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REVIEW ARTICLE

Nucleolus as an emerging hub in maintenance of genome stability and cancer pathogenesis

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Abstract

The nucleolus is the major site for synthesis of ribosomes, complex molecular machines that are responsible for protein synthesis. A wealth of research over the past 20 years has clearly indicated that both quantitative and qualitative alterations in ribosome biogenesis can drive the malignant phenotype via dysregulation of protein synthesis. However, numerous recent proteomic, genomic, and functional studies have implicated the nucleolus in the regulation of processes that are unrelated to ribosome biogenesis, including DNA-damage response, maintenance of genome stability and its spatial organization, epigenetic regulation, cell-cycle control, stress responses, senescence, global gene expression, as well as assembly or maturation of various ribonucleoprotein particles. In this review, the focus will be on features of rDNA genes, which make them highly vulnerable to DNA damage and intra- and interchromosomal recombination as well as built-in mechanisms that prevent and repair rDNA damage, and how dysregulation of this interplay affects genome-wide DNA stability, gene expression and the balance between euchromatin and heterochromatin. We will also present the most recent insights into how malfunction of these cellular processes may be a central driving force of human malignancies, and propose a promising new therapeutic approach for the treatment of cancer.

Introduction

The nucleolus is the most prominent nuclear substructure involved in production of ribosomes, large and complex ribonucleoprotein machines responsible for translation of mRNAs into proteins (Fig. 1a) [1]. The human 80 S ribosome is composed of a 40 S subunit consisting of 18 S ribosomal RNA (rRNA) and 33 distinct ribosomal proteins (RPs), and a 60 S subunit containing 5 S, 5.8 S, and 28 S rRNAs and 47 distinct RPs [2, 3]. Nucleoli form around the nucleolar organizer regions (NORs) on acrocentric chromosomes 13, 14, 15, 21, and 22 that contain clusters of ~ 300 ribosomal DNA gene repeats (rDNA) mostly arranged in a head-to-tail manner, although inverted rDNA repeats or palindromes are also present [4]. The number of rDNA repeat units varies significantly across the human population, suggesting that these loci are susceptible to intrinsic recombinational instability [5]. The rDNA clusters consist of modules of ~ 13 kb transcribed by RNA polymerase I (Pol I) into the 47 S pre-rRNA, and an intergenic spacer (IGS) of ~ 30 kb (Fig. 1b). The 47 S pre-rRNA transcript is processed into 18 S, 5.8 S, and 28 S rRNAs. The IGS contains: spacer promoters, repetitive enhancer elements, the 47 S pre-RNA promoter, origins of DNA replication, terminator elements (T) downstream and upstream of the pre-rRNA transcription unit that can bind transcription termination factor (TTF-I), replication fork barriers (RFBs), tandem repeats and non-coding RNA genes transcribed by Pol II under certain stress conditions (Fig. 1b) [6]. DNA sequences proximal and distal to rDNA arrays are highly conserved among the five acrocentric chromosomes. Whereas proximal sequences, termed proximal junctions

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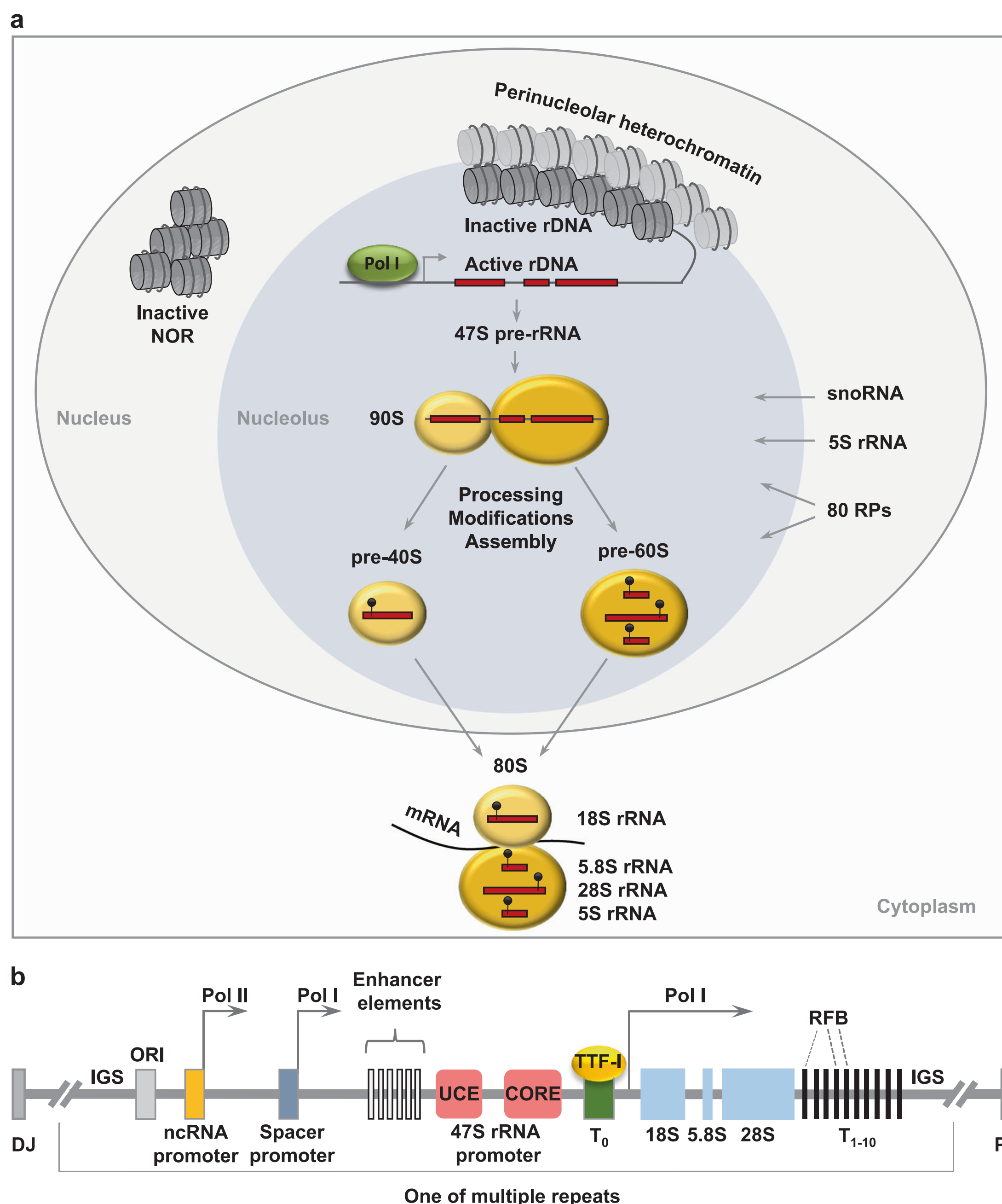

Fig. 1 Nucleolus and rDNA genes. **a** Intranucleolar heterochromatic inactive rDNA repeats are tightly connected with perinucleolar heterochromatin, whereas euchromatic active rDNA genes are transcribed into the 47 S pre-rRNA by Pol I. The 47 S pre-rRNA is co-transcriptionally assembled into the 90 S processome with RPs and 5 S rRNA and modified by ~200 snoRNPs. Following cleavage of the pre-rRNA, the 90 S processome separates into a pre-60S subunit and a pre-40S subunit that largely follow independent maturation pathways in the nucleolus, the nucleoplasm and the cytoplasm. Modifications of rRNA are indicated with lollipops. **b** Schematic diagram of the rDNA array. Shown are coding regions for 18 S, 5.8 S, and 28 S rRNA as well as IGS with the rDNA core promoter and upstream control element (UCE), enhancers, spacer promoter, and Pol I promoter, ORIs as well as T0 that binds TTF-I and T1, T4, and T5 that act as RFBs. DJ and PJ sequences that flank the rDNA array are indicated

(PJ) are segmentally duplicated and resemble the regions flanking centromeres on other chromosomes, the distal junctions (DJ) are unique to the five acrocentric chromosomes and contain a large inverted repeat and 48-bp satellite repeats (Fig. 1b) [7]. Each arm of the inverted repeat can be transcribed by Pol II into long non-coding RNAs implicated in chromatin regulation [7].

In proliferating cells, the nucleolus disassembles during prophase and begins to reassemble in telophase [1]. Upstream binding factor (UBF)-dependent mitotic

bookmarking of NORs is responsible for the open chromatin state, visible as achromatic gaps on DAPI-stained metaphase chromosomes and the establishment of a transcriptionally active state essential for post-mitotic nucleolar formation [8]. Conversely, NORs unbound by UBF possess epigenetic features characteristic of constitutive heterochromatin; they are transcriptionally incompetent and not associated with the nucleoli (Fig. 1a) [4]. Importantly, both transcriptionally active (euchromatic) and inactive (heterochromatic) rDNA repeats are present in "active" NORs associated with the nucleoli (Fig. 1a) [4]. Transcriptionally active rDNA genes represent ~ 50% of all rDNA repeats and localize at the surface of the so-called fibrillar center where they are transcribed by Pol I into the 47 S pre-rRNA. Inactive rDNA repeats form intranucleolar heterochromatin [9]. The ratio between active and inactive rDNA genes is regulated through recruitment of either Cockayne syndrome B protein (CSB) or nucleolar remodeling complex (NoRC) to TTF-I bound to the promoter-proximal terminator T_0 [10]. Whereas the CSB-loaded TTF-I recruits histone methyltransferase G9a and HP1γ, leading to the establishment and maintenance of transcriptionally active state, binding of NoRC subunit TIP5 (TTF-I-interacting protein 5) to TTF-I leads to recruitment of enzymes that induce CpG DNA methylation and heterochromatic histone modifications on rDNA. However, the molecular mechanisms responsible for the differential recruitment of CBS or NoRC to TTF-I at T_0 remain unknown [10]. Intranucleolar heterochromatin containing inactive rDNA repeats is tightly connected with a shell of heterochromatin surrounding the nucleolus, known as perinucleolar heterochromatin (Fig. 1a) [9]. The establishment and maintenance of perinucleolar heterochromatin is also regulated by NoRC, suggesting functional interdependence between these two heterochromatic domains [11], partly mediated by the DJ sequences embedded in adjacent perinucleolar chromatin to anchor the NORs to nucleoli [7].

Ribosome biogenesis is the most energy consuming process in human cells [12]. rDNA genes are the most actively transcribed genes, collectively accounting for more than half of total transcription in proliferating cells [13]. The production of the 47 S pre-rRNA was thought to be exclusively controlled by changing the rate of Pol I transcription. However, recent evidence indicates that epigenetic mechanisms also regulate this process by altering the ratio of active and inactive rDNA genes in response to specific cues [6, 14, 15]. The 47 S pre-rRNA is co-transcriptionally assembled into the 90 S processome, with numerous RPs and 5 S rRNA synthesized by Pol III, where it is subjected to ribose 2′-O-methylation and pseudouridylation, folding and processing steps depending on ~200 snoRNAs (Fig. 1a) [16, 17]. Following cleavage within the internal transcribed spacer the 90 S processomes are separated into pre-40S and pre-60S ribosomal subunits that follow largely independent maturation pathways in the nucleolus, the nucleoplasm and the cytoplasm (Fig. 1a) [18]. Most research on the nucleolus has focused on understanding ribosome biogenesis and the impact of altered ribosome biogenesis on the development of human diseases, notably ribosomopathies and cancer [19]. However, proteomic studies have identified some 4500 nucleolar proteins, mostly participating in processes unrelated to ribosome biogenesis [20]. Based on results of these and functional studies, the nucleolus has been implicated in the regulation of additional cellular processes: genome integrity maintenance, epigenetic control, cell-cycle regulation, diverse stress responses, cellular senescence, genomic organization, nuclear architecture, and maturation, assembly, and export of the signal recognition particle, telomerase RNP and processing of precursor transfer RNAs [19, 21]. Collectively these studies demonstrate that the nucleolus performs many more functions than originally appreciated.

Several features make rDNA genes highly vulnerable to DNA damage and intra- and interchromosomal recombination, including high transcription rate, challenging DNA replication, repetitive nature, and presence in clusters on five different chromosomes [4, 22]. This review will discuss rDNA damage and repair mechanisms, and how dysregulation of these processes affects genome-wide DNA stability, gene expression and the balance between euchromatin and heterochromatin. We will also review the recent advances in understanding how malfunction of these processes may contribute to malignancy, and opportunities to exploit this emerging knowledge for improvements of anticancer treatments.

Systematic proteomic and genomic studies implicate the nucleolus in promoting genome stability

Based on studies of the nucleolar proteome and the human protein atlas database, Ogawa and Baserga assembled a list of 166 DNA repair proteins that localize to the nucleolus [23]. However, whether the nucleolus is just a storage depot for DNA-damage response (DDR) proteins that operate in the nucleus or they indeed play some specific nucleolar roles, remains unclear. A number of DDR proteins accumulate in the intranucleolar body (INB) and nucleolar cap structures upon DNA damage, suggesting that these structures provide a platform for recruitment of specific factors that sense and repair rDNA damage in the nucleolus [24]. Furthermore, genetic screens to identify human ribosome biogenesis factors revealed that a subset of these factors are co-classified as DDR proteins [25–27]. Moreover, a mass spectrometry analysis of proteins phosphorylated in

response to DNA damage by ataxia-telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinases revealed 700 target proteins, 98 of which are nucleolar proteins involved in various aspects of ribosome biogenesis, ribosome function and epigenetic regulation of rDNA genes, thus further supporting the notion that the nucleolus is an important hub of the DDR [28, 29]. A recent genetic screen in yeast revealed that almost 15% of 4876 non-essential genes, most of which have not been linked to nucleolar functions so far, are required for the maintenance of rDNA stability, revealing an unexpected complexity of this process. Lastly, in HeLa and HT1080 cells, ~ 4% of the genome, including tandemly arrayed genes, tRNAs, highly repetitive centromeric, pericentromeric, telomeric, and satellite sequences are embedded in the perinucleolar heterochromatin [30, 31]. The close proximity between the nucleolus and these so-called nucleolus-associated chromosomal domains further suggests a role for the nucleolus in spatial genome organization. Altogether, these systematic approaches provide a wealth of information that can serve as a strong foundation for future mechanistic studies to elucidate the interplay between the nucleolus, the DDR, chromatin and spatial genome organization in the maintenance of genome stability and cancer prevention.

Conflicts between replication and transcription machineries at the rDNA genes as a source of genomic instability

Mounting evidence indicates that DNA replication machinery frequently encounters obstacles, which can slow down or stall replication fork progression, leading to DNA damage and recombination. Insults causing such replication stress (RS) include: DNA secondary structures, heterochromatin, DNA lesions, torsional stress in DNA, tightly DNA-bound proteins and transcribed genes [32, 33]. The transcriptional machinery presents a particularly severe impediment to DNA replication, and clashes between these two molecular machineries represent the major source of genomic instability, an event fueling the acquisition of all cancer hallmarks [34–37]. Given that rDNA genes are the most highly transcribed genes in eukaryotes, conflicts between transcription and replication are especially frequent at rDNA gene clusters [38]. To better understand how conflicts between rDNA transcription and replication machineries occur and how they contribute to genome instability, we will first briefly describe what is known about the causes and consequences of these conflicts at the other genomic regions [39].

Head-on convergence between DNA replication forks and transcription machineries may trigger local accumulation of positive DNA supercoils, which likely block replication fork progression, leading to fork slowing and stalling [37, 40]. Such persistent fork stalling can lead to occurrence of long stretches of single-stranded DNA (ssDNA) and single-strand DNA breaks (SSBs), events which trigger a checkpoint surveillance program orchestrated by the ATR-Chk1 kinase signaling module [41–43]. These kinases phosphorylate many factors that function to prevent genome instability by: stabilizing the replication fork, release of RNA polymerase and termination of transcription, activating dormant origins of replication around the stalled forks to facilitate the replication of affected regions, blocking late origin activation, orchestrating DNA repair with restart of stalled forks and cell-cycle inhibition [28, 32, 44]. Under persistent RS or when the ATR-dependent RS-response fails, stalled forks may collapse, leading to dissociation of replisome proteins and conversion of SSBs into double-strand breaks (DSBs), the most deleterious of DNA lesions [32]. DSBs trigger activation of the ATM kinase, which orchestrates cell-cycle arrest and DSB repair. If repair is successful, DNA replication resumes [45, 46]. However, when the ATR checkpoint is malfunctional and/or error-prone DNA repair processes are engaged, various genomic alterations may result from RS, including: point mutations, deletions or amplifications, loss of heterozygosity, gross chromosomal rearrangements, and structural and numerical chromosomal aberrations if unreplicated DNA or unresolved repair intermediates persist until mitosis [32, 47].

Recent studies suggest that genomic instability upon clashes of transcriptional and replication machineries depends on transcription-associated R-loop formation [48–51]. R-loops form owing to local negative supercoiling behind a transcription bubble, followed by opening of the DNA and formation of highly stable hybrids between nascent RNA and template DNA strands (RNA:DNA hybrid) [48, 49]. The overall load of R-loops increases under perturbed replication and fuels DNA damage and genomic instability (Fig. 2) [34, 52]. Persistent R-loops can also impede RNA Pol elongation, resulting in the pileup of Pol molecules, ultimately provoking replication fork slowing and stalling [50, 53]. The number and length of R-loops increase upon dysregulation of post-transcriptional processes, including packaging of nascent RNAs into ribonucleoprotein particles, mRNA splicing, export or degradation, explaining the association of dysregulation of these post-transcriptional processes and genomic instability [49, 54]. R-loop formation is counteracted by Topoisomerases 1 and 2 (Top1/2), which relieve positive and negative supercoils ahead of and behind transcribing RNA polymerases, respectively, DNA helicases Senataxin that unwinds RNA:DNA hybrids, and RECQ5 that counteracts replication fork stalling in RNA pol I and II transcribed genes, acting at sites of replication-transcription collisions,

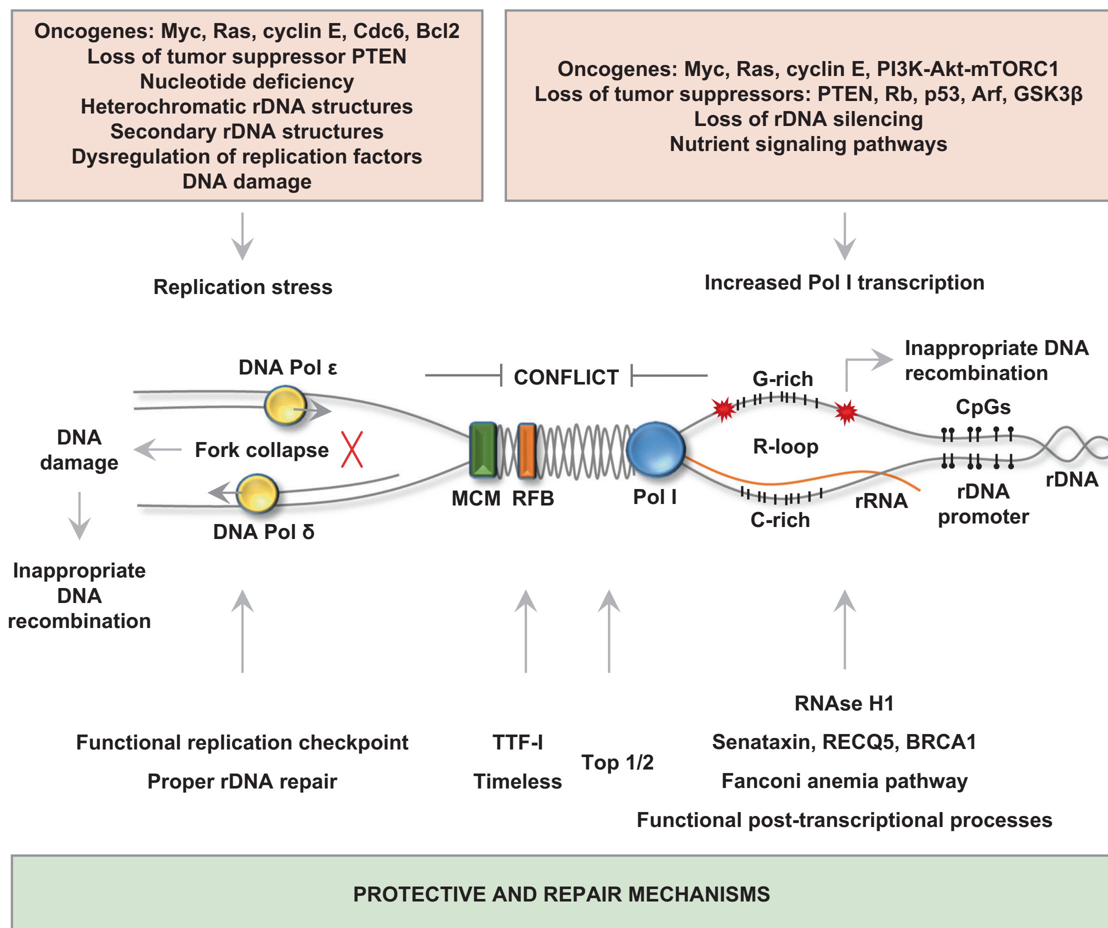

Fig. 2 Causes and consequences of conflicts between Pol I transcriptional and replication machineries. The indicated oncogenic signaling pathways can lead an aberrant increase in the rate of Pol I transcription. Convergence of Pol I and replication machineries in head-on orientation leads to the accumulation of positive DNA supercoiling, which slows down both machineries. Negative DNA supercoiling forming behind transcription bubble leads to opening of rDNA strands and the formation of highly stable rRNA:rDNA hybrids, leaving the displaced non-template strand. The rDNA genes are predilection sites for the formation of R- loops owing to the presence of a region of GC skewing, downstream of unmethylated CpG island rDNA promoters. Clashes of Pol I transcriptional machinery and R-loops with replication fork may cause its stalling or collapse. Perturbing replication of rDNA may further potentiate conflicts between replicating forks and Pol I transcriptional machinery as well as generation and stabilization of co-transcriptional R-loops and R-loop dependent rDNA damage. Several protective and DNA repair mechanisms are shown that preserve genome stability upon conflicts between Pol I transcriptional and replication machineries, thus preventing tumorigenesis. Asterisks indicate DNA damage

and RNAse H1 which cleaves the RNA component of the RNA:DNA hybrid [55–59]. Importantly, BRCA1 and the Fanconi anemia pathway, that stabilize stalled replication forks, also help resolve transcription-associated R-loops, thus allowing continuation of DNA replication (Fig. 2) [60, 61]. Apart from directly or indirectly impairing progression of the replication machinery and causing RS, R-loops can also enhance genomic instability by serving as substrate for transcription-coupled nucleotide excision repair (NER) factors, which generate DSBs or through generation of various types of DNA lesions in the displaced non-template DNA strand by DNA-damaging agents, DNA modifying and repair enzymes [48, 62–64].

Active rDNA genes are transcribed by >100 Pol I transcriptional complexes [65]. This, together with the fact that rDNA transcription occurs also in early S phase, suggests that the Pol I transcriptional machinery may represent a barrier for replication fork progression [1, 66–68]. However, recent research has suggested the spatial separation between these machineries on rDNA genes in early S phase [68, 69]. Furthermore, upon binding TTF-I and a replisome factor timeless to RFBs in the IGS regions of the rDNA loci obstruct replication fork progression and thereby prevent potential clashes of transcription and replication machineries (Figs. 1b and 2) [70]. The highest RFB activity is in early S phase when actively transcribing rDNA genes replicate [66, 70]. The rDNA region is a frequent site for R-

loop formation [71], owing to an unmethylated CpG island in rDNA promoters making the non-template strand G-rich (Fig. 2). A G-rich pre-rRNA outcompetes a G-rich non-template rDNA for binding to a C-rich template DNA strand, forming a stable rRNA:rDNA hybrid. Under physiological conditions, R-loop formation at rDNA promoters prevents DNA methylation of CpG islands by DNA methyltransferase DNMT3B1, thus facilitating rDNA transcription [71]. R-loops are formed co-transcriptionally over the Pol I promoter, the 5′ external transcribed sequence and the 5′ region of the 18 S rDNA in yeast cells. Loss of Top1 and RNase H enhances this inherent formation of R-loops, which then cause severe Pol I pileups and consequent inhibition of pre-rRNA synthesis (Fig. 2) [56]. A subsequent study demonstrated that RS caused by aphidicolin, an inhibitor of replicative DNA polymerases, results in the formation of co-transcriptional R-loops at the 5′ ends of active human rDNA genes, and consequent DNA damage via R-loop-dependent mechanisms (Fig. 2) [71]. This model is further supported by experiments in which depletion of a negative regulator of rDNA transcription, the PHD finger protein 6 (PHF6), led to the formation R-loops and accumulation of R-loop-dependent rDNA damage in human nucleoli [72]. Mutations in the *PHF6* gene have been associated with the X-linked mental retardation disorder Borjeson–Forssman–Lehmann syndrome as well as with acute myeloid leukemia, T-cell acute lymphoblastic leukemia and hepatocellular carcinoma, implying that R-loop-mediated rDNA damage might contribute to the pathogenesis of these diverse diseases [73, 74]. Whether DSBs in spontaneous human cancers that frequently localize to transcriptionally active rDNA genes are generated through this mechanism remains untested [75]. Mutations in RP genes or ribosome biogenesis assembly factors can cause both inherited and sporadic human cancers [16, 76]. Given that excessive accumulation of nascent mRNAs facilitates the R-loop formation [49, 54], it is plausible that mutations impacting ribosome biogenesis impair pre-rRNA processing, leading to accumulation of unprocessed or partially processed pre-rRNA (Fig. 2). This may facilitate R-loop formation, and thereby interference with DNA replication, rDNA damage, genomic instability, and cancer.

An important question is whether oncogenes and loss of tumor suppressors contribute to cancer pathogenesis by causing rDNA damage. The rate of Pol I transcription is directly or indirectly upregulated by activation of a number of oncogenic pathways, including Myc, PI3K-Akt-mTORC1, Ras-MAPK, and cyclin E, the loss of tumor suppressors such as PTEN, pRb, p53, ARF, GSK3β, or dysregulation of factors that mediate epigenetic events at the rDNA locus (Fig. 2) [77]. It will be critical to assess whether dysregulation of different oncogenic pathways generates damage at specific sites within rDNA genes, given their differential impact on the rate of rDNA transcription, DNA replication process, ROS production and the rate of cell division (Fig. 2) [78–83]. Copies of rDNA that replicate during mid/late S phase show compact heterochromatic structure suggesting that they are difficult to replicate, particularly under conditions of RS [67]. Consistently, recent work suggests that old hematopoietic stem cells, which characteristically display RS owing to decreased expression of the mini-chromosome maintenance replicative helicase, feature enhanced chromosome breakage in late-replicating, heterochromatic rDNA clusters [84]. Such aberrant replication in rDNA could lead to accumulation of unreplicated or unresolved repair intermediates in mitosis and consequently structural and numerical chromosomal aberrations, as observed elsewhere in the genome [47].

Epigenetic status of the rDNA impacts the susceptibility of rDNA to breakage upon RS caused by folate deficiency, an essential factor for nucleotide biosynthesis. Folate deficiency enhanced DNA damage in the whole-genome [85], but it was particularly prominent in several specific regions of IGSs within rDNA clusters [86]. These breaks sites correspond to histone H3K4me1 mark sites and positively correlate with increased recruitment of UBF to rDNA genes and their enhanced transcription [86]. One could speculate that DNA breakage under these conditions is the consequence of clashes between transcriptional and replication machineries [86]. Despite this recent progress, however, mechanisms leading to rDNA damage remain poorly understood.

Repair of rDNA

Repair of DSBs in rDNA shows some atypical features, reflecting a combination of the more faithful homologous recombination (HR) repair pathway and the more error-prone non-homologous end-joining (NHEJ) pathway [87]. HR repair preserves rDNA stability when the rDNA template is the sister chromatid, yet such rDNA repair can also result in rDNA copy number alterations and genomic rearrangements if it is mediated by unequal sister chromatid recombination or recombination with homologous NOR-bearing chromosomes [88]. Unequal sister chromatid recombination may reflect malfunctional mechanisms that normally restrict the movement of sister chromatids relative to each other. For example, in yeast strains harboring low-rDNA-copy number most rDNA copies are actively transcribed, and the inability of condensin to bind transcriptionally active rDNA genes interferes with cohesion between sister chromatids at the rDNA loci, resulting in unequal sister chromatid recombination and rDNA instability [89]. In contrast, sufficient numbers of

Table 1 Inappropriate rDNA repair may lead to chromosomal rearrangements that contribute to cancer

Chromosomal rearrangements involving rDNA	Consequences
rDNA gain	Increased ribosome biogenesis
	Collision between Pol I transcription and replication machineries
rDNA loss	Increased transcription of the remaining rDNA genes
	Loss of nucleolar heterochromatin
	Alteration of the global epigenetic landscape
	Centromeric, pericentromeric and telomeric instability
	Altered condensin-dependent DNA repair
	Genome-wide dysregulation of gene expression
	Faster replication of the genome
	Increased sensitivity to DNA-damaging agents
Translocations *(dicentrinc, multiple jumping)*	Unknown

heterochromatically silent rDNA repeats facilitates condensin association and proper sister chromatid cohesion [89]. It is tempting to speculate that dysregulated balance between active and inactive rDNA genes could also affect the mode of HR at the rDNA genes in human cells via a similar mechanism.

The critical importance of proper regulation of replication and repair at rDNA clusters for tumor suppression was supported by studies of two human cancer-prone syndromes: Ataxia telangiectasia (A–T) and Bloom syndrome, which show 10-fold and 100-fold elevations of the rate of spontaneous alterations in the rDNA gene cluster architecture, respectively [75, 90]. A-T is caused by mutations in the *ATM* gene, the kinase product of which orchestrates DSB repair by HR, whereas Bloom syndrome is caused by mutations in the *BLM* gene encoding a helicase critical for the dissolution of Holliday junctions and abortive HR intermediates, structures that facilitate exchange of chromosome segments between sister chromatids or homologous chromosomes [91, 92]. rDNA gene clusters are also recombinatorial hot spots in human cancers, with over 50% of lung and colorectal carcinomas showing rDNA rearrangements [75, 93]. Furthermore, genomic rearrangements involving rDNA, including insertions, amplifications, and jumping translocations occur frequently in Hodgkin's lymphoma [94]. These observations indicate that the genomic rearrangements are positively selected for during tumor evolution and that the maintenance of rDNA stability may represent a major tumor-suppressive mechanism.

Notably, besides endogenous rDNA damage, rDNA recombination in human cancer may also reflect exogenously caused DNA damage, a notion consistent with the higher frequency of rDNA alterations in adult solid cancers compared with pediatric cancers [75]. Given that a small proportion of rDNA genes are arranged as inverted (palindromic) repeats, intermolecular recombination between such repeats on the various acrocentric chromosomes might generate dicentric chromosomal translocations linked by an rDNA cluster, somewhat reminiscent of the Robertsonian translocations [7]. It can also be hypothesized that the almost complete nucleotide sequence identity of DJ and PJ sequences surrounding rDNA clusters on acrocentric chromosomes, could predispose these regions as sites of recombination involving these chromosomes [7]. However, direct experimental evidence in support of these hypotheses is still lacking. As discussed above, illegitimate junctions of broken DNA ends during inappropriate DNA repair events may also result in rDNA copy number alterations [88, 95].

The impact of dysregulated rDNA copy number in cancer

It is plausible that more copies of rDNA genes may confer upon incipient cancer cells a competitive advantage over untransformed cells by providing an increased capacity for ribosome biogenesis and proteosynthesis [96]. Although this may occur in some tumors, recent studies identified lower rDNA copy numbers in several human cancer types compared with adjacent normal tissues [97, 98]. Interestingly, deletion of the mTORC1 negative regulator, tumor suppressor PTEN, in hematopoietic stem cells resulted in a lower rDNA copy number, which preceded their malignant transformation. Notably, these cells showed increased rates of rDNA transcription, proteosynthesis and proliferation, suggesting a compensatory increase in transcriptional activity of the remaining rDNA genes [97]. Furthermore, the finding that specific cancer types harboring lower rDNA copy number show mutational activation of mTORC1, suggests that increased mTORC1 activity might fuel rDNA damage and consequent inappropriate recombination and loss of rDNA copies, likely via mTORC1-dependent

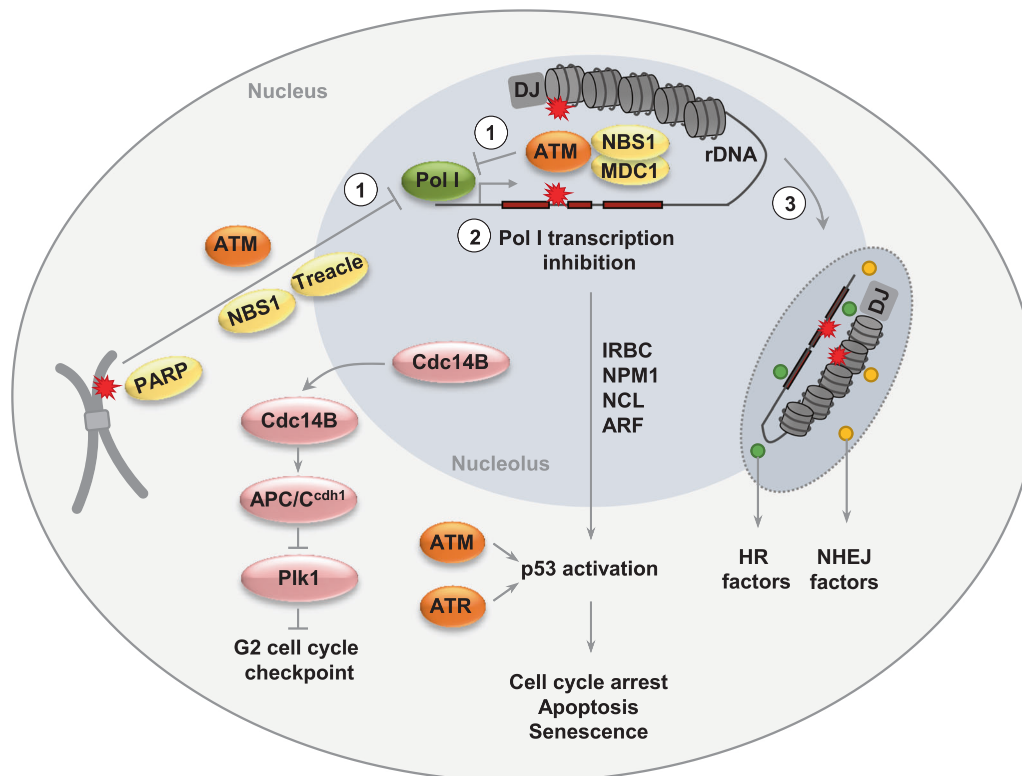

Fig. 3 Nucleolar response to nuclear or nucleolar DNA damage. DSBs in the nuclear chromatin cause Pol I inhibition in the nucleolus through an ATM-dependent signaling pathway, which includes NBS1, PARP, and Treacle. Pol I is also inhibited upon direct rDNA damage in an ATM-NBS1-MDC1-dependent manner. Inhibition of Pol I transcription is most likely responsible for the formation of nucleolar caps and relocalization of damaged rDNA to this structures, which are enriched in HR and NHEJ factors, thus providing an optimal environment for proper rDNA repair. Notably, cap formation was not observed upon nuclear DNA damage is several studies, in spite of Pol I inhibition. DDR and associated Pol I inhibition activate p53 through the impaired ribosome biogenesis checkpoint (IRBC), NMP1, NCL, ARF, ATM, and ATR. Upon genotoxic stress, Cdc14B is released from the nucleolus to the nucleoplasm where it promotes APC/C^{Cdh1}-dependent Plk1 degradation, triggering a G2-phase cell-cycle checkpoint. Asterisks indicate DSBs

upregulation of rDNA transcription [97, 99]. Consistent with such model, hyperactivation of mTOR in *Drosophila melanogaster* by mutations in components of the insulin/mTOR signaling axis or excessive nutrient uptake instigated loss of rDNA copies [100]. Mutations in the *TP53* gene have also been positively correlated with decreased rDNA copy numbers in several cancer types [98].

These studies provoke the question: how would cancer cells benefit from a decrease in rDNA copy number? A lower rDNA copy number could potentially decrease rDNA heterochromatin levels and alter the balance of nuclear heterochromatin/euchromatin, increase transcription of the remaining rDNA genes, dysregulate gene expression genome-wide and promote illegitimate recombination between highly repetitive DNA sequences (Table 1) [89, 101, 102]. Any or all of these features may explain why the rDNA loss could be positively selected for during cancer evolution [97]. Furthermore, mutations of genes required for heterochromatin formation in *Drosophila* and human cells led to decreased rDNA copy numbers and promoted global genome instability [11, 103]. These studies underscore the intimate link between rDNA copy number, global heterochromatin, and regulation of genome stability, with important implications for cancer pathogenesis. An additional explanation for how would cancer cells benefit from decreased rDNA copy numbers is that the loss of heterochromatic rDNA genes, which are hard-to-replicate, may allow faster replication of the genome, thereby providing the incipient cancer cells with a growth advantage (Table 1) [67, 97].

Ample evidence implicates dysregulated insulin/mTORC1 signaling and *TP53* mutations in cancer pathogenesis [104–108]. Indeed, decreased rDNA copy number may contribute to tumorigenesis driven via increased insulin/mTOR signaling activity or *TP53* aberrations [97, 98]. However, lower rDNA copy numbers may be necessary but not sufficient to drive cancer or they may be just a consequence of malignant transformation. These possibilities are consistent with the observation that many healthy individuals harbor low-rDNA copy number [97, 109].

DDR coordinates Pol I inhibition with rDNA repair

DDR signaling regulates multiple cellular activities, including inhibition of Pol I transcriptional activity (Fig. 3) [29], and promotion of nucleolar cap formation with recruited DNA repair factors, thereby preventing collisions with DNA repair machineries and providing an optimal environment for rDNA repair [29, 110, 111]. Two modes of DSB-induced Pol I transcriptional inhibition depend on ATM [29]. First, signaling from ionizing radiation (IR)-induced DSBs transiently represses rDNA transcription via ATM, Nijmegen breakage syndrome 1 (NBS1) and mediator of DNA-damage checkpoint protein 1 (MDC1) (Fig. 3) [112], accompanied by segregation of the nucleolar protein UBF into nucleolar caps. When DSBs were locally introduced using laser micro-irradiation in individual nucleoli, inhibition of rDNA transcription did not spread to surrounding nucleoli. Inhibition of Pol I was absent in ATM-null murine fibroblasts, NBS1- or MDC1-depleted cells upon IR [112]. Molecularly, the ATM-NBS1-MDC1 signaling interfered with Pol I initiation complex assembly followed by displacement of the elongating Pol I holoenzymes from rDNA [112]. Second, more recent studies described how inhibition of Pol I transcription occurs in response to DSBs introduced in chromatin outside the nucleoli (Fig. 3) [110, 113, 114]. This response featured ATM-dependent accumulation of NBS1 specifically in actively transcribed nucleolar regions of rDNA, temporally correlating with inhibition of rDNA transcription [114]. In this setting, inhibition of rDNA transcription occurred in all nucleoli of a cell even when DSBs were introduced outside of the nucleoli. Mechanistically, NBS1 recruitment into the nucleoli required interaction with the nucleolar protein Treacle (encoded by the *TCOF1* gene), but not the usual NBS1 partner, MRE11 (Fig. 3) [114].

Pol I activity can also be inhibited upon DNA damage in DNA-PK- and PARP-1-dependent manner [115]. Finally, the proteins TopBP1 and ATR also co-operate to inhibit pre-rRNA synthesis upon DNA damage and RS [116]. Collectively, available data suggest roles for ATM, ATR, and DNA-PK kinases in downregulation of rDNA transcription following DNA damage.

Where and how are DSBs in rDNA repaired in mammalian cells? Induction of DSBs within the rDNA repeats by either CRISPR-cas9 technology or I-PpoI expression resulted in nucleolar cap formation and downregulation of Pol I transcription in an ATM-dependent manner (Fig. 3) [110, 111]. Damaged rDNA moves from the nucleolar interior to the periphery where it is accessible to DNA repair factors (Fig. 3). NHEJ was the predominant mode of DSB repair [110] but DSBs within rDNA may also recruit the HR machinery throughout the cell cycle (Fig. 3) [95, 111]. DNA synthesis indicative of HR at damaged NORs was observed in G1 suggesting that damaged rDNA that cannot be re-joined by NHEJ factors is recognized and repaired by the HR machinery at the nucleolar periphery (Fig. 3) [111]. Chromosomal context may impact nuclear DSB signaling to nucleoli, as DSBs in the distal junction, a genomic sequence immediately distal to the rDNA arrays, did not inhibit rDNA transcription [111].

There are several putative nucleolar ATM targets of relevance for rDNA silencing, including Pol I subunits RPA34 and TAF1C [29]. PHF6 is another candidate ATM substrate, known to interact with UBF at the rDNA promoter region [29]. The importance of these ATM-dependent mechanisms is apparent in cells from A-T patients, which show a 10-fold increase in spontaneous rDNA alterations over normal cells [75, 90]. One can also speculate that a failure to mount these protective DDR responses in cancer cells may increase their vulnerability to genotoxic treatments.

Nucleolar stress responses triggered by DNA damage and oncogenic stress

The nucleolus responds to different types of DNA damage and oncogenic stressors by activating diverse signaling pathways that affect cell-cycle progression in both p53-dependent and -independent manners, trigger cellular senescence and promote DNA repair [117]. It is well established that upon impairment of ribosome biogenesis RPL11 (uL5) and RPL5 (uL18), trigger p53 activation [16, 118–124]. RPL11 and RPL5 activate p53 as constituents of the 5 S ribonucleoprotein particle that also contains 5 S rRNA [124–126]. This response was recently termed the Impaired Ribosome Biogenesis Checkpoint (IRBC) [127]. Mechanisticly, impairment of any step of ribosome biogenesis leads to redirection of the nascent IRBC from the assembly into pre-60S ribosomes towards inhibition of MDM2, the E3 ligase that negatively regulates p53 [124–126]. DDR signaling often converges on the inhibition of rDNA transcription, which may trigger activation of p53 by the IRBC-dependent mechanism (Fig. 3) [19]. In addition, oncogenic stresses also trigger the IRBC-mediated cellular senescence by enhancing rDNA transcription and delaying rRNA processing, further highlighting the complexity of the nucleolar stress signaling [128]. However, further research should uncover the role(s) of the IRBC-mediated mechanisms in cancer pathogenesis caused by DNA-damaging and oncogenic stressors.

In addition to the IRBC, DNA damage triggeres additional nucleolar signaling pathways, which may or may not co-operate with the IRBC in p53 activation (Fig. 3). For example, the protein interacting with carboxyl terminus 1

regulates the IRBC via interaction with RPL11 under impaired ribosome biogenesis [129] but also links ATM signaling to the IRBC upon DNA damage [130].

The tumor suppressor protein ARF acts as key activator of the p53 stress signaling pathway by inhibiting MDM2 in response to oncogene activation but is also involved in controlling nucleolar structure and limiting protein synthesis (Fig. 3) [131, 132]. Despite it was originally suggested that the ARF-MDM2 and IRBC-MDM2 pathways are distinct, it appears that the IRBC also sets the level of p53 activation by ARF [125].

The abundant nucleolar protein nucleophosmin (NPM1) interacts constitutively with ARF, sequestering it in the nucleolus [133]. Upon genotoxic stress, modifications of NPM1 lead to the release of ARF which then translocates to the nucleoplasm, where it inhibits MDM2. Notably, NPM1 directly binds MDM2 and p53 to regulate the MDM2-p53 pathway in an ARF-independent manner. For example, UV damage triggers NPM1 translocation from the nucleolus to the nucleoplasm where NPM1–MDM2 complexes are formed [134]. Interestingly, NPM1–ARF interaction may have an important role upon DNA damage in the absence of ATM. Active ATM phosphorylates protein phosphatase 1, which dephosphorylates NPM1 in the nucleolus, thereby weakening the binding between ARF and NPM1 [135]. This allows ARF to translocate to the nucleus where it is degraded by the ubiquitin ligase of ARF (ULF) [135]. In contrast, ATM loss promotes ARF accumulation, which may activate p53 and inhibit ribosome biogenesis, acting as a potential tumor suppressor back-up mechanism following DNA damage in cells with compromised ATM function.

Several other examples of the interplay between NPM1 and DDR mechanisms, particularly DNA repair pathways, have been identified. NPM1 is essential for embryonic development and its loss was associated with DNA damage and genome instability [136, 137]. Interestingly, NPM1 colocalizes with γH2AX following γ-irradiation (IR) and contributes to DSB repair [138]. NPM1 phosphorylated at residue Thr199 is recruited to IR-induced DSBs through K63-linked ubiquitination mediated by RNF8/RNF168 [138]. Furthermore, NPM1 is involved in DNA repair of ssDNA lesions by BER and NER repair pathways, reviewed in ref139. Similar to NPM1, another abundant nucleolar protein, nucleolin (NCL), associates with a number of DDR proteins near DSBs [140]. Reduced levels of NCL negatively affect accumulation of specific DDR proteins, DSB repair and weaken ATM-dependent cell-cycle checkpoints [140]. Analogous to NPM1, NCL interacts with MDM2, disrupting its association with p53 [141]. Furthermore, NCL binds both 5′ and 3′-UTR of p53 mRNA to repress its translation, but is outcompeted by ribosomal protein RPL26 following IR to boost p53 mRNA translation [142]. Other molecular interactions between NCL and the DDR machinery have been reviewed elsewhere [143]. Whether NPM1 and NCL are also involved in sensing and repair of potential rDNA damage and/or in sensing defective pre-rRNA processing remains to be determined.

Furthermore, dual specificity phosphatase cell division cycle 14B is also released from the nucleolus upon genotoxic stress to dephosphorylate and activate the anaphase-promoting complex/cyclosome/(APC/C^{Cdh1}) ubiquitin ligase in the nucleoplasm, leading to polo-like kinase degradation, and the consequent execution of a G2-phase cell-cycle checkpoint [144].

On the other hand, several signaling pathways previously linked to DNA damage are activated in cells upon specific impairments of ribosome biogenesis. The most convincing evidence so far points to activation of the ATR-Chk1-dependent G2/M cell-cycle checkpoint pathway in cells treated with low concentrations of actinomycin D, which specifically inhibits Pol I activity [145]. Additional studies showed that both the ATR-Chk1 and ATM-Chk2 signaling pathways are active in RP-deficient cells, for example in RPS19-depleted cells [146]. What could be the trigger for activation of these canonical DDR pathways under such conditions? The accumulation of pre-rRNA upon pre-rRNA processing defects may facilitate the formation of R-loops, leading to DNA RS and rDNA damage as discussed in Section 3 [147]. Moreover, the increased demand for rRNA in RP-deficient cells could lead to compensatory transcriptional activation of silent rDNA genes, with the ensuing DNA topological stress, R-loop formation and rDNA damage [147]. Finally, an imbalance in nucleotide pools owing to defects in pre-rRNA synthesis may also cause DNA RS and activation of the ATR/Chk1-signaling pathway [146].

In view of the extensive interaction between DDR and ribosome biogenesis it comes as no surprise that their functions are dysregulated in many cancer types and that the mechanism of action of many DNA-damaging chemotherapeutics used for cancer treatment involves either the inhibition of pre-rRNA synthesis or its maturation in both DDR-dependent and -independent manners [16, 19, 148]. However, the relative contribution of these ribosome biogenesis impairments to their anticancer efficacy still remains to be determined.

Role of the nucleolus in chromatin and genome organization

In addition to a decrease in rDNA copy number, disruption of the heterochromatin architecture surrounding nucleoli is seen in cells depleted of specific RPs, indicating that

alterations of the fine balance between ribosome biogenesis and chromatin organization could be an unanticipated mechanism by which RP-deficiencies mediate pathological phenotypes [76, 149].

Different protein complexes, in part through association with RNA genes transcribed by RNA pol III (5 S rRNA, tRNA), and RNA species (e.g., long non-coding RNA) may be involved in the establishment and maintenance of nucleolus-chromatin domain interactions. One nucleolus-chromatin tethering candidate is CCCTC-binding factor (CTCF), a sequence-specific DNA binding protein that acts as a transcriptional repressor, and delimits juxtaposed domains of active and inactive chromatin [150]. CTCF loss in HeLa cells resulted in nucleolar fragmentation and reduced rDNA silencing [151]. Similarly, in *Drosophila* cells, CTCF loss resulted in nucleolar fragmentation and activation of silent rDNA [152].

Some members of the nucleoplasmin/NPM1 family of histone chaperones [153] including NPM1, NPM2, and dNLP share the nucleolar localization and various nucleolar activities. In a landmark paper, the *Drosophila* nucleoplasmin-like protein (dNLP), CTCF, and the nucleolar protein Modulo (homolog of mammalian NCL), proved to be essential for the positioning of centromeres near the nucleolus [154]. Centromere clustering during interphase occur in different organisms and cell types, and often around the nucleolus. dNLP and CTCF were sufficient for clustering, whereas Modulo served as the nucleolar anchor. Interestingly, in this case, unclustering of centromeres resulted in spatial destabilization of pericentric heterochromatin, partial defects in the silencing of repetitive elements, defects during chromosome segregation, and subsequent genome instability [154]. Of particular interest is that NPM1 also interacts with CTCF in mammalian cells and may function in a similar fashion [150].

Another example comes from studies on the mammalian maternal oocyte nucleolus, a structure essential for early embryonic development in mammals [155, 156]. Zygotes derived from enucleolated oocytes exhibit abnormal heterochromatin formation around parental pericentromeric DNA, causing mitotic delay and frequent chromosome missegregation in the first mitotic division [156]. It is well known that the mouse nucleoplasmin-like protein Npm2 accumulates in oocyte nuclei and nucleoli, and its persistence there in preimplantation embryos is critical for nucleolar formation and embryonic development [157]. Npm2-deficient oocytes lack normal nucleolar structure, not being surrounded by heterochromatin, and display chromosome segregation defects similar to those in enucleolated oocytes [156]. Remarkably, expression of Npm2 alone was sufficient to reconstitute the nucleolar structure in enucleolated embryos and rescued the first mitotic division and full-term development [156].

Interestingly, the nucleolar phenotype in NPM1-depleted cells displays similarities to that observed in Npm2-deficient oocytes [158]. Moreover, cells derived from Npm1 knockout mice exhibit extensive genome instability and mitotic defects in analogy with Npm2 [158]. Chronic and acute NPM1 loss disrupts normal nucleolar morphology and triggers rearrangement of perinucleolar heterochromatin indicating a function of NPM1 in the spatial organization of nucleolus-associated heterochromatin similar to NPM2 [159].

All together, these findings further highlight the importance of the nucleolus as a platform for the organization of chromatin and regulation of genomic stability.

Conclusions

Intense research efforts over the last decade have suggested that the maintenance of rDNA stability is critical for normal structure and functions of the nucleoli but also for genome maintenance, global chromatin regulation, and genome-wide gene expression. It is also becoming evident that instability of rDNA gene clusters may be an early event in tumorigenesis and a driving force in human cancer. Quantification of rDNA copy number could be used in the future to gain a more precise insight into the extent of rDNA copy number variations in various cancer types [97]. Similar to yeast, the loss of rDNA copies increases the sensitivity of cancer cells to DNA-damaging agents (Table 1) [89, 97]. Thus, rDNA copy number could be a cancer patient stratification marker to guide genotoxic therapies. The observations that almost 10% of yeast genes are involved in the maintenance rDNA stability suggests that the mechanisms contributing to rDNA instability described in this review are most likely just the tip of the iceberg [160]. These genes are involved in numerous cellular processes including DNA repair, DNA metabolic processes, epigenetic regulation, transcription, amino-acid transport, protein modifications, ribosome biogenesis, protein synthesis, and regulation of cell cycle. Addressing roles of the human homologs of these genes in genome maintenance and cancer pathogenesis may be another fruitful avenue for future investigations. Given the observation that upregulation of mTOR activity by excessive nutrient uptake in *D. melanogaster* instigated loss of rDNA copies [100], future studies of this phenomenon are warranted in humans, particularly because the well documented links between overnutrition, obesity, high insulin levels and dyregulation of the mTORC1-signaling pathway with the pathogenesis of cancer [104–107]. Taken together, future studies on the role of the nucleolus in the regulation of genome stability could have huge implications for anticancer therapies as well as cancer prevention measures.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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