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NEURODEGENERATION-ASSOCIATED INSTABILITY OF RIBOSOMAL DNA

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B.S. Western Michigan University 2006
M.S. Indiana State University 2009

A Thesis Submitted to the Faculty of the
School of Medicine of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Master of Science

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ABSTRACT

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Justin L. Hallgren

January 16, 2014

Homologous recombination-mediated instability of the repetitively organized ribosomal DNA has been proposed as a mediator of cell senescence in yeast triggering the DNA damage response. High individual variability in the content of human ribosomal DNA suggests that this genomic region remained relatively unstable throughout evolution. Therefore, quantitative real time PCR was used to determine the genomic content of ribosomal DNA in *post mortem* samples of parietal cortex from 14 young and 9 elderly individuals with no diagnosis of a chronic neurodegenerative/neurological disease. In addition, ribosomal DNA content in that brain region was compared between 10 age-matched control individuals and 10 patients with dementia with Lewy bodies which involves neurodegeneration of the cerebral cortex. Probing ribosomal RNA-coding regions of ribosomal DNA revealed no effects of aging on the ribosomal DNA content. Elevated ribosomal DNA content was observed in Dementia with Lewy Bodies. Conversely, in the Dementia with Lewy Bodies pathology-free cerebellum, lower genomic content of ribosomal DNA was present in the Dementia with Lewy Bodies group. In the parietal cortex, such a Dementia with Lewy Bodies-associated instability of ribosomal DNA was not accompanied by any major changes of CpG methylation of the

ribosomal DNA promoter. As increased cerebro-cortical ribosomal DNA content was previously reported in Alzheimer's diseases, neurodegeneration appears to be associated with instability of ribosomal DNA. The hypothetical origins and consequences of this phenomenon are discussed including possibilities that the DNA damage-induced recombination destabilizes ribosomal DNA and that differential content of ribosomal DNA affects heterochromatin formation, gene expression and/or DNA damage response.

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INTRODUCTION

The age related accumulation of DNA damage, and the subsequent genomic instability which follows, has been proposed as a mechanism for proliferative senescence, cancer, and neurodegeneration, all of which are associated with aging or aging related disorders [1-3]. DNA damage is well documented in neurodegeneration and in some cases it may be sufficient to cause disease [1, 4]. Moreover, significant increases in oxidative DNA damage are known to occur in Alzheimer's disease, Parkinson's disease, and Dementia with Lewy Bodies [5, 6]. Additionally, multiple congenital diseases involving mutations of DNA repair proteins often involve neurodegeneration. These diseases include ataxia with oculomotor apraxia 1, spinocerebellar ataxia with axonal neuropathy 1 and xeroderma pigmentosum [1, 4, 7-9]. Moreover, DNA damage is well known to induce cell death by a number of mechanisms including inhibition of nucleolar transcription and genomic instability [4, 10].

Homologous recombination is believed to be the preferred DNA damage repair mechanism as it produces the highest fidelity, while other repair mechanisms such as non-homologous end joining are known to frequently generate single nucleotide polymorphism [11]. Homologous recombination utilizes homologous templates, usually a sister chromatid, to repair DNA double strand breaks, inter-strand crosslinks, and stalled replication forks. The major limitation is that sister chromatids are only available during the late S and G2 phases of the cell cycle [11]. Interestingly in yeast, the

mechanisms for increasing ribosomal DNA content have been proposed to be homologous recombination with unequal sister chromatid exchange [12].

Homologous recombination may potentially act as a double-edged sword in regards to genomic stability. Efficient and accurate DNA repair allows the cells to resist replication stress and maintain genomic stability, thus preventing oncogenic transformation [13]. Conversely, a growing body of evidence suggests that homologous recombination can be a source of genomic instability in response to regulatory failure [14-16]. For example, it has been suggested that the Rec Q helicases BLM and WRN, whose mutations are linked to Bloom Syndrome and Werner Syndrome respectively, act as negative regulators of homologous recombination [17]. Additionally, in Werner syndrome, Bloom syndrome, and several subsets of Rothmund-Thomson syndrome, which is a third Rec Q linked disease, individuals have a predisposition for cancer [18]. Moreover in yeast, mutation of the SGS1 helicase creates a hyper recombination phenotype which is associated with frequent segmental deletions. Overexpression of the WRN or BLM Rec Q helicases can produce a partial rescue from this hyper recombination. [19]. Thus, while homologous recombination is of great importance due to its high fidelity DNA repair capabilities, tight regulation is necessary to prevent promiscuous potentially oncogenic recombination.

Recent evidence suggests the dysregulation of homologous recombination in neurodegenerative diseases. The gradual loss of telomeres is a well-documented aspect of cellular senescence, but interestingly both expansions and contractions of telomere content have been documented in neurodegenerative disease. Accelerated telomere loss has been documented in the leukocytes of patients with Alzheimer's disease and

Dementia with Lewy Bodies [20-23]. Conversely, increased telomere content has been documented in the hippocampus of Alzheimer's disease patients [20]. Moreover, we have previously documented an increase in the ribosomal DNA content of parietal- and prefrontal- cortex of Alzheimer's disease donor tissues [24]. Changes in the genomic content of highly repetitive sequences, especially those which occur in terminally differentiated cells which do not express telomerase, strongly suggest homologous recombination as a potential mechanism.

The tandem repeat arrangement of ribosomal DNA is well known for its relative instability compared to other regions of the genome [25-27]. The combination of this arrangement and the close physical proximity of all ten arrays inside the nucleolus potentially make them hot spots for homologous recombination. Uneven homologous recombination between clusters or within a cluster may lead to expansions or contractions of ribosomal DNA content and such a process is common in yeast [12]. In 1972, Bernard Strehler proposed the loss of ribosomal DNA copies in post-mitotic cells, such as neurons and muscle, as a mechanism of senescence [28]. Strehler proposed that this loss would subsequently lead to a deficiency in the ribosomal supply and insufficient levels of protein translation. While his initial results were promising, they were later refuted as experimental artifact as subsequent studies could not reproduce these observations [29, 30]. Moreover, mammalian cell culture based studies of replicative senescence did not document changes in ribosomal DNA content [31, 32].

Cellular growth and maintenance requires enormous amounts of protein synthesis. The ribosome is the center of all cellular translation and as such ribosomal quantities are linked to the cells protein synthesis capacity [33]. To accommodate the demand for

increased protein synthesis, cells have amplified the ribosomal DNA gene which encodes for pre-rRNA, a transcript which is processed into the 18S, 5.8S, and 28S ribosomal RNAs (rRNAs). In eukaryotes, ribosomal DNA content is believed to range from 100-10,000 copies depending on the species [33]. Humans are estimated to have approximately 300 copies per haploid genome, which are organized in clusters of long tandem arrays on the terminal ends of chromosomes 13, 14, 15, 21 and 22 [26]. Interestingly, in mature cells only about half of the ribosomal DNA copies are transcriptionally active while the other half is epigenetically silenced [34]. This may suggest two possibilities. First, the cellular demand for protein synthesis may vary greatly throughout a lifespan. For example, such a large amount of copies may be essential for rapid proliferative growth and early development, but unnecessary upon maturity. Alternatively, excess ribosomal DNA copies may function to allow cells to tolerate the loss of damaged copies. Such a condition may be of great importance for post-mitotic neurons which survive for an organism's entire life span. Moreover, these excess ribosomal DNA copies have evolved to have functions unrelated to ribosomal biogenesis, such as regulating chromatin structure, gene expression, and stress response [35-39].

Alternatively, in yeast the age related loss of ribosomal DNA is known to occur and has been proposed as the mechanism behind replicative senescence. Originally, it was thought that ribosomal DNA instability lead to the production of extra chromosomal circles a ribosomal DNA cleavage product composed of 8 ribosomal DNA units [40]. It was proposed that these ERCs would titrate out the factors necessary for ribosomal biogenesis and genome maintenance. However, more recent work suggests that ERCs

are simply a marker of ribosomal DNA instability and that the loss of ribosomal DNA itself triggers the DNA damage response [37, 41]. Kobayashi has proposed that ribosomal DNA may act as a sink for DNA damage proteins, and thus release them in response to ribosomal DNA copy loss [3]. He went on to propose that such a function could monitor genomic integrity in a manner somewhat analogous to telomeres [3].

Previously our lab has documented an increase in ribosomal DNA copy number in the parietal cortex tissue of Alzheimer's disease positive tissue donors using a qPCR based approach [24]. Moreover, the increased copy number was associated with an increase in ribosomal DNA promoter methylation, which is a marker of inactive copies. Interestingly in a murine cell line, decreased epigenetic silencing correlated with loss of ribosomal DNA content [38]. This led to two mutually exclusive possibilities. First, increased ribosomal DNA content could be the result of disease associated amplification or second, the increased methylation could be preventing an age associated loss of ribosomal DNA. The latter possibility would suggest that the ribosomal DNA stability studies conducted in the 1970s and 1980s which disproved Strehler's hypothesis of age related ribosomal DNA loss may have been inaccurate.

Despite the negative data generated several decades ago, it should be noted that the experimental design of these studies utilized a hybridization based competitive binding assay and as such did not allow for a high resolution picture of the ribosomal DNA gene. The human ribosomal DNA gene is fairly large with a length of 43kb, of which roughly three fourths is the untranscribed intergenic spacer. The other one fourth is composed of the promoter and 3 exons which include the 18S, 5.8S, and 28S rRNAs (Fig. 1). The results created by the prior hybridization based approaches do not

necessarily exclude segmental losses inside the ribosomal DNA unit. In support of segmental ribosomal DNA loss a more recent study which used a quantitative PCR (qPCR)-based approach documented a modest, but still statistically significant, age related decrease in 5.8S and 28S ribosomal DNA content, but not 18S [42]. Moreover, a study which utilized *in situ* hybridization with fluorescent probes for 18s and 28s unexpectedly demonstrated that in healthy controls approximately one third of ribosomal DNA copies are arranged in a head-to-head or tail-to-tail orientation and not strictly in tandem arrays as the canonical model suggests [43]. Additionally, they found significant heterogeneity in the length of the intergenic spacer. In fibroblasts obtained from Werner patients the frequency of this non-canonical arrangement increased to approximately 50% of total ribosomal DNA copies [43]. As Werner syndrome is known to be caused by a Req Q helicase mutation, this may suggest homologous recombination is involved in these abnormal rearrangements. Thus, higher resolution techniques have suggested that segmental or directional variation of ribosomal DNA units may not be uncommon.

Independent of age related ribosomal DNA changes, multiple studies suggest that ribosomal DNA is relatively unstable compared to other regions of the genome as its tandem repeat arrangement potentially makes them hot spots for homologous recombination mediated fluctuations in both yeast and higher organisms, including humans. Meiotic ribosomal DNA recombination rates are believed to be as high as 10% and this subsequently causes significant heterogeneity in ribosomal DNA content even among closely related individuals [44]. In human cells, mitotic ribosomal DNA instability is common in both cancer and congenital diseases linked to impaired DNA damage repair. For example, in human cancers approximately 50% of lung and

colorectal solid tumors demonstrate ribosomal DNA restructuring [26]. Moreover, this restructuring was also seen in fibroblasts from Ataxia Telangiectasia and Bloom syndromes, which are known to be caused by mutations in a DNA double strand break repair protein ATM and the BLM Rec Q helicase mentioned above [26, 27].

Surprisingly, mutations in the Rec Q helicase WRN, which is implicated in Werner syndrome, did not alter the length of ribosomal DNA clusters [27]. Furthermore, as mentioned previously, the methylation status of the ribosomal DNA promoter and subsequent heterochromatin formation was directly linked to ribosomal DNA stability in a mammalian cell line [38]. Lastly, amphibian and insect oocytes amplify ribosomal DNA as extra chromosomal circles presumably to enable exceptionally high rates of rRNA synthesis necessary for early development [45-47]. Taken together these facts strongly suggest that ribosomal DNA content is unstable under both physiological and pathological circumstances in higher organisms.

Given our previously described increase in the 18S ribosomal DNA content in Alzheimer's disease parietal cortex tissue and the increased ribosomal DNA promoter methylation which accompanied it, we investigated age related segmental changes in parietal cortex ribosomal DNA content. Thus we acquired parietal cortex samples from 14 young and 9 elderly brain tissue donors, all of which were free of neurological and neurodegenerative conditions and probed all 3 ribosomal DNA exons. Moreover, to determine if our prior findings are exclusively a characteristic of Alzheimer's disease or alternatively a general correlate of neurodegeneration, parietal cortex samples from 10 Dementia with Lewy Bodies tissue donors and 10 healthy age matched controls were probed in an identical manner. Moreover, we acquired cerebellar tissue from Dementia

with Lewy Bodies and control subjects to assess if the documented ribosomal DNA increases are confined to areas with pathology.

Dementia with Lewy Bodies is an age related neurodegenerative disease and is the 2nd most common form of dementia in the elderly after Alzheimer's disease. The major symptom of Dementia with Lewy Bodies is memory loss, but fluctuations in alertness, Parkinsonian motor deficits, and visual hallucinations are also common [48]. It is associated with the loss of dopaminergic neurons and frequently contains reactive gliosis [49, 50]. Pathologically, Dementia with Lewy Bodies is similar to Parkinson's disease as both are classified as synucleinopathies, diseases which are associated with intracellular inclusions of aggregated α -synuclein referred to as Lewy Bodies which develop in both neurons and glia [48]. Some have speculated that Dementia with Lewy Bodies and Parkinson's disease may be the same disease as they share significant symptomatic and pathological overlap. For example, the major diagnostic difference between Dementia with Lewy Bodies and Parkinson's disease dementia for clinicians is only the rate of dementia onset. Dementia with Lewy Bodies is associated with the widespread accumulation of Lewy Body pathology throughout the cortex, including the parietal- and prefrontal cortices which were probed in our prior Alzheimer's disease study [51]. The cerebellum is predominately pathology free in Dementia with Lewy Bodies. Thus, Dementia with Lewy Bodies should serve as an ideal specificity control for Alzheimer's disease. Moreover, synucleinopathies have been documented to contain increased amounts of oxidative stress and in particular increased amounts of oxidative DNA damage [5, 52]. Such DNA damage could potentially lead to the loss of ribosomal DNA copies.

METHODS AND MATERIALS

Subjects and sample preparation. For aging studies fresh flash frozen samples of parietal cortex (Brodmann areas 39 and 40) were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The young donor group consisted of 7 men and 7 women (1-25 years old, median age: 20); the old donor group consisted of 4 men and 5 women (73-90 years old, median age: 79). The donors had no prior history of a chronic neurodegenerative- or neurological disease. The post mortem intervals were comparable for both groups averaging 7.5 h (Post mortem intervals were always less than 14 h). For Dementia with Lewy Bodies studies, the donors were participants of the IRB-approved University of Kentucky Alzheimer's Disease Center cohort and were followed for at least 2 years before death [53]. The follow up included annual Minimental State Examination as well as neurological and physical examinations. The donors had no history of substance abuse, head injury, encephalitis, meningitis, epilepsy, or stroke/transient ischemic attack. The Minimental State Examination score closest to death was used as an indicator of overall cognitive status. During autopsy (usually 5 or less hours after death), tissue samples including parietal cortex and cerebellum were processed for neuropathological evaluations or flash-frozen in liquid nitrogen and stored at -80°C, as described previously [53, 54]. All included Dementia with Lewy Bodies subjects met the clinical and histopathological criteria for diagnosis of Dementia with Lewy Bodies [55]. The control

subjects received Minimental State Examination scores ≥ 23 with Braak staging at ≤ 2 . Detailed donor information is presented in Tables 1 and 2.

Analysis of genomic ribosomal DNA content. Genomic qPCR using standard curve based analysis was utilized to determine ribosomal DNA content. Amplicons targeting 18S, 5.8S, and the 28S coding regions of ribosomal DNA were used (Fig 1). For normalization, amplicons corresponding to the coding regions of the *tRNA^{K-CTT}* gene or the albumin gene (*ALB*) were amplified.

Bisulfite mapping of ribosomal DNA promoter methylation was performed as described previously [24]. Briefly, two hundred ng of DNA/sample was treated with bisulfite to convert unmethylated cytosines to uracil. The ribosomal DNA promoter region was PCR-amplified and cloned into the pGEM-T vector. Following bacterial transformation, individual clones were isolated and sequenced. Only clones with unique methylation patterns were included in the analysis to avoid potential PCR/cloning artifacts. Clones with incomplete bisulfite conversion were also discarded. Thus, twenty fully converted, and unique clones were analyzed for each individual.

DNA methylation analysis using the methyl-CpG sensitive restriction enzyme HpaII.

Methylation of the HpaII site located at position -9 relative to the transcription start site (CpG #23 of the human ribosomal DNA promoter) was analyzed with a quantitative real-time PCR of the HpaII-digested genomic DNA as described previously [24] with modifications. Importantly, as a reference, the HpaII-digested DNA was used as template

to determine the content of an amplicon adjacent to the one with the HpaII site. Such normalization resulted in reproducible methylation values as opposed to using a non-digested DNA with the HpaII amplicon.

Evaluation of methylation effects on qPCR efficiency. An unmethylated DNA template for the 5.8S amplicon was obtained by PCR on genomic DNA (primer sequences: forward cccgtggtgtgaaacctt, reverse agctagctgcgttcttcatc). The PCR product was then *in vitro* methylated in a buffer containing 6 ng DNA/ μ L, 0.16 mM S-adenosylmethionine and 0.2 units M.SssI/ μ L (New England Biolabs) at 37°C for 2 hours followed by M.SssI inactivation at 65°C for 20 minutes. Methylated and unmethylated templates were used for qPCR with the 5.8S amplicon primers. To verify the extent of methylation, both methylated and unmethylated templates were incubated with the methyl sensitive restriction enzyme HpaII. Template resistance to HpaII was determined by qPCR.

Statistical analysis was performed using the non-parametric Kruskal-Wallis one way ANOVA and linear regression fitting. In addition, comparisons of individual ribosomal DNA methylation sites were accomplished using a modified significance analysis of microarrays (SAM), as reported previously [24].

Table 1. Young and old donors of the parietal cortex samples that were used for the aging studies.

Group	Age (years)	PMI (h)¹	Sex
Young	2	10	Male
	15	9	Female
	16	7	Male
	18	8	Female
	19	7	Female
	20	6	Male
	20	5	Male
	21	7	Male
	22	7	Male
	22	10	Female
	24	9	Female
	24	7	Female
	24	9	Male
	25	4	Female
Old	73	13	Female
	76	3	Male
	76	3	Female
	77	8	Female
	79	5	Male
	79	10	Male
	88	8	Female
	89	14	Male
	90	4	Female

¹PMI, *post mortem* interval

Table 2. Control and Dementia with Lewy Bodies donors of the parietal cortex and the cerebellum samples that were used for the Dementia with Lewy Body studies.

Group	Age (years)	PMI (h)¹	Sex	Parietal cortex	Cerebellum
Control	77	3.5	Male	+	Unavailable
	81	2	Male	+	Unavailable
	87	2.4	Male	+	Unavailable
	82	2.1	Male	+	Unavailable
	85	2.5	Female	+	+
	90	2	Female	+	+
	79	1.8	Female	+	+
	78	1.2	Male	+	+
	81	2.8	Male	+	+
	96	2.1	Female	+	+
	86	2.5	Female	Not included	+
	90	3.8	Female	Not included	+
	80	2.5	Female	Not included	+
	95	2.8	Female	Not included	+
	DLB²	78	2.5	Male	+
78		3.8	Male	+	+
81		2.5	Male	+	+
72		2.8	Male	+	+
65		9.5	Male	+	+
87		2	Female	+	+
68		3.8	Male	+	+
90		2.5	Female	+	+
82		2.3	Male	+	+
91		10.5	Female	+	+

¹PMI, *post mortem* interval, ²DLB, Dementia with Lewy Bodies

Table 3. qPCR primers used for aging, Dementia with Lewy Bodies, and HPAII based methylation Studies.

Primer Set	Direction	Sequence 5' to 3'
18S	Forward	agcctgagaaacggctacca
	Reverse	ggtcgggagtggttaattgc
5.8S	Forward	gaggcaaccccctctctctt
	Reverse	gagccgagtgatccaccgcta
28S	Forward	gacctcagatcagaggtggcga
	Reverse	ttcactcgccgttactgagggaaat
tRNA^{K-ctf}	Forward	ctagctcagtcggtagagcatg
	Reverse	ccaacgtggggctcgaac
ALB	Forward	cggcggcgggcggcggggctgggcggaaatgctgcacagaatccttg
	Reverse	gcccggcccggcggcccgtcccggaaaagcatggtcgctgtt
HPAII	Forward	gtatatcttctgctccgagtcg
	Reverse	acaggtcgccagaggacag
HPAII Reference	Forward	acggacgttcgtggcga
	Reverse	ggagaggagagacgagggg

RESULTS

Stability of the cerebro-cortical ribosomal DNA copy number in aging. To assess the effects of aging on ribosomal DNA copy number in the brain, genomic DNA was isolated from *post mortem* parietal cortex samples that were collected from two groups of donors. The young donor group consisted of 14 individuals with a median age of 20 ± 1.6 (range 1-25, Table 1). The old donor group included 9 individuals with a median age of 79 ± 2.1 (range 73-90, Table 1). None of the donors were known to suffer from a chronic neurodegenerative and/or neurological disease. The genomic content of 18S- and 28S rRNA coding regions of ribosomal DNA was analyzed by a qPCR assay (Fig. 1). For normalization, a coding region of the multi-copy gene *tRNA^{K-ctt}* was used. There are 17 almost identical copies of this gene in the haploid human genome. However, in contrast to ribosomal DNA, they are not clustered together [56] and therefore, are less likely than ribosomal DNA to undergo recombination-associated instability.

As the investigated ribosomal DNA regions are physically linked, a direct correlation between their genomic content for each analyzed sample is expected (Fig. 1). Indeed, the individual values obtained for the 18S- and the 28S amplicons were strongly correlated fitting to a linear model of a direct correlation (Fig. 2A, $a=0.7327$, $R^2=0.8103$). These results support accuracy of the applied qPCR methodology for determination of the genomic ribosomal DNA content.

However, genomic content of 18S- and 28S- regions of ribosomal DNA did not significantly differ between the young- and the old subjects (Fig. 2B, C). Usage of a

coding region of the albumin gene (*ALB*) as an alternative genomic reference produced similar results (Fig. 2D). Therefore, in the cerebral cortex, genomic content of ribosomal DNA appears to be stable throughout the lifespan.

Dementia with Lewy Bodies-associated instability of brain ribosomal DNA. To assess whether aging-related neurodegeneration affects genomic ribosomal DNA content in the brain, *post mortem* parietal cortex samples from ten patients who were diagnosed with Dementia with Lewy Bodies were analyzed (Table 2). The control group included ten age-matched individuals who died of non-neurological diseases and whose brains did not display any evidence of neurodegeneration (Table 2). Similar to the midbrain-focused Parkinson's disease, Dementia with Lewy Bodies is a form of a synucleinopathy [57, 58]. Their common characteristic is the presence of intracytoplasmic α -synuclein-containing inclusions, the Lewy Bodies. In addition, Dementia with Lewy Bodies is associated with neuronal atrophy, neuronal death and reactive gliosis in the cerebral cortex including the parietal region [57]. Oxidative damage of macromolecules including DNA is observed in both conditions [5, 59]. Hence, Dementia with Lewy Bodies and Parkinson's disease may represent a similar pathological process that affects different areas of the brain.

Three ribosomal DNA coding regions were analyzed including probes corresponding to the 18S-, 5.8S-, and 28S exons. As expected for a tight physical linkage, the Dementia with Lewy Bodies- and the control group-derived individual values obtained with these probes correlated with each other fitting a linear model of direct correlation (28S(18S), $y=1.7416*x-0.6165$, $R^2=0.8495$; 5.8S(18S), $y=1.4776*x-0.4126$, $R^2=0.8957$; 28S(5.8S), $y=1.1008*x-0.0133$, $R^2=0.8274$). Interestingly, genomic content of ribosomal DNA was

higher in Dementia with Lewy Bodies. In this group, the 18S-, 5.8S-, and 28S probes revealed ribosomal DNA content that was 1.6-, 2.0-, and 2.3 fold controls respectively (Kruskal-Wallis ANOVA, $p < 0.01$, Fig. 3A-C). Similar results were obtained when *ALB* was used as an alternative genomic reference (Fig. 3D). To determine whether this Dementia with Lewy Bodies-associated effect on ribosomal DNA content was directly related to pathological changes in the cortex, samples from the Dementia with Lewy Bodies pathology-free cerebellum were analyzed. In samples from this structure, direct correlations between individual values for various ribosomal DNA probes were observed confirming accuracy of our determinations (data not shown). Surprisingly, in the cerebellum, ribosomal DNA content was lower in the Dementia with Lewy Bodies group. Significant decreases of 0.55- and 0.42 fold control were observed for the 5.8S- and 28S probes, respectively (Kruskal-Wallis ANOVA, $p < 0.01$, Fig. 4). While the 18S probe did not detect significant differences between the two groups, the genomic 18S content also showed a downward trend in Dementia with Lewy Bodies (Fig. 4). Therefore, Dementia with Lewy Bodies appears to be associated with instability of brain ribosomal DNA.

Effects of Dementia with Lewy Bodies on methylation of the ribosomal DNA

promoter region. At least in cancer cell lines, amplification of ribosomal DNA may be associated with CpG hypermethylation [60]. As CpG methylation of the ribosomal DNA is associated with epigenetic silencing of ribosomal DNA copies a combination of amplification and hypermethylation may help to keep the number of active ribosomal DNA copies constant [34]. Moreover, epigenetic silencing of ribosomal DNA may stabilize the inactive genes preventing recombination; conversely, de-silencing may have

an opposite effect [3, 38]. Thus, methylation of the ribosomal DNA promoter region was analyzed in the parietal cortex of the Dementia with Lewy Bodies- and control groups using the CpG methylation-sensitive restriction endonuclease HpaII or bisulfite sequencing. Methylation of the ribosomal DNA promoter was similar in the Dementia with Lewy Bodies- and the control group (Fig. 5). Therefore, in the Dementia with Lewy Bodies pathology-affected cerebral cortex, ribosomal DNA amplification is not associated with changes in CpG methylation of the ribosomal DNA promoter.

Effects of template methylation on qPCR efficiency. Methylation of ribosomal DNA is observed not only in the promoter region but also in the rRNA-coding exons that have been targeted for qPCR-based ribosomal DNA quantification. Therefore a possibility exists that the apparent Dementia with Lewy Bodies-associated changes in ribosomal DNA content are due to altered methyl-CpG content in those regions if methylation affects qPCR amplification efficiency. To exclude such a possibility, non-methylated ribosomal DNA fragment containing the 5.8S amplicon template was produced by PCR. As the 5.8S amplicon contains 19 CpG sites including 3 in the reverse primer target sequence methylated template was generated *in vitro* using a non-selective CpG DNA methyltransferase M.SssI (Fig. 6). As compared to the unmethylated template, methylation only slightly increased qPCR efficiency resulting in about 10% overestimation of the template content. Therefore, even dramatic changes in CpG methylation of the ribosomal DNA templates are unlikely to explain up to 130%-increases or decreases of ribosomal DNA content that were observed between control and Dementia with Lewy Bodies samples.

DISCUSSION AND CONCLUSIONS

In this study, we probed for changes in ribosomal DNA content in the brain of old vs young subjects and Dementia with Lewy Bodies patients vs. controls using a qPCR based approach. No difference was detected in the parietal cortex ribosomal DNA content of young and old individuals. Our results are in agreement with a study which attempted to address this question using a hybridization based approach. Significant increases in ribosomal DNA content were detected in the parietal cortex of Dementia with Lewy Bodies individuals compared to age matched controls with increases ranging from approximately 1.5 to 2.2 fold. The ribosomal DNA content of the cerebellum, a region which does not show pathological changes in Dementia with Lewy Bodies also contained altered ribosomal DNA content, however the direction of change was unexpectedly opposite that of the parietal cortex with a decrease of approximately 50% in two of the three probed regions.

Our prior Alzheimer's disease study produced similar results for the parietal cortex as both Dementia with Lewy Bodies and Alzheimer's disease contained elevated ribosomal DNA content [24]. Moreover, the magnitude of 18S increase, approximately 50%, was similar to what has been documented in mild cognitive impairment and late stage Alzheimer's disease which contained increases of 50% and 69%, respectively. In contrast, the Dementia with Lewy Bodies' cerebellum contained a significant decrease in ribosomal DNA content, a finding which was not apparent in Alzheimer's disease. However, it is important to note that this decrease was documented in the 5.8S and 28S

ribosomal DNA regions which were not probed in our prior Alzheimer's disease study. The 18S region trended down in Dementia with Lewy Bodies but did not reach statistical significance. Thus, comparing the Dementia with Lewy Bodies and Alzheimer's disease cerebellum is at least partially inconclusive. Interestingly, a similar trend has been documented in the genomic content of another highly repetitive sequence, the telomere. In both Alzheimer's disease and Dementia with Lewy Bodies telomere length was decreased in peripheral blood leukocytes, and, at least in the case of Alzheimer's disease, increased in the hippocampus, a brain structure directly affected by Alzheimer's disease pathology [20-22].

It is important to note that all data obtained in this study are based on genomic DNA which was extracted from tissue containing a mixed population of cells. Thus, it is unclear which cell type or types are contributing to these findings. As homologous recombination is believed to be the major mechanism behind ribosomal DNA instability and readily occurs during both mitosis and meiosis, this suggests inappropriate activation of homologous recombination is occurring in cycling cycles. [44]. Recent literature suggests this may not be the only possibility for our findings. We propose three potential explanations.

First, this phenomenon could be occurring in cycling cells. This may suggest our findings are the result of reactive gliosis, a condition which is involved in both Alzheimer's disease and Dementia with Lewy Bodies [61, 62]. Reactive gliosis involves mitosis and the rapid proliferation of glia, thus allowing homologous recombination to occur. Theoretically, expansion of ribosomal DNA in reactive glia could aid in their

ability to rapidly proliferate. A similar phenomenon is known to occur in amphibian and insect oocytes [45-47].

A second potential explanation is that homologous recombination occurs in response to reactivation of the cell cycle in post-mitotic neurons. Multiple studies have documented at least partial cell cycle reactivation in Alzheimer's disease pathology associated areas [63-65]. Proteins from all phases of the cell cycle have been shown to be increased in Alzheimer's disease. Moreover, the frequency of aneuploidy has also been shown to be increased in Alzheimer's disease [66]. Such a condition could potentially be sufficient for homologous recombination to occur.

Lastly, canonical homologous recombination may not be necessary for fluctuations in ribosomal DNA copy number. Interestingly, emerging data have identified homologous recombination-like activity in post-mitotic cells [67, 68]. In these cells, UV radiation induced double strand breaks which were repaired using a mechanism that involved short segmental contact between homologous chromosomes in a transcription- and ATM- dependent manner. Theoretically, such a repair mechanism could be sufficient to produce effects similar to canonical homologous recombination. Moreover, the unique repetitive nature of the ribosomal DNA and the general structure of the nucleolus may allow for recombination in the absence of mitosis. The cell's 600 copies of ribosomal DNA, which are arranged as tandem arrays on 5 separate chromosomes, are all in close physical proximity due to their nucleolar localization. Such an atypical orientation and the relatively high rates of transcription which occur in this region may make the generation of sister chromatids unnecessary for homologous recombination like activity.

While the effects of altered ribosomal DNA content in the human brain are unclear, findings from lesser species and mammalian cell lines may potentially provide insight. Surprisingly, the rate of ribosomal biogenesis may not be subject to large alterations under the documented circumstances. It is known that most multicellular species contain a large excess of ribosomal DNA copies of which a large percentage is epigenetically silenced [69]. Thus, an expansion of ribosomal DNA copies would most likely add to this epigenetically silenced pool and cause little, if any, alterations in the rate of ribosomal biogenesis. Moreover, cells appear capable of withstanding limited ribosomal DNA loss. Germ line deletions of significant ribosomal DNA quantities in both flies and chickens still produced viable organisms [70, 71]. For example in the chicken study, embryos with 66% of the normal ribosomal DNA content grew and developed as expected [70]. Thus, a portion of the cellular ribosomal DNA content is unnecessary for ribosome synthesis even during the organism's most demanding periods of growth. Taken together, these reports suggest that any potentially detrimental effects of ribosomal DNA copy loss are potentially related to functions other than ribosomal biogenesis.

Interestingly, in lower organisms, manipulation of ribosomal DNA content has identified several non-coding functions. In the aforementioned fly studies, ribosomal DNA variation directly influenced heterochromatin content [35]. Moreover, in a mouse cell line, loss of the nucleolar silencing complex component TIP5 lead to a decrease in both heterochromatin content at major- and micro- satellite DNA and loss of ribosomal DNA content [38]. Taken together, such findings suggest that ribosomal DNA instability may affect mammalian heterochromatin content. Moreover in ribosomal DNA deficient

flies, changes in euchromatin and gene expression changes were detected [36]. Furthermore, clusters of genes linked to both mitochondrial function and lipid metabolism were prominent in the population of altered genes [36]. Interestingly, alterations in both of these areas are common to both Alzheimer's disease and Dementia with Lewy Bodies [52, 72-74].

In yeast it has been proposed that silent ribosomal DNA copies function as a sink for mediators of the DNA damage response. Kobayashi proposed a model in which ribosomal DNA loss, which is known to be associated with replicative senescence, would release DNA damage response mediators in a manner analogous to telomere shortening triggering the DNA damage response [3]. Alternatively, increased ribosomal DNA content could potentially raise the threshold for DNA damage response activation. In support of Kobayashi's theory, it was demonstrated that yeast with larger ribosomal DNA content were more resistant to the DNA damaging agent methyl methanesulfonate than those with less ribosomal DNA content. While this possibility has not been addressed in mammalian cells, nucleolar sequestration of other stress mediators is known. Heat Shock Protein 70, Murine Double Minute 2, and the Von Hippel Lindau protein have been documented to be sequestered in the nucleolus via their affinity for non-coding RNAs generated from the intergenic spacer region of the ribosomal DNA unit [39]. Therefore, changes in ribosomal DNA content may affect the cellular stress response.

While more work needs to be done to identify the consequences of ribosomal DNA instability in the human brain, we propose a working model based on the effects of altered ribosomal DNA documented in the literature (Fig.7). We propose DNA damage induced genomic instability as a general component of the degenerating brain. Under

these conditions, ribosomal DNA content would be disproportionately affected due to its tandem array arrangement, the high rate of ribosomal DNA transcription, and the physical proximity of all 10 ribosomal DNA arrays. In this model homologous recombination or “homologous recombination like” activity would produce instability in ribosomal DNA content. This would ultimately alter heterochromatin levels, euchromatin gene expression, and the DNA damage response. Increases in ribosomal DNA content would increase the level of heterochromatin, increase ribosomal DNA’s ability to influence euchromatin gene expression, and increase DNA damage response resistance. Conversely, loss of ribosomal DNA would produce the opposite effect. We propose that genomic instability is induced by DNA damage. Additionally, the application of a selection pressure, such as the enhanced DNA damage which is common to the Dementia with Lewy Bodies and Alzheimer’s disease parietal cortex, would select for increased ribosomal DNA content and promote cell survival. Thus in the Dementia with Lewy Bodies cortex, which experiences significantly larger levels of oxidative stress and synucleinopathy, the magnitude of DNA damage would be large enough to select for increased quantities of DNA. In the case of the Dementia with Lewy Bodies cerebellum, low level potentially undetectable DNA damage would still induce genomic instability. However, no such selection pressure would exist and genomic instability would present as a loss of ribosomal DNA. Moreover, neurodegeneration associated cell loss would remove less resilient cells thus further enriching the total population with cells containing higher ribosomal DNA content.

In summary, we have not detected any age related changes in ribosomal DNA content. However, we have identified a second neurodegenerative disease, Dementia with

Lewy Bodies, with increased ribosomal DNA content in the parietal cortex. Moreover, we unexpectedly documented decreased ribosomal DNA content in the pathology free cerebellum specifically for the 5.8S and 28S segments. Therefore, we propose that genomic instability is present in the degenerating brain.

Figure 1. The qPCR-based assay to determine the genomic content of ribosomal DNA. The ribosomal DNA copies are organized as long tandem repeats located on five acrocentric chromosomes. Each copy consists of the ribosomal RNA gene and the intergenic spacer. Each ribosomal RNA gene includes a Pol1-dependent promoter and exons that correspond to 18S-, 5.8S- and 28S ribosomal RNAs. They are separated by introns (5'ETS, ITS1, ITS2 and 3'ETS). The positions of the analyzed ribosomal DNA amplicons are indicated by the thick black lines. The schematics are not drawn in scale.

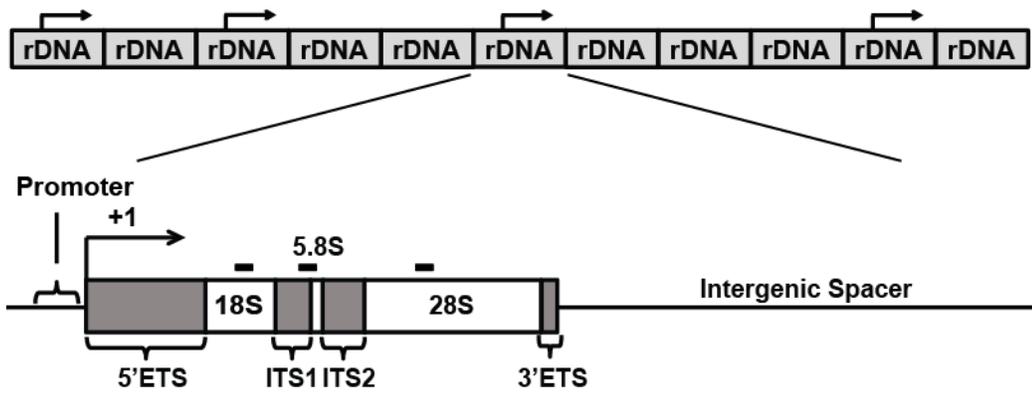


Figure 2. Effects of age on the cerebro-cortical content of ribosomal DNA. Genomic ribosomal DNA content was quantified in *post mortem* samples of the parietal cortex from young- and old individuals without any neurodegenerative conditions (n=14, median age 20, and n=9, median age 79, respectively). The 18S- and 28S- amplicons were used for ribosomal DNA content determinations; the reference genes are indicated. For each amplicon, the ribosomal DNA content was normalized to average value of young individuals. **A**, Regression analysis of individual values of ribosomal DNA content as determined with 18S- or 28S probes. As expected for a close physical linkage between these genomic templates, a direct correlation is present supporting validity of the qPCR assay. Filled or opened diamonds indicate young or old individuals, respectively. **B-D**, Content of ribosomal DNA is similar in young- and old individuals. Individual values are depicted by squares; mean values are indicated by the lines intersecting the error bars (SEM); *p* values of the Kruskal-Wallis ANOVA are shown.

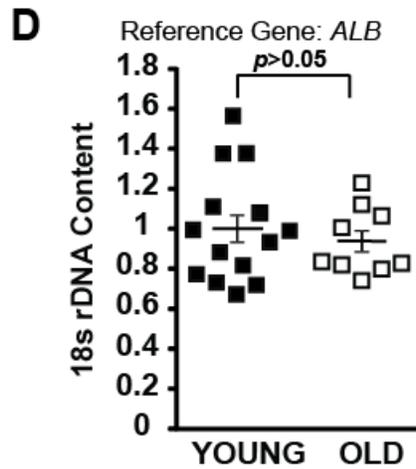
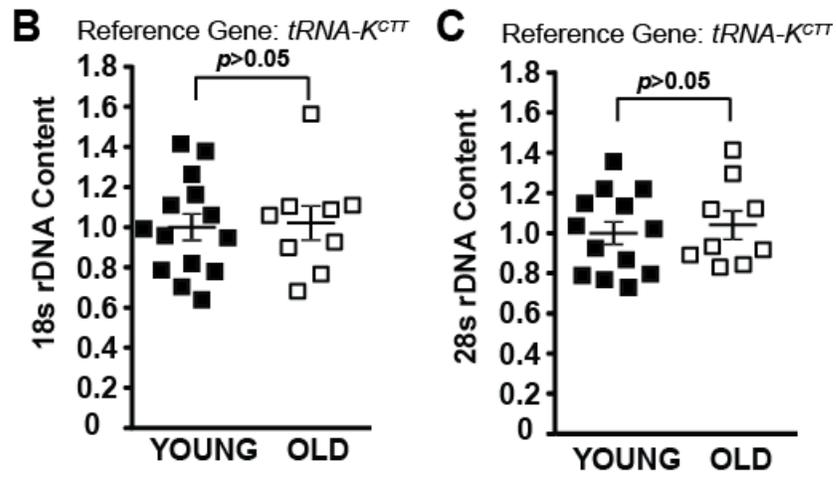
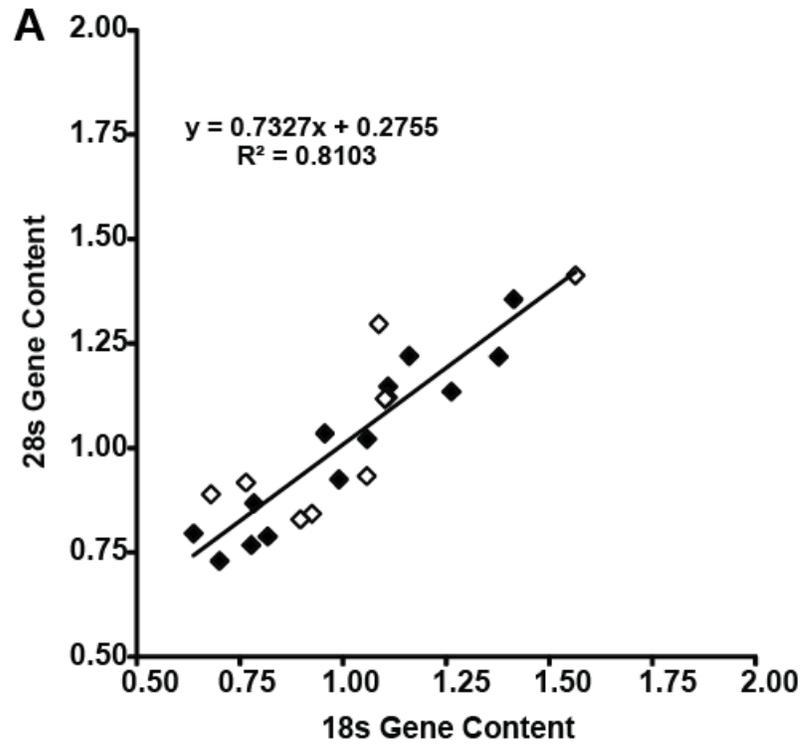


Figure 3. Increased ribosomal DNA content in the Dementia with Lewy Bodies parietal cortex. Genomic ribosomal DNA content was quantified in *post mortem* samples of the parietal cortex from Dementia with Lewy Bodies- and age-matched control individuals without any neurodegenerative conditions (n=10 each). The 18S-, 5.8S- and 28S amplicons were used for the determinations; the reference genes are indicated. Regression analysis of individual values for pairs of ribosomal DNA amplicons revealed strong correlations similar to those in Fig. 2A (see the Results section for more details). In the Dementia with Lewy Bodies group, significant increases of ribosomal DNA content were detected using the 18S/*tRNA*^{K-ctt} (A), 5.8S/*tRNA*^{K-ctt} (B), 28S/*tRNA*^{K-ctt} (C) and 18S/*ALB* (D) ratios. Individual values are depicted by squares; mean values are indicated by the lines intersecting the error bars (SEM); *p* values of the Kruskal-Wallis ANOVA are shown.

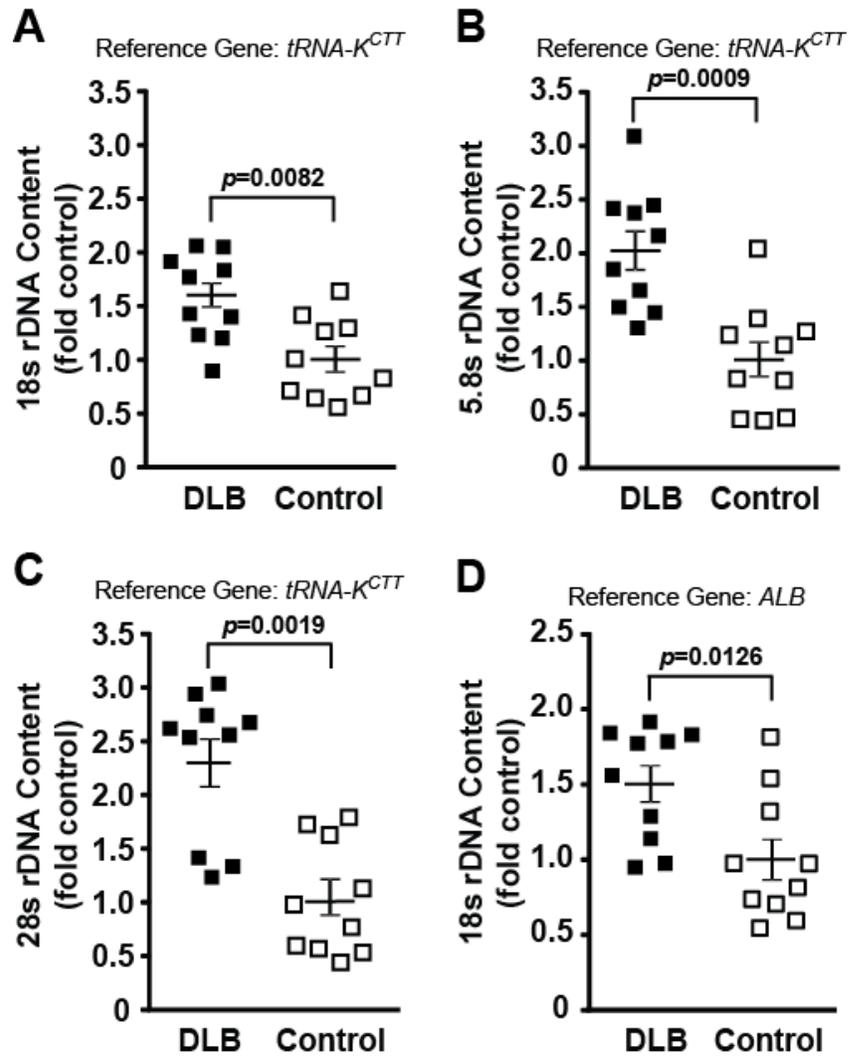


Figure 4. Decreased ribosomal DNA content in the Dementia with Lewy Bodies

cerebellum. Genomic ribosomal DNA content was quantified in *post mortem* samples of the cerebellum from 10 Dementia with Lewy Bodies- and 10 aged-matched control individuals. Regression analysis of individual values for pairs of ribosomal DNA amplicons revealed strong correlations similar to those in Fig. 2A (data not shown). While a downward trend was observed for the 18S amplicon (A), the 5.8S- and the 28S probes revealed significant decreases of ribosomal DNA content in Dementia with Lewy Bodies samples (B-C). Individual values are depicted by squares; mean values are indicated by the lines intersecting the error bars (SEM); *p* values of the Kruskal-Wallis ANOVA are shown

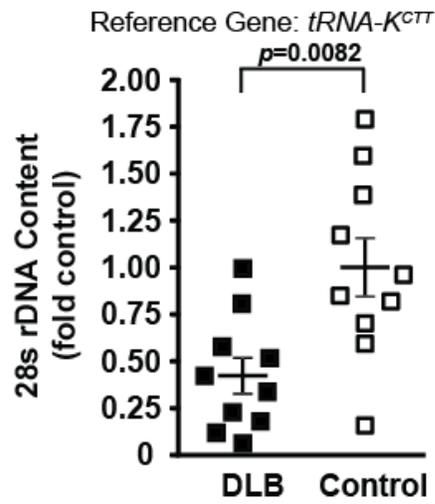
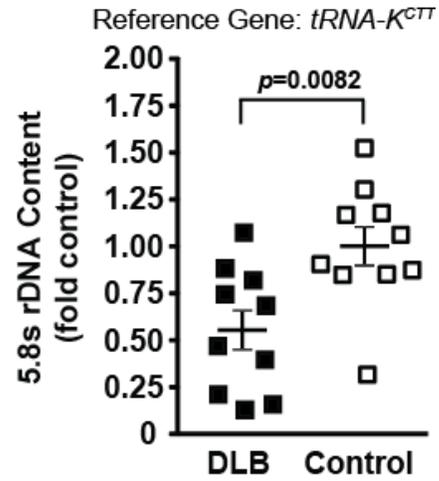
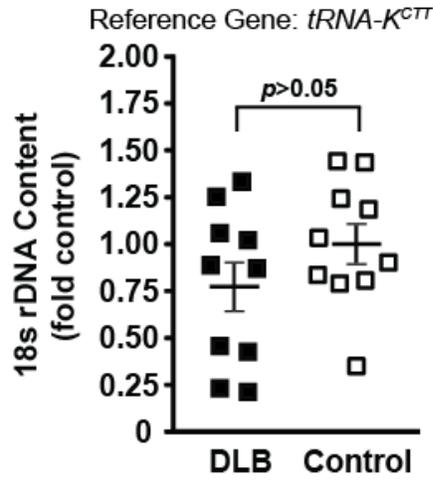


Figure 5. Similar methyl-cytosine content of the ribosomal DNA promoter region in the control- and Dementia with Lewy Bodies parietal cortex. *A*, Methylation of the ribosomal DNA promoter CpG#23 (position -9 from the transcription start site) content was determined using a methylation-sensitive enzyme HpaII and qPCR. *B-C*, Methylation of all 26 CpG sites within the human ribosomal DNA promoter region (positions -186 to +26) was investigated using bisulfate sequencing. *B*, Average CpG methylation in the ribosomal DNA promoter. *C*, Effect of Dementia with Lewy Bodies on distribution of CpG methylation across the ribosomal DNA promoter. The data represent averages \pm SEM from 10 Dementia with Lewy Bodies- and 10 control- individuals. Individual values are depicted by squares; mean values are indicated by the lines intersecting the error bars. In *A* and *B*, *p* values of the Kruskal-Wallis ANOVA are shown; in *C*, *, *p*<0.05 (SAM statistics). Although CpG#7 and 17 appeared hypermethylated in Dementia with Lewy Bodies, the overall trend of CpG methylation across the 26 ribosomal DNA promoter CpGs was not significantly affected by Dementia with Lewy Bodies (local regression analysis, data not shown).

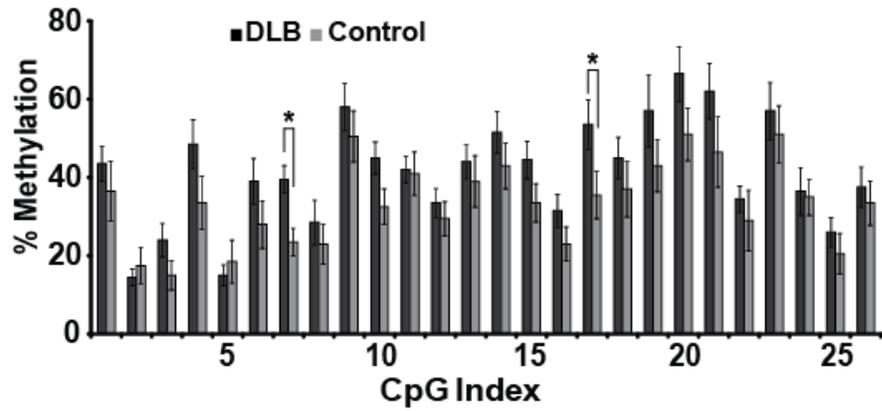
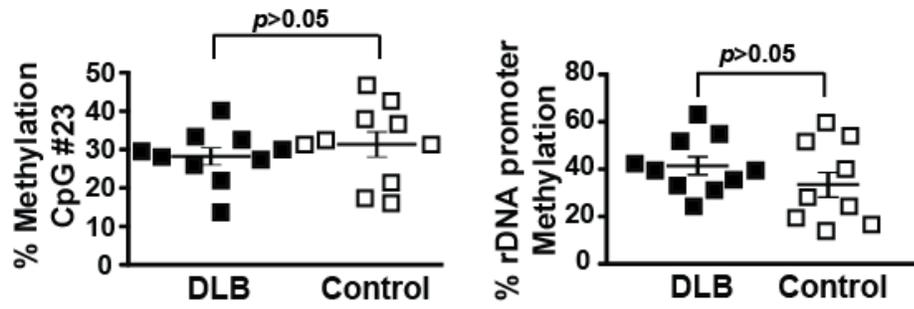


Figure 6. Effects of template methylation on qPCR efficiency. **A**, PCR on genomic DNA was used to produce an unmethylated DNA fragment including a template for the 5.8S qPCR amplicon that revealed ribosomal DNA instability in Dementia with Lewy Bodies (red box, qPCR primers indicated by red arrows, PCR primers indicated by black arrows). DNA was then *in vitro* methylated with M.SssI DNA methyltransferase that indiscriminately targets all CpG sites. Note presence of multiple CpGs in the 5.8S amplicon (opened and filled circles correspond to CpGs or methyl-CpGs, respectively; one of the CpGs is a part of an HpaII site). **B**, Methylation of a CpG that was part of an HpaII site was confirmed using HpaII-qPCR assay. When methylated DNA fragment was used as a template for 5.8S qPCR, ribosomal DNA content was overestimated by just 10% as compared to unmethylated template. Hence, differential methylation of ribosomal DNA templates of the qPCR amplicons is unlikely to account for Dementia with Lewy Bodies-associated changes in ribosomal DNA content. Data represent two independent experiments; error bars are SDs.

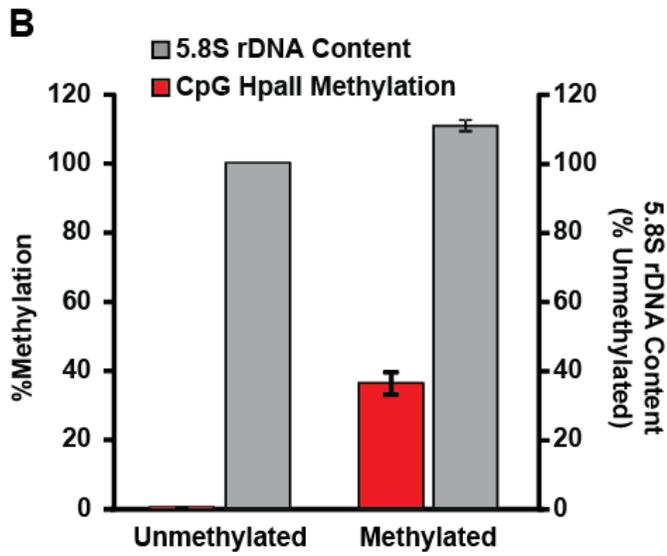
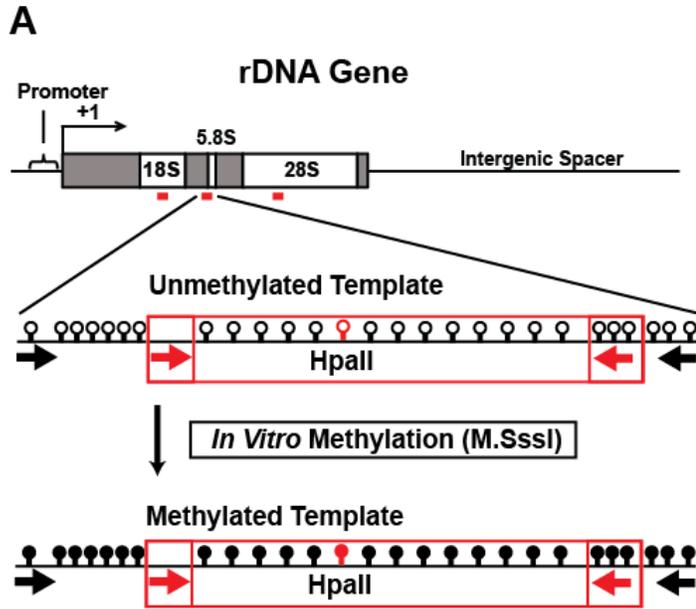
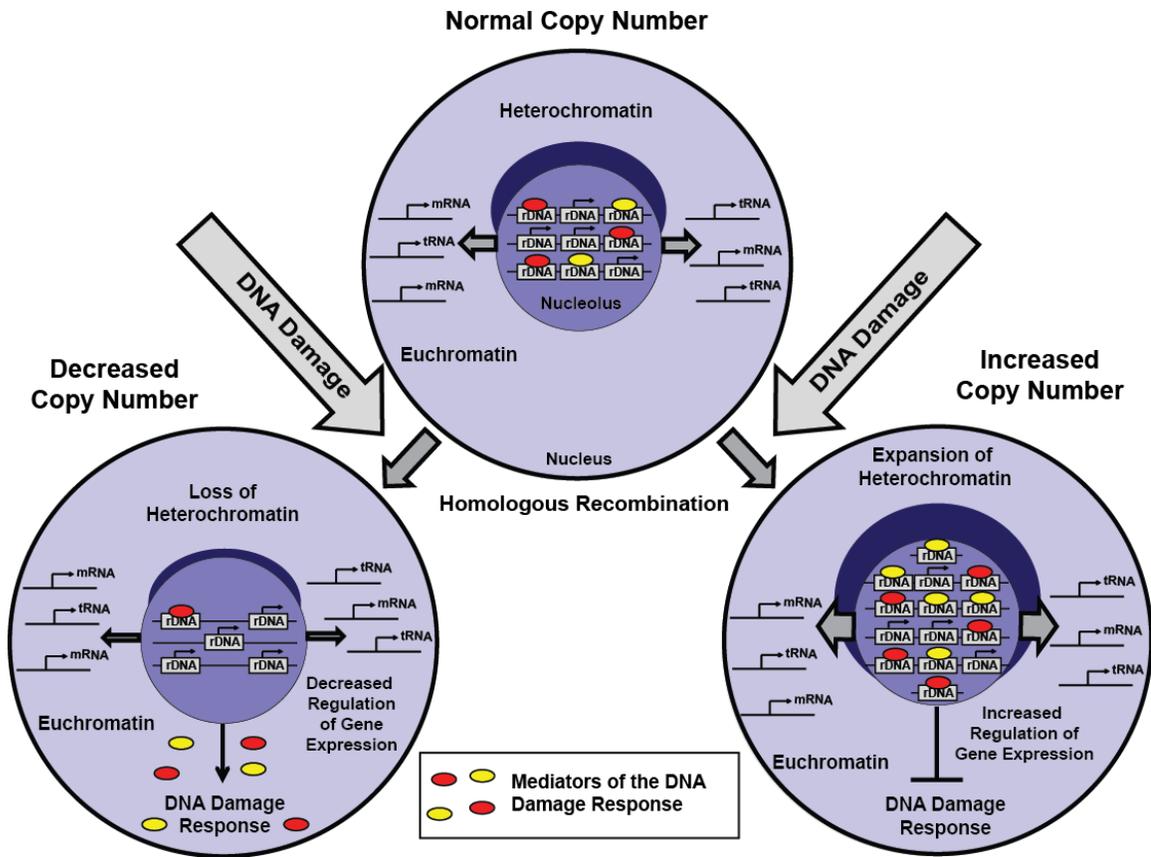


Figure 7. A hypothetical model summarizing possible causes and consequences of ribosomal DNA instability. The DNA damage-stimulated homologous recombination is a likely cause of ribosomal DNA instability in the degenerating brain. The moderate changes in ribosomal DNA copy number as those reported here are not expected to significantly affect ribosomal biogenesis. Instead, ribosomal biogenesis-independent functions of ribosomal DNA may be affected including control of the DNA damage response, maintenance of heterochromatin, and expression regulation of the non-ribosomal RNA genes that are located in the euchromatin. One can speculate that higher content of ribosomal DNA may support cell survival by suppressing the DNA damage response, stabilizing chromatin and stronger regulatory control of the euchromatic genes. Conversely, lower ribosomal DNA content may have opposite effects on cell sensitivity to DNA damage as well as other forms of stress by enhancing the DNA damage response, reducing control over euchromatin gene expression and destabilizing the chromatin architecture. Thus, ribosomal DNA amplification in the degenerating regions of the Dementia with Lewy Bodies- or Alzheimer's- disease brain may be a consequence of increased genotoxic stress. In turn, cells with the higher genomic content of ribosomal DNA may become overrepresented due to lower sensitivity to subsequent injuries.



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LIST OF ABBREVIATIONS

albumin gene	<i>ALB</i>
cytosine phosphate guanine	CpG
Dementia with Lewy Bodies	DLB
lysine tRNA-ctt gene	<i>tRNA^{K-ctt}</i>
quantitative PCR	qPCR
ribosomal RNA	rRNA

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