Chapter 30

Nucleolar Contributions to DNA-Damage Response and Genomic (In)Stability in the Nervous System

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Chapter Outline

1. INTRODUCTION

While cancer is recognized as a genomic instability disease, neurodegenerative disorders may also involve disturbed maintenance of the genome. For instance, DNA damage is extensively documented in various neurodegenerative conditions including Alzheimer's disease [1]. Moreover, mutations in various components of DNA-repair machinery produce neurodegenerative phenotypes in animals and humans [2,3]. In addition, despite being postmitotic cells, neurons are highly sensitive to various DNA-damaging agents [4]. Lastly, genomic instability may produce disease-causing mutations of specific loci triggering neurodegeneration [5]. Except these latter cases, role of DNA damage/genomic instability in neurodegeneration is not clear. In particular, neurotoxic mechanisms of DNA damage that are relevant for degeneration of mature neurons are poorly understood.

In proliferating cells, the nucleolus is a prominent subnuclear structure whose best recognized role is ribosomal biogenesis [6]. The nucleolus is not limited by a membrane but, instead, is formed around transcribed genes for nucleolar rRNA (47S rRNA in humans, rDNA). The rDNA is organized as clusters of repeated genes which in a human haploid genome include about 300 copies in 5 clusters that are located on acrocentric chromosomes. RNA-polymerase-1 (Pol1) transcribes rDNA initiating ribosomal biogenesis. The nucleolus is also a site of further steps of this critical process including rRNA processing and assembly of ribosomes. Inhibition of Pol1 disrupts ribosomal biogenesis and nucleolar localization of many proteins [7]. Such a nucleolar stress also leads to activation of the stress-activated transcription factor p53 [8]. Hence, the nucleolus may serve as a sensor for such stressors that inhibit Pol1. In addition, nucleolar accumulation of various components of ribonucleoprotein biogenesis machinery underlies its involvement in such nonribosomal roles as spliceosome assembly or generation of the signal recognition particle (SRP) [9]. By regulated sequestration of various important

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regulatory proteins of the cell cycle and/or differentiation programs, nucleolus affects these processes [9]. Finally, the nucleolus appears to play a structural role in genome organization [10–12].

In this review, the literature is discussed that suggests a role for the nucleolus in DNA-damage response (DDR) and genome maintenance in the brain. In the first part, nucleolar involvement in sensing DNA damage is presented. In the second part, the case for the nucleolus as a site of neurodegeneration-associated genomic instability is laid out.

2. NUCLEOLUS AS A SENSOR OF NEURONAL DNA DAMAGE

2.1 Effects of DNA Damage on the Nucleolus

The stress-sensing function of the nucleolus relies on the requirement of Pol1 activity for the maintenance of the nucleolar structure [8]. The Pol1-dependent nucleolar compartmentalization of various proteins provides a number of potential stress mediators which can be released upon Pol1 inhibition [8]. Importantly, the stress-sensing function of the nucleolus is able to operate in a wide range of cells provided that they contain an active nucleolus. Of note, many types of mature neurons including those highly prone to neurodegeneration (motoneurons, dopaminergic neurons of the substantia nigra, cerebellar Purkinje neurons) contain prominent nucleoli. Hence, the nucleolus-based stress-sensing mechanism could remain active in many types of differentiated cells including postmitotic neurons in the adult brain.

Consistent with such a concept, the structure of the neuronal nucleolus is sensitive to various types of injuries including DNA-damaging anticancer drugs, ionizing irradiation, hypoxia, and oxidants [13–18]. In many of these instances, it is likely that nucleolar disruption is a consequence of DNA lesions that directly interfere with rDNA transcription [4]. Alternatively, stress-activated signaling cascades including hydrogen peroxide-activated JNK2 or DNA double-strand break (DSB)-activated ATM may inhibit Pol1-driven transcription to induce the nucleolar stress [19–21].

2.2 Nucleolar Stress-Mediated Responses to Neuronal DNA Damage

A DNA damage–sensing role of Pol1 inhibition and the subsequent nucleolar stress have been proposed in developing neurons that are challenged with the DNA-damaging anticancer drug camptothecin (CPT) [22]. Camptothecin inhibits DNA topoisomerase-1 (Topo1) and induces DNA single-strand breaks (SSBs) as well as DNA-Topo1 adducts [23]. In cultured rat cortical neurons that were isolated from newborn animals, CPT blocked Pol1 activity and triggered nucleoplasmic translocation of the nucleolar marker protein nucleophosmin/B23 (Npm) [22]. Nmp translocation is a convenient marker of Pol1 inhibition in many cell types including proliferating cancer cell lines, cultured primary neurons and mature neurons in whole animal brain [17,24,25]. The CPT-induced nucleolar stress was followed by activation of the DNA damage–regulated pro-apoptotic transcription factor p53 and the p53-dependent apoptosis. Importantly, nucleoplasmic Npm translocation that indicates nucleolar stress also occurred if p53 and/or apoptosis were blocked [22]. Inhibition of Pol1 by knockdown of the specific Pol1 coactivator TIF1A was sufficient to induce p53-dependent neuronal apoptosis. Such a response required protein synthesis suggesting that the p53-mediated transcription of protein-coding genes was involved. This conclusion is also supported by observations that neuronal apoptosis was induced by the transcriptional inhibitor actinomycin D if it were applied at low concentrations which were relatively selective against Pol1 than the mRNA-transcribing RNA-polymerase-2 (Pol2). Of note, conditional knockout of TIF1A that was limited to the neuroprogenitor cells of the developing mouse brain was also followed by p53-dependent apoptosis [26]. Therefore, in immature neurons that are challenged with DNA damage that blocks Pol1-driven transcription, the p53-dependent apoptosis seems to be a direct response to the nucleolar stress.

One should emphasize the apparent specificity of the nucleolar stress response to only certain types of DNA damage. For instance, in cultured rat cortical neurons etoposide that blocks DNA toposisomerase-2 (Topo2) and produces SSBs, DSBs, and, Topo2-DNA adducts triggers nucleolar stress only at high concentrations that exceed 10 μM [25]. Conversely, the low concentration of 1 μM that is sufficient to induce strong DSB response does not induce nucleolar stress [25]. In addition, induction of DSBs within the 28S rRNA-coding region of rDNA using the *Physarum* endonuclease I–Ppo1 was insufficient to induce Pol1 inhibition and disrupt the nucleolus [25]. Such findings could be interpreted as evidence that in neurons, nucleolar stress is not a consequence of DSBs. Instead, SSBs and/or Topo–DNA adducts that accumulate in sufficient amounts after CPT and/or high concentration etoposide treatments are capable of triggering nucleolar stress, most likely by direct damage to rDNA and the subsequent interference with Pol1-driven transcription.

Interestingly, unlike neurons, proliferating cells were reported to show signs of nucleolar stress after DSB induction [20,21]. Such a response appeared to be caused by inhibition of Pol1 and required the DSB-activated DNA damage-signaling kinase ATM. Insensitivity of the neuronal nucleolus to DSBs may be part of the mechanism that underlies the well-established tolerance of such lesions by mature neurons [27]. Conversely, single-strand breaks and DNA adducts

that interfere with Pol1 activity and trigger nucleolar stress appear to be highly neurotoxic (for review see Ref. [4]). For instance, a 2014 study has documented the role of unrepaired Topo–DNA adducts as a likely trigger for neurodegeneration that is a major phenotypic manifestation of ATM deficiency in humans [28]. Moreover, it has been proposed that such a phenotype is directly related to transcriptional inhibition. Likewise, accumulation of DNA adducts was proposed as a trigger for neurodegeneration that is associated with deficiencies in nucleotide excision repair including Cockayne syndrome or xeroderma pigmentosum [3]. Finally, SSBs and various forms of oxidative adducts are present in the degenerating brain [1]. Therefore, these transcription-interfering lesions may trigger a nucleolar stress response in brain cells.

2.3 DNA Damage–Induced Nucleolar Stress in Intact Brain

Some forms of the DDR are developmentally restricted. For instance, DNA damage–induced apoptosis occurs in neuroblasts and early postmitotic neurons but not in mature neurons [25,29]. However, no developmental restriction was observed for nucleolar stress as it was present in brain neurons of either neonate or adult rats that received intracerebroventricular- or intracarotid etoposide injections, respectively [25]. Therefore, the nucleolus may participate in sensing at least some forms of DNA damage in both developing and mature neurons. Such a role is supported by observations of dispersed NPM in dopaminergic neurons of substantia nigra of adult mice that received the pro-oxidant neurotoxin MPTP [17]. Likewise, in human postmortem samples from that region, NPM dispersion was associated with Parkinson's diseases in which oxidative DNA damage is also documented [17,30].

2.4 Mediators of the Nucleolar Stress Response

The p53-mediated neuronal apoptosis is among consequences of DNA damage–induced nucleolar stress [22] (Fig. 30.1). Hence, the key question is that of the identity of the nucleolar stress–specific activators of the p53 pathway that trigger this response. While there are no published reports on such mediators in neurons, there is abundant literature on the mechanisms linking nucleolar stress and the p53 pathway in proliferating cells. Most attention in this respect was focused on regulation of the p53 ubiquitin ligase MDM2 (as reviewed in Ref. [31]). Under basal conditions, this protein promotes degradation of p53 preventing its accumulation and the p53-dependent responses including apoptosis (Fig. 30.1). Nucleolar stress triggers nucleoplasmic release of several proteins that may bind to MDM2 inhibiting its negative effects on p53. As a result, p53 becomes stabilized and capable of activating apoptosis and/or cell cycle arrest. Various nucleolar proteins were demonstrated to bind and inhibit MDM2 including Npm, nucleolin, and several ribosomal proteins (RPs). RPs are highly abundant components of the ribosomes. However, they also occur in relatively smaller quantities as free proteins. Such a free pool of

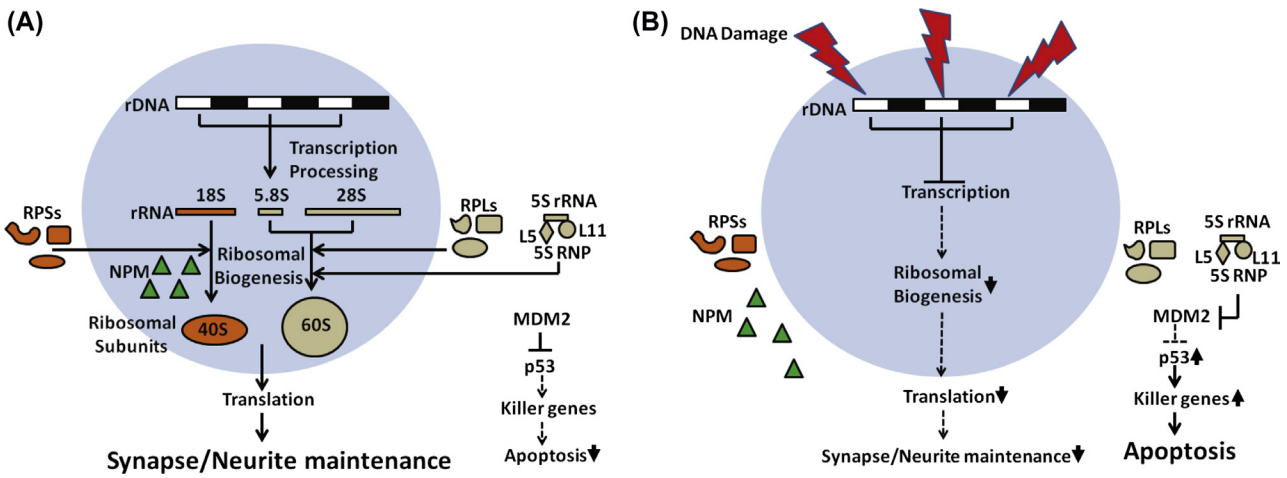

FIGURE 30.1 A hypothetical model illustrating role of the nucleolar stress in neuronal DNA–damage response. (A) Under normal conditions, nucleolus is maintained by ongoing transcription of the active copies of rDNA (white boxes) that initiates ribosomal biogenesis. Ribosomal proteins (RPSs and RPLs) as well as 5SrRNA are used for that process; under such conditions, MDM2 suppresses the p53 pathway including apoptosis. In addition, sufficient supply of ribosomes supports translation-mediated maintenance of neuronal structure and function. (B) Damage of rDNA inhibits its transcription disrupting ribosomal biogenesis. Ribosomal components including the 5S ribonucleoprotein (5S RNP) that is composed of 5S rRNA, RPL5, and RPL11 are no longer used for ribosome assembly. Thus, the 5S RNP inhibits MDM2 producing activation of p53. In immature neurons, that leads to the p53-mediated apoptosis. In the absence of apoptosis, chronic nucleolar stress may compromise neuronal translation and negatively affect structural as well as functional integrity of the neuron.

RPs is concentrated in the nucleolus and may be released when ribosomal biogenesis is blocked. It appears that L5 and L11 are two RPs playing a central role in p53 activation [31] (Fig. 30.1).

Together with 5S rRNA, these RPLs form the 5S ribonucleoprotein complex (5S RNP) that may inhibit MDM2. The MDM2 region that binds RPL11 has been identified and MDM2 knock-in mice were generated with a MDM2 point mutation (C305F) that selectively disrupts the RPL11 interaction [32]. In these mice, MDM2 ability to sense blockage of ribosomal biogenesis is impaired [32]. At least proliferating fibroblasts as well splenocytes and thymocytes from such animals did not appear to have any issues with activating p53 in response to various DNA-damaging agents including the Topo-1 inhibitor doxorubicin or SSB/DSB-inducing γ-irradiation. However, their p53 response to inhibition of ribosomal biogenesis was impaired. As neurons are postmitotic cells, their ability to engage the cell cycle–checkpoint-based sensing of DNA damage may be reduced. Therefore, it is tempting to speculate in these cells, relative contribution of the nucleolar stress to DNA damage–mediated activation of p53 may be greater than in fibroblasts. Indeed, preliminary observations suggest that L11 knockdown reduces neuronal apoptosis in response to not only TIF1A knockdown, but also to the Topo1 inhibitor CPT (M. Hetman and J. Hallgren, unpublished observations). Clearly, future studies are needed to determine which components of the nucleolus including specific RPs are involved in the neuronal responses to the DNA damage–induced nucleolar stress and whether p53 activation in such cells involves inhibition of MDM2.

Beyond p53, nucleolar stress may also engage other effector mechanisms to modulate neuronal survival. For instance, Npm has been shown to act as a chaperone for the pro-apoptotic protein Bax [33]. Moreover, in an experimental model of stroke, mitochondrial translocation of Npm has been proposed to increase Bax abundance/activity at this critical location for its death-promoting effects. Of note, in 2013, beneficial effects of a peptide that blocks Npm/Bax interactions was demonstrated using renal ischemia model [34]. PARP is another nucleolar protein which is released after nucleolar stress [35]. Also, in nonneuronal cells, PARP release from the nucleolus, sensitized cells to DNA damage [35]. It is tempting to speculate that a similar PARP-mediated sensitization may occur in neurons that are challenged with DNA damage that induces nucleolar stress. Lastly, Npm was shown to limit pro-excitotoxic activity of the GAPDH–SIAH1 complex in NMDA-treated cortical neurons [36]. Hence, one can consider a possibility that nucleolar stress may also promote neuronal survival.

In this context, one should note that the p53-dependent apoptosis is a consequence of nucleolar stress that is restricted to developing neurons [22,25,26]. Moreover, adult mouse neurons with conditional deletion of TIF1A survive for months despite absence of Pol1 activity, inhibition of mTOR signaling, mitochondrial impairment, and presence of oxidative stress [17,26,37]. Likewise, no apoptosis was found after transient disruption of neuronal nucleoli in adult rats that were treated with etoposide [25]. Hence, nucleolar stress may also activate a survival program that allows mature neurons to cope with unfavorable conditions. Finally, under hypoxia and/or oxidative damage, a transient inhibition of Pol1 may support survival by conserving energy [38]. Taken together, nucleolar stress may mediate neuronal responses to DNA damage that are not limited to cell death. In particular, one can consider a possibility that the nucleolar stress helps neurons to survive DNA damage.

2.5 Ribosomal Deficiency and Neurodegeneration as Consequences of Persistent Nucleolar Stress

While the DNA damage–induced nucleolar stress may activate stress-signaling response, it is also tempting to consider a possibility that if unrepaired, DNA damage may chronically impair ribosomal biogenesis compromising neuronal translation (Fig. 30.1). Since many critical processes including synaptic plasticity and synapse maintenance require translation, chronic ribosomal deficits could have profound negative effects on neuronal structure and function [39,40]. Thus, an interesting possibility emerges that neurodegeneration-associated accumulation of DNA damage induces neuronal atrophy and impairs neuronal function by inducing chronic deficiency of ribosomal biogenesis (Fig. 30.1). Indeed, conditional deletions of TIF1A in various populations of mature neurons produced chronic neurodegeneration [17,26,37]. Moreover, dendritic atrophy was observed after inhibition of ribosomal biogenesis in cultured hippocampal neurons with established dendritic trees [40a]. In that case, dendritic degeneration was accompanied by appearance of RNA stress granules which mark translational inhibition and are often found in various neurodegenerative diseases including AD [41]. Likewise, a causative connection has been proposed between oxidative DNA damage, nucleolar disruption, ribosomal deficits, and neurodegeneration that have been documented in cerebellar Purkinje neurons of the *pcd*-mutant mice [18].

Interestingly, reduced nucleolar size indicative of Pol1 inhibition has been reported in neurons from brain regions that are affected by AD [42,43]. Conversely, in PD, reduced nucleolar size and nucleolar stress have been observed in the *substantia nigra* [17,44].

Importantly, analysis of postmortem samples of AD brain revealed reduced numbers of ribosomes and/or extensive oxidation of rRNA in the AD-affected regions including in hippocampus and the parietal cortex [45,46]. Such deficits were associated with decreased protein synthesis [45,46]. Oxidation of rRNA as well as ribosomal defects have been found in mild cognitive impairment (MCI) which often represents an early stage AD [46]. AD-associated hypermethylation of the rDNA promoter was also observed [47]. Such epigenetic silencing of the nucleolar rRNA genes was most pronounced at the early stages of AD.

However, causative relationship between DNA damage, nucleolar deficits, and neurodegeneration remains to be established. Although, currently, such a relationship is not certain, one can consider an interesting possibility that rescue of nucleolar defects can improve structural integrity and function of the brain regions that are challenged with neurodegenerative pathologies such as AD. Indeed, nucleolar hypertrophy has been found in cortical and hippocampal neurons from asymptomatic patients with AD pathology [43].

3. NEURODEGENERATION-ASSOCIATED INSTABILITY OF rDNA

Various consequences of aging including proliferative senescence, cancer, and neurodegeneration have been proposed to be caused by accumulation of DNA damage and subsequent genomic instability [27,48]. For instance, loss of telomeres as well as de-regulated homologous recombination (HR) have been associated with aging-related disorders [48,49]. In AD, telomere shortening that in proliferating cells triggers DDR and cell cycle arrest may have complex effects including neuroprotective reduction of inflammation but also increased neuronal dysfunction [50]. The nucleolar rDNA is another site of genomic instability whose consequences started to emerge only recently. In addition, instability of rDNA has also been documented in human neurodegeneration [47,51]. Here, potential mechanisms and consequences of rDNA instability in the degenerating brain will be presented.

3.1 Consequences of rDNA Instability in Nonneuronal Systems

In early 1970s, it has been proposed that in nondividing mammalian cells including neurons, aging is associated with reduced number of rDNA copies [52]. It has been further hypothesized that such a reduction may underlie aging-associated decline in protein synthesis by reducing ribosomal biogenesis due to insufficient number of rRNA genes. However, other studies revealed no aging-associated changes in rDNA copy number in mouse or human brain [53,54]. Moreover, in chickens, fruit flies, and yeast, it was demonstrated that relatively few rRNA genes are fully sufficient to meet all the ribosomal biogenesis needs including periods of rapid proliferative growth which requires high rates of ribosomal generation [55–57].

In 1990s, the concept of rDNA instability as a component of aging got new support from work in a yeast model of replicative senescence. In that model, a correlation has been established between excessive rDNA recombination and the senescence-associated inability by the mother cell to produce offspring [58]. Moreover, accumulation of extrachromosomal rDNA circles (ERCs) rather than deficient ribosomal biogenesis has been proposed as a toxic mechanism underlying such a phenotype [58].

Subsequent studies confirmed that instability of rDNA contributes to yeast replicative aging (for review see Ref. [12]). However, such an effect appeared to be a direct consequence of rDNA loss rather than the secondary accumulation of ERCs [59]. While deficient ribosomal biogenesis prolongs replicative life span of yeast [60], a hypothesis has been proposed that loss of rDNA induces aging not by impairment of ribosome generation but by activation of the DDR [61]. According to this hypothesis, DDR activation would be a consequence of insufficient buffering capacity of DDR mediators that is provided by rDNA and would be similar to the DDR activation following loss of telomeres. In addition, structural role of rDNA in stabilization of yeast genome has been also proposed. Such an effect could be mediated by promoting chromatid cohesion to enable recombination-mediated amplification countering rDNA loss. Thus, DDR but not ribosomal insufficiency would lead to growth arrest and cell senescence. In support of such a possibility, yeast strains with decreasing content of rDNA were shown to have increased sensitivity to DNA damage [57]. Interestingly, such sensitivity was dependent on increased transcription of the few remaining rDNA genes as the high rate of transcription interfered with rDNA repair.

These and other yeast studies prompted a proposal of the rDNA hypothesis of aging [12]. This hypothesis claims that due to its high transcriptional activity, rDNA is hypersensitive to aging-associated DNA damage which in turn triggers its instability due to activation of DNA repair. Then, the rDNA instability becomes a direct cause for the aging-associated DDR and the replicative senescence.

Fruit fly is another experimental system in which consequences of rDNA instability have been directly investigated. By stimulating rDNA recombination with the rDNA-specific endonuclease I-CreI, a series of fly strains was generated with a defined content of rDNA [56]. While ribosomal supply defects were obvious when rDNA copy number was below the

minimally required threshold for appropriate levels of ribosomal biogenesis, additional nonribosomal effects were also identified in those strains with adequate number of ribosomes. For instance, changes in rDNA copy number affected the general content of heterochromatin [10]. Specifically, high or low rDNA content was associated with high or low levels of heterochromatin markers, respectively. Likewise, the strength of heterochromatin-mediated regulation of gene expression was directly correlated with the amount of rDNA.

Effects of variation in rDNA copy number were also observed in the euchromatin [62]. For instance, relatively small but widespread changes of gene expression were observed in fly strains with different rDNA content. Similar effects were observed not only in the engineered mutants of rDNA, but also strains with natural variation in rDNA copy number. The strongest influence of rDNA copy number was noted on genes involved in mitochondrial function and lipid metabolism.

Finally, there are some correlative data that suggest role of rDNA in heterochromatin maintenance in mammals. Thus, in a mouse cell line, loss of heterochromatin coincided with loss of rDNA [63]. Such effects were observed after perturbing rDNA silencing by knocking down the critical component of the nucleolar repressive complex (NoRC), Tip5/Baz2b [63]. Interestingly, loss of constitutive heterochromatin including that at centromere and telomere regions was also observed in human cell lines with knock down of NoRC [11]. Growth arrest, defects in chromosome segregation, and genomic instability were among outcomes of such a deficiency. In addition, in human cancer cell line HeLa, analysis of nucleolus-associated chromatin revealed enrichment for repetitive elements as well as gene families with monoallelic expression limited to highly specialized cells including immunoglobulin receptors, T-cell antigen receptors, and odorant receptors [64]. Genes that are expressed during brief periods of embryonic development were also overrepresented suggesting that nucleolar association characterizes silent chromatin. Indeed, this nucleolus-associated domain (NAD) was enriched in repressive histone marks suggesting its heterochromatic nature [64]. Of note, differences have been observed between NAD and another well-established heterochromatin region, the lamina-associated domain (LAD). For instance, NAD also contained transcriptionally active RNA genes that are transcribed by RNA-Polymerase-3 (Pol3) including tRNA and 5S RNA. Hence, in mammals, rDNA may be of particular importance for maintenance of specific domain of heterochromatin that combines silenced and active genes. It remains to be determined whether such role of rDNA is by providing a nearby source of NoRC activity for the maintenance of this domain, or by supplying noncoding RNAs that are required for heterochromatin formation or by structural effects including interactions with cohesins.

3.2 Mechanisms of rDNA Instability in Nonneuronal Systems

In yeast, instability of rDNA is mediated by HR [65]. These mechanisms operate during DNA replication and involve unequal sister chromatid exchange leading to rDNA loss or rDNA expansion. The rDNA loss and expansion appear to balance each other in nonsenescent yeast cells [61].

The key determinants of rDNA instability include DNA sequences in the noncoding portion of the yeast rDNA unit (the intergenic spacer, IGS) (for review see Refs. [12,61]). First, the replication fork block (RFB) site binds the nuclease Fob1 that induces a recombinogenic DSB to stimulate HR. Second, RNA-Polymerase-2-mediated transcription from the E-Pro promoter displaces cohesins. It has been proposed that such events lead to unequal sister chromatid recombination leading to expansion of rDNA. In addition, the noncoding IGS transcript appears to promote unequal sister chromatid recombination by forming the recombinogenic RNA:DNA hybrid (R loop) which may collide with the replication fork [66]. Stability of rDNA is, therefore, regulated by the activity of another IGS site with the replication origin activity [12]. Its strength is closely correlated with rDNA stability and lifespan of yeast strains. In addition, Sir2 stabilizes rDNA by silencing the E-Pro-driven transcription. Likewise, the yeast ataxin-2 Pbp-1 prevents R-loop formation stabilizing rDNA [66]. Thus, loss of Fob1 or Sir2 and Pbp1 extends or shortens yeast life span, respectively [12,66]. In addition, deletions of HR components counteract increased DNA damage sensitivity in yeast strains with low copy number of rDNA copies [57]. Noncoding antisense transcription of rRNA has been also shown to be a source unequal sister chromatid recombination within rDNA [67]. Such a recombinogenic activity was inhibited by Dicer [67]. Finally, rDNA stabilizing role of the yeast chromatin structure is illustrated by instability of rDNA after disruption of its tethering to the nuclear membrane [68].

Critical role of HR control within rDNA is further illustrated by compartmentalization of HR machinery. Thus, its critical component, Rad52 is generally excluded from the nucleolus where the rDNA is located [69]. Rad52 interacts with rDNA DSBs only after a transient exit of the affected rDNA region from the nucleolus. Such a compartmentalization is at least in part due to sumoylation of Rad52. Conversely, anti-recombinogenic regulators including DNA helicase Srs2 have been found in proximity of rDNA [69].

Mechanisms of rDNA instability in higher eukaryotes are not clear. However, RFB activity was demonstrated in human rDNA IGS [70]. As in yeast, loss of HR control appears to destabilize human rDNA as its remarkable instability has been reported in cell lines from patients with the defective human DNA helicase BLM [71]. Like Srs2, BLM inhibits HR.

Moderate instability of human rDNA that was present in ATM-deficient cells may be also a result of increased HR as unrepaired DSBs may provide an additional stimulus for that process [71]. Finally, a study conducted in 2015 using targeted DSBs inside rDNA revealed similar HR-based repair compartmentalization as that observed in yeast [21]. Therefore, HR-based instability of mammalian rDNA appears to be suppressed by anti-recombinogenic activity, efficient DSB repair, and restricted access of HR to transcriptionally active regions of the nucleolus. Last but not least, epigenetic silencing and the resulting changes in chromatin structure appear as important factors promoting stability of mammalian rDNA.

Interestingly, nutrient availability affects rDNA stability of the Y-chromosome-linked rDNA cluster of fruit flies [72]. Thus, diet enriched in yeast extract has been shown to induce rDNA instability resulting in rDNA loss. Such changes were persistent and occurred both in germ line and in somatic cells. Interestingly, mTOR was required for this effect. Increased insulin receptor signaling mimicked effect of enriched diet on rDNA stability. Lastly, inhibition of rDNA transcription with actinomycin D improved rDNA stability. Such observations suggest that HR-based destabilization of rDNA is promoted when high nutrient supply increases Pol1 activity. In addition, it has been proposed that rDNA stabilization may at least partially contribute to antiaging effects of caloric restriction.

3.3 Evidence of rDNA Instability in the Brain

Early evidence that suggested rDNA loss in the brains of aged dogs, mice, and people has not been confirmed by follow-up studies [53,54]. Moreover, in 2014 a study using quantitative real-time PCR methodology revealed no apparent loss of rDNA in human parietal cortex [51]. Therefore, unlike in yeast, brain rDNA seems to be stable during normal aging. There are, however, some limitations to this conclusion. First, the number of individuals that were included in this study was relatively low including 14 young and 9 old individuals. While such sample size allows excluding big effects of aging on rDNA content, smaller changes may require greater number of cases to reach statistical significance. Indeed, far larger group ($n = 120$) was investigated to demonstrate modest age-associated declines in rDNA content of human adipose tissue [73]. In addition, age-associated rDNA loss may be limited to only some cell types such as neurons. In such a case, tissue homogenate-based assay would miss and/or underestimate rDNA loss as in mature human brain neurons are outnumbered by glia. Future studies using DNA from selectively dissected neurons and glia could unequivocally solve the issue of aging-associated rDNA instability in these cells.

However, the qPCR-based analysis of total genomic DNA from cerebrocortical tissue revealed instability of rDNA in two different forms of age-associated neurodegeneration. AD is the most common form of dementia [74,75]. Alzheimer's disease (AD) is associated with excessive production of amyloid-β leading to tau pathology, synapse loss, neuronal atrophy, and subsequent neuronal death. Such changes occur throughout the forebrain including the hippocampus and the cortex. AD pathology appears to develop first in the temporal lobe, then in the parieto-occipital cortex and finally in other cortical areas including the prefrontal cortex. In postmortem cerebrocortical samples from AD patients, increased genomic content of the rDNA 18S-coding region was observed in both the parietal and the prefrontal cortex [47]. In addition, similar expansion of rDNA was also found in parietal cortex samples from patients with mild cognitive impairment (MCI), which, in most cases, represents early stages of AD. The relative increase of rDNA ranged between 1.5- and 2.4-fold of age-matched controls as compared to the genomic content of the *tRNA-K* (CTT anticodon) gene whose 17 copies are dispersed throughout several human chromosomes (http://gtrnadb.ucsc.edu/).

To determine whether such a change may be unique for AD, rDNA content was analyzed in dementia with Lewy bodies (DLB) [51]. DLB is the second most common type of dementia after AD [76] (http://www.omim.org/entry/127750). It is associated with intraneuronal accumulation of α-synuclein-containing inclusions, the Lewy bodies. In DLB, Lewy bodies accumulate in the cerebral cortex including parietal cortex. Progressive neurodegeneration appears to be a consequence of such a cortical synucleinopathy as α- or β-synuclein mutations have been associated with rare familial cases of DLB. As the causative relationship between the synuclein pathology and neurodegeneration is also present in Parkinson's disease (PD), DLB and PD may represent the same neurodegenerative process that affects different regions of the brain. Interestingly, PD and DLB may also coincide with pathology found in both the nigral and cortical neurons. Therefore, although DLB affects similar brain areas as AD, the underlying pathologies of these two forms of dementia are different.

In postmortem parietal cortex samples of DLB patients, increased genomic content of rDNA was observed using three different rDNA amplicons that probed 18S-, 5.8S-, or 28S-coding regions of rDNA [51]. Importantly, similar results were obtained when a multiplied- or a single locus gene was used as a total genomic DNA normalizer (*tRNA-K*^{ctt} or *ALB*, respectively). The magnitude of rDNA amplification was estimated between 1.5 and 2.3-fold of age-matched controls. Surprisingly, when the DLB pathology-free cerebellum was analyzed, nearly 50% reduction of rDNA content was found in DLB patients using 5.8S and 28S amplicons. Therefore, similarly to AD, DLB appears to be associated with rDNA instability.

As in AD, rDNA expanded in the pathology-affected cerebral cortex. In contrast, rDNA loss was found in the pathology-free cerebellum. These findings suggest that at least in DLB, rDNA becomes unstable in neurodegeneration-affected and unaffected brain regions. In the degenerating areas, the rDNA expansions would be the predominant product of such a destabilization; in the nondegenerating areas, rDNA instability would result in rDNA loss. Similar divergence has been reported for another unstable region of the genome, the telomere. Telomere contraction has been found in the peripheral blood leukocytes in AD, and DLB [77–79]. However, at least in AD, telomere expansion has been observed in the hippocampus that is affected by AD pathology at the early stages of this disease [78].

Although somatic rDNA instability appears to be the most probable source of the observed differences between the DLB and the control group, one cannot formally exclude a possibility that in addition to rDNA instability, the observed effects of neurodegeneration on brain rDNA content are also affected by germ line-derived rDNA copy number polymorphisms that are associated with an increased risk of AD or DLB. Thus, expanding the sample sizes and including material from other nonbrain tissues could help to examine such a possibility. However, at least for DLB, the demonstrated divergence between the cerebral cortex and the cerebellum argues for somatic instability as a major source of the observed variation. Indeed, mitotic recombination of human rDNA has been previously reported in normal individuals [80]. Interestingly, mitotic instability of rDNA has been also found in at least 50% of human colon and lung cancer samples [81].

3.4 Potential Mechanisms and Significance of Neurodegeneration-Associated Instability of rDNA

Because HR has been identified as a major mechanism of rDNA recombination [61,65], findings of neurodegeneration-associated instability in this region suggest that HR becomes activated in the brain. HR is thought to occur during or after DNA replication peaking in the S phase of the cell cycle [82]. Hence, the observed rDNA instability may be localized to reactive glia that underwent divisions in response to neuronal loss [83]. At least in AD-affected brain regions, neuronal cell–cycle reentry has been also documented including DNA synthesis [84]. Therefore, neurons that reenter the cell cycle could also activate the HR.

As human rDNA is present in five clusters on five distinct chromosomes that all reside in close physical proximity within the nucleolus and the perinucleolar heterochromatin, rDNA may also undergo HR in the absence of the replication-generated sister chromatids. Such a "nonreplicative" recombination could engage homologous rDNA units within the same chromatid or from different chromosomes [49]. Years 2012 and 2013 reports have documented HR-like activity of nonmitotic human somatic cells in G_0 [85,86]. Because such activity required the recombining DNA to be transcribed, this noncanonical HR may be possible at the active rDNA units. In addition, in G_1 HeLa cells, rDNA DSBs can trigger activation of HR and unscheduled DNA synthesis in the perinucleolar region where such lesions are translocated [21]. Therefore, HR of rDNA may also occur in cells with unreplicated genomes including most neurons.

Although major components of the HR machinery are not expressed in normal mature mammalian brain (http://www.brain-map.org/), there are no data on their expression/activity in the degenerating brain. Moreover, the reported expansion of telomeres in the AD hippocampus suggests HR is active in that tissue as HR is presumed to be a major positive regulator of telomere length in most somatic cells in which telomerase is not expressed [78,82].

While rDNA instability is a direct result of rDNA damage, recombinogenic potential of this region may be regulated by changes in chromatin structure, Pol1 activity, and noncoding RNAs as discussed in Section 3.2. Thus, in the neurodegeneration-affected human brain tissue, altered epigenetic status of rDNA and/or changes to Pol1 activity and/or altered ncRNA expression/processing may co-operate with DNA damage to destabilize rDNA.

Loss of heterochromatin was reported in AD and proposed to drive neurodegeneration in a fruit fly tauopathy model [87]. However, rDNA methylation analysis revealed increased presence of this repressive chromatin mark in rDNA from AD brains and no changes in DLB [47,51]. Thus, it remains to be tested whether neurodegeneration-associated instability of rDNA is due to loss of perinucleolar heterochromatin. At least in AD, increased rDNA promoter methylation and ribosomal deficits could suggest transcriptional insufficiency of Pol1 as a possible trigger of instability of rDNA [45–47]. Conversely, nucleolar hypertrophy that was observed in some cells of AD brain suggests that in some cells Pol1 activity increases [43]. Such an increase could also promote rDNA instability. Finally, as RNA metabolism changes are well documented in various neurodegenerative diseases including AD, excessive R-loop formation in rDNA could contribute to rDNA instability in the degenerating brain [41].

Recombination of rDNA is initiated by DNA damage [65,82]. DSBs that are the major recombinogenic form of DNA damage have been reported in normal mouse forebrain neurons following periods of increased physiological neuronal activity [88]. Moreover, DSB repair appeared to be impaired in a mouse model of AD-like amyloidosis. In addition, bulky DNA adducts and single-strand DNA gaps that are generated by the adduct removal via the nucleotide

excision repair may also activate the HR pathway [82,89]. Therefore, in the degenerating brain, instability of rDNA may be initiated by excessive DNA damage (Fig. 30.2).

What may be possible consequences of rDNA destabilization in human brain? The reported changes in rDNA content are unlikely to affect ribosomal biogenesis as only a fraction of rDNA units is required for efficient ribosome supply even during accelerated growth in development [55,56]. Thus, one can consider the ribosome-unrelated effects of altered rDNA content as discussed in Section 3.1. For instance, rDNA amplification may increase structural support for cohesion- and/or NoRC-mediated stabilization of the genome (Fig. 30.2). Indeed, the recently suggested AD-associated loss of heterochromatin could promote defense mechanisms supporting its expansion (Fig. 30.2). Effects of rDNA content on euchromatin gene expression are another interesting possibility of rDNA interaction with the neurodegenerative pathology [62]. Enrichment of rDNA-regulated genes that affect mitochondrial function and lipid metabolism fit well with the AD- and DLB-associated dysfunction of the mitochondria and/or dysregulation of brain lipid homeostasis [90–93]. Last but not least, yeast studies led to a proposition that the inactive copies of rDNA sequester mediators of the cytotoxic DDR pathway [57,61]. Of note, in mammalian cells, stress-induced sequestration of various proteins has been demonstrated by the rRNA noncoding regions of rDNA [94]. Therefore, changes in rDNA copy number may affect cellular stress response including that to DNA damage.

A working model may be proposed that increasing content of rDNA would be beneficial for the brain cells by promoting heterochromatin formation, tighter regulation of euchromatic genes and stronger capacity to regulate the DDR. The opposite may be true if rDNA content declines. Therefore, increases of rDNA in the degenerating cerebral cortex may support cell survival. Such an effect could explain the apparent discrepancy between the cerebellar- and the cerebrocortical rDNA content in DLB. As in DLB, the cerebellum is not confronted with the pathology/cell loss, there is no selection factor against suboptimal genomic arrangements resulting in overall reduction of rDNA content. In the cortex, cell death would produce enrichment of most resistant cells with expanded rDNA. One could speculate that similar mechanisms may promote rDNA copy number variability in cancer cells helping them to adapt to changes in cellular environment including therapy resistance.

4. CONCLUDING REMARKS

The nucleolus-based process of ribosomal biogenesis emerges as an important participant in the neuronal DDR acting both as a sensor and a source of transducers for various DNA damage signals. In addition, changes of rDNA content my further modulate brain tissue response to stress including DNA damage and neurodegeneration. Thus, the nucleolus may exert multilayer influence on the nervous system that is coping with an injury. Such an influence may involve its most canonical function of making ribosomes and its core structure of rDNA. The key challenge for the future research is to directly evaluate contributions of the nucleolar stress pathway as well as rDNA instability to the nervous system maintenance in physiological and pathological conditions. Thus, identification of nucleolar stress mediators could set a stage for experiments to directly evaluate role of nucleolar stress in animal models of neurodegenerative diseases. Moreover, transgenic technologies could be harnessed to determine how changes in rDNA content determine outcome of neurodegeneration. As such studies address the fundamental question of the mechanisms that underlie age-associated neurodegenerative diseases, they deserve significant attention of the research community.

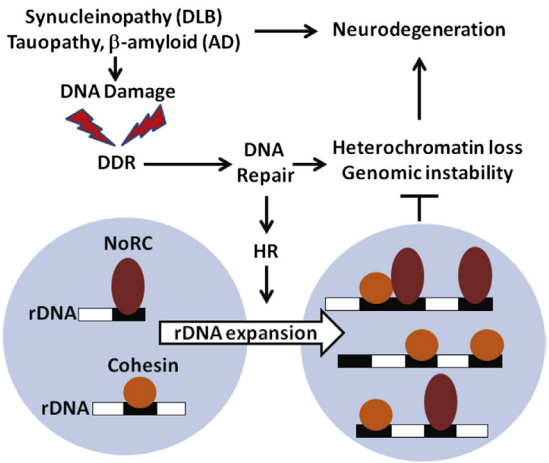

FIGURE 30.2 A hypothetical model presenting potential mechanisms and consequences of the neurodegeneration-associated rDNA expansion. The rDNA expansion that occurs in Alzheimer's diseases (AD) and in dementia with Lewy bodies (DLB) may be a consequence of increased DNA damage that activates the DNA-damage response (DDR) including rDNA repair via homologous recombination (HR). Additional copies of rDNA may provide stronger structural support for the chromatin. Such a support could include cohesion-mediated inter- and intrachromosomal interactions and/or increased supply of silencing complexes such as the NoRC. As in yeast, structural role of rDNA may be fulfilled by the inactive copies of rDNA (black). Thus, expanded rDNA would promote genomic stability and heterochromatin maintenance counteracting cytotoxic consequences of the neurodegenerative pathologies.

GLOSSARY

Extrachromosomal rDNA circles This is a form of extrachromosomal DNA that is observed in yeast. It is made of circular rDNA fragments that are generated by homologous recombination and which replicate during cell divisions.

Mild cognitive impairment (MCI) A brain function disorder involving cognitive impairments beyond those expected based on the age of the individual, but which are not severe enough to interfere with daily activities. It often represents a transitional stage between normal aging and dementia.

rDNA Ribosomal DNA, rRNA genes.

LIST OF ABBREVIATIONS

5S RNP 5S ribonucleoprotein
AD Alzheimer's disease
CPT Camptothecin
DDR DNA-damage response
DLB Dementia with Lewy bodies
DSB Double-strand break
HR Homologous recombination
mTOR Mammalian target of rapamycin
NoRC Nucleolar-repressive complex
Npm Nucleophosmin
PD Parkinson's disease
Pol1 RNA polymerase-1
Pol2 RNA polymerase-2
rDNA Ribosomal DNA
RP Ribosomal protein
SSB Single-strand break
TIF1A Transcription initiation factor-1A
Topo1 DNA topoisomerase-1
Topo2 DNA topoisomerase-2

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