacher Collins syndrome in which mutations in *TCOF1*, the gene encoding Treacle, activate a p53-dependent apoptotic mechanism in neuronal crest cells in mice [74]. One might reasonably conclude that efficient repair of induced rDNA DSBs by the nucleolar DDR response protects the majority of cell types in developing *Xenopus* embryos.

Outstanding Questions

How do nucleoli respond to more physiological levels of rDNA damage? To date, all studies have used high levels of DSBs, as all rDNA repeats are potential targets for either I-PpoI or Cas9. By exploiting genome-editing technology, it should be possible to engineer a situation where subsets of rDNA repeats are potential DSB targets.

What is the precise mechanism that drives nucleolar reorganisation in response to rDNA DSBs, including cap formation?

How does transcription influence the repair pathway selection? Are active and silenced rDNA repeats/NORs treated differentially?

What are the short-term and long-term downstream consequences of rDNA DSBs, especially at low levels of damage? Are checkpoints activated and are cells programmed for either apoptotic or senescence pathways?

Do rDNA DSBs alter the cellular response to DSBs elsewhere in the genome and *vice versa*?

How does chromosomal context influence the stability of rDNA arrays? Throughout eukaryotic organisms, NORs are located in isolated chromosomal regions, close to either telomeres or centromeres. In mouse and human genomes, NORs are located on dedicated short chromosome arms.

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