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The role of the nucleolus in neurodegeneration.

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THE ROLE OF THE NUCLEOLUS IN NEURODEGENERATION

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M.S. Indiana State University 2009
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ABSTRACT

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August 26, 2014

The overarching goal of my studies was to determine the role of the nucleolus in neurodegenerative disease. Numerous studies suggest the impairment of ribosomal biogenesis in neurodegenerative disease and such a condition may contribute to neuronal dysfunction in two ways, loss of function and stress response. Ribosomal biogenesis has been demonstrated to play an essential role in both neuronal growth and maintenance. Therefore insufficient levels of ribosomal biogenesis would be expected to produce atrophy and synapse loss. Moreover, the fidelity of ribosomal biogenesis is monitored by nucleolar and ribosomal stress responses which induce apoptosis and/or cell cycle arrest. Therefore, I have reviewed the literature demonstrating nucleolar involvement in neurodegeneration and investigated three important aspects of nucleolar dysfunction which are relevant to both general mechanisms of neuronal death and specifically neurodegenerative disease.

The studies contained in this document address four important questions regarding the nucleolus and neurodegeneration. First, is rDNA content stable throughout aging? Second, is increased rDNA promoter methylation specific to Alzheimer's disease? Third, are human brain tissue-based assessments of nucleolar stress

mechanistically feasible? Fourth, is RPL11 a bona fide nucleolar and ribosomal stress mediator in primary cortical neurons?

I demonstrate several important results. rDNA content is stable throughout aging and increased rDNA promoter methylation appears to be specific to Alzheimer's disease. Nucleolar stress markers are at least moderately stable in the mammalian post mortem brain. Lastly, RPL11 is indeed a bona fide mediator of nucleolar stress, ribosomal stress and neuronal death.

Three conclusions contained in this document provide a foundation for future studies investigating nucleolar and ribosomal stress in neurodegenerative disease. First, I provided weight of evidence support for the validity of post mortem brain tissue-based studies. Second, the identification of bona fide stress mediators is an essential step for designing novel therapeutic interventions. Lastly, my results suggest that in primary neurons RPL11-mediated stress does not require catastrophic inhibition of RNA Polymerase I. Therefore, RPL11-mediated stress may indeed be a significant contributor to neurodegeneration despite the lack of canonical nucleolar stress markers.

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CHAPTER I

INTRODUCTION

While Wagner is believed to be the first person to formally describe the nucleolus in 1835, it was Gabriel Valentine who first described it in neurons one year later [91, 201]. The nucleolus is a non-membrane bound organelle that forms over the rDNA-containing nucleolar organizer regions [59]. Interestingly, the nucleolus has been referred to as “an organelle formed by the act of building a ribosome” [171]. Such a description is based on findings which suggest that RNA Polymerase I mediated transcription is necessary for its assembly [171].

While many think of the nucleolus as simply a ribosome factory, a considerable amount of evidence suggests it has important roles in numerous other cellular functions [91]. Perhaps the most exciting of these non-canonical functions is its role in stress response [91, 162]. The nucleolus possess the ability to sequester numerous proteins within its borders, including but not limited to stress mediators and cell cycle regulators [202]. Impairment of RNA Polymerase I is sufficient to induce changes in nucleolar morphology which allow for the escape of these previously sequestered stress mediators. In addition to stress response, recent literature suggests that the nucleolus also regulates total cellular transcription through modulation of global heterochromatin content and cellular metabolism through interactions with p53 [145, 195]. Lastly, the nucleolus is known to facilitate the processing of non-ribosomal RNAs and contribute to the assembly

of ribonucleoprotein complexes such as U6 and the signal recognition particle (SRP) [208]. Thus, the nucleolus appears to be significantly more than just a ribosome factory.

rDNA is unlike other portions of the genome. Ribosomal biogenesis begins with RNA Pol I transcribing the 47S pre-rRNA and importantly this is believed to be the rate limiting step in ribosomal biogenesis [82, 163]. To accommodate protein synthesis demands, cells evolved to have hundreds, and in some cases thousands, of rDNA copies [76, 163]. The diploid human genome contains approximately 600 copies of rDNA, but, interestingly, this number is subject to considerable inter-individual variability [233]. The rDNA units are arranged as tandem repeat head-to-tail arrays on the short arms of the 5 acrocentric chromosomes [236]. Surprisingly, at any given time, only about half of the cells total rDNA copies are believed to be active [76, 233].

The 47S pre-rRNA is transcribed as one continuous transcript containing the 18S-, 5.8S-, and 28S-rRNAs along with 4 spacers regions[76] (Fig.1). Two of these spacers, the internal transcribed spacers 1 and 2 (ITS1 and ITS2) are interspersed between the three rRNAs [81]. The other two spacers, the 5'- and 3'-external transcribed spacers (5'ETS and 3'ETS), are located on the extreme upstream and downstream ends of the transcript. The last ribosomal RNA, the 5S rRNA is transcribed outside the nucleolus by RNA Polymerase III [199]. All of the approximately 80 ribosomal proteins are transcribed in the nucleus by RNA Polymerase II, translated in the cytoplasm, and trafficked back to the nucleolus for ribosomal assembly [163]. Ribosome assembly begins in the nucleus. Prior to large subunit assembly, RPL5, RPL11 and the 5S RNA form a sub-complex referred to as the 5S ribonucleoprotein

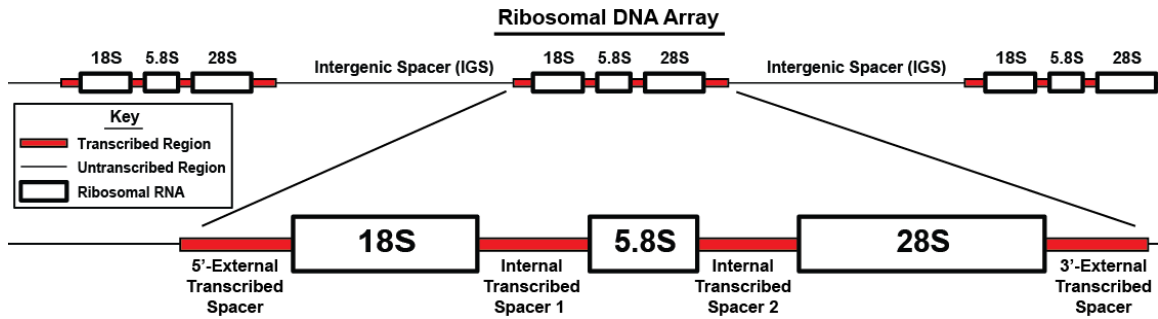


Figure 1. Depiction of a canonical ribosomal DNA Array.

rDNA units are arranged as tandem head-to-tail arrays. The transcribed region is illustrated by the thick red line. Ribosomal RNAs are depicted by rectangles. The transcribed region contains 4 spacers, which are removed during pre-rRNA processing, and 3 ribosomal RNAs. The last ribosomal RNA, 5S, is transcribed outside the nucleolus by RNA polymerase III.

particle (5S RNP) [225]. Importantly, this sub complex has been implicated in the ribosomal stress response [24, 64]. The 5S RNP then joins with the 5.8S and 28S rRNAs and the remaining large subunit ribosomal proteins (RPLs) to form the large subunit. The 18S rRNA and the small ribosomal subunit proteins (RPSs) join to form the small subunit. Both subunits are trafficked out of the nucleus by chromosome region maintenance 1 (CRM1) mediated nuclear export and are chaperoned by nucleophosmin [104]. Interestingly, the export mechanism for each subunit is not identical as exportin 5 (EXP5) is necessary for export of the large subunit, but not the small subunit [255]. Lastly, the joining of the two subunits occurs in the cytoplasm [91].

Ribosomal biogenesis is dynamically regulated on multiple levels by a number of factors including nutrient availability, oncogenes, growth factors, tumor suppressors, genotoxic stress, starvation, metabolic stress, and viral infection [76]. In general, stimuli that increase metabolism or promote growth will enhance ribosomal biogenesis. Conversely, signals which decrease metabolism or growth will inhibit ribosomal biogenesis [126, 182, 260]. The master regulator for this process appears to be RNA Polymerase I as early studies have determined this to be the rate limiting step in ribosomal biogenesis [163]. In support of Pol I as the master regulator, unbound ribosomal proteins have been linked to the inhibition of their own translation through a p53- phosphatase and tensin homolog (PTEN)-mammalian target of rapamycin (mTOR) cascade [44, 96, 213]. Thus, ribosomal proteins appear to adjust their production based on the availability of pre-rRNA and not vice versa.

Regulation of pre-rRNA production is believed to happen in two ways, first through modulating the activity of RNA Polymerase I and second by modulating the

number of active rDNA copies [76]. Not surprisingly, regulation of RNA Polymerase I activity is an especially intricate and convoluted process with connections to numerous kinases, phosphatases, tumor suppressors, and oncogenes converging on many of its cofactors either altering their levels of expression or changing their activity through post-translational modification [76]. Traditionally, it was believed that modulating RNA Polymerase I activity was responsible for transient changes in activity, such as in response to nutrient availability, whereas alteration in the number of active rDNA copies was reserved for long term conditions such as following cell differentiation. However, recent studies suggest this may not be the case. For example, epigenetic silencing of rDNA copies was shown to occur in response to low cellular energy levels [182, 260].

Ribosomal biogenesis is believed to be the number one consumer of cellular energy in rapidly dividing cells [113]. Building a ribosome is an enormous task which requires the concerted effort of all three RNA polymerases to produce approximately 80 ribosomal proteins, 200 non-ribosomal proteins, 4 large ribosomal RNAs and 75 small nucleolar RNAs [62, 124, 163]. To give perspective, in yeast RNA Polymerase I is believed to account for 60% of total cellular transcription [250]. Moreover, at any given time, ribosomal RNA can account for as much as 80% of total cellular RNA, with tRNAs and mRNAs accounting for 15% and 5%, respectively [250]. Thus, ribosomal biogenesis is a major undertaking which consumes enormous amounts of cellular resources.

Given the extensive transcriptional and energetic commitment, cells have evolved a ribosomal stress response for monitoring the fidelity of ribosome synthesis. Traditionally, the field has used the term nucleolar stress to refer to nucleolar disintegration and perturbed ribosomal biogenesis. However, current reports suggest that

nucleolar disintegration and perturbed ribosomal biogenesis are not synonymous. Ribosomal biogenesis can be perturbed in many ways which do not cause nucleolar disintegration. Thus, it is important to differentiate between the concepts of nucleolar stress and ribosomal stress (Fig.2).

In general, the term nucleolar stress describes an inhibition of RNA polymerase I which disrupts nucleolar morphology and triggers a p53 mediated stress response. However, over the last decade it has become apparent that the p53 response is centered on stoichiometric errors in ribosome assembly [44, 114]. Essentially, “extra-ribosomal” ribosomal proteins, meaning ribosomal proteins which are not incorporated into the ribosome, may be recruited to perform various “extra-ribosomal” functions [251]. The most extensively documented of these extra-ribosomal functions is the inhibition of the p53 negative regulator mouse double minute homologue 2 (MDM2) by the ribosomal proteins L5 and L11 [24, 63, 64]. However, numerous other extra-ribosomal functions have been described in the literature [251].

In addition to nucleolar stress-associated reductions of rRNA there are several additional mechanisms for disrupting stoichiometry of ribosomal biogenesis (Fig.3). Therefore, ribosomal stress is a separate entity from the canonical concept of nucleolar stress. Haploinsufficiency of a single ribosomal protein gene is one such mechanism and is linked to diseases such as Diamond Blackfan anemia and 5q- Syndrome. Oncogenic signaling may be a third possibility [114, 153]. The ribosomal stress pathway appears to be tumor suppressive as mice lacking ribosomal protein-MDM2 interaction display accelerated leukemogenesis in a c-Myc driven leukemia model [153]. This suggests that

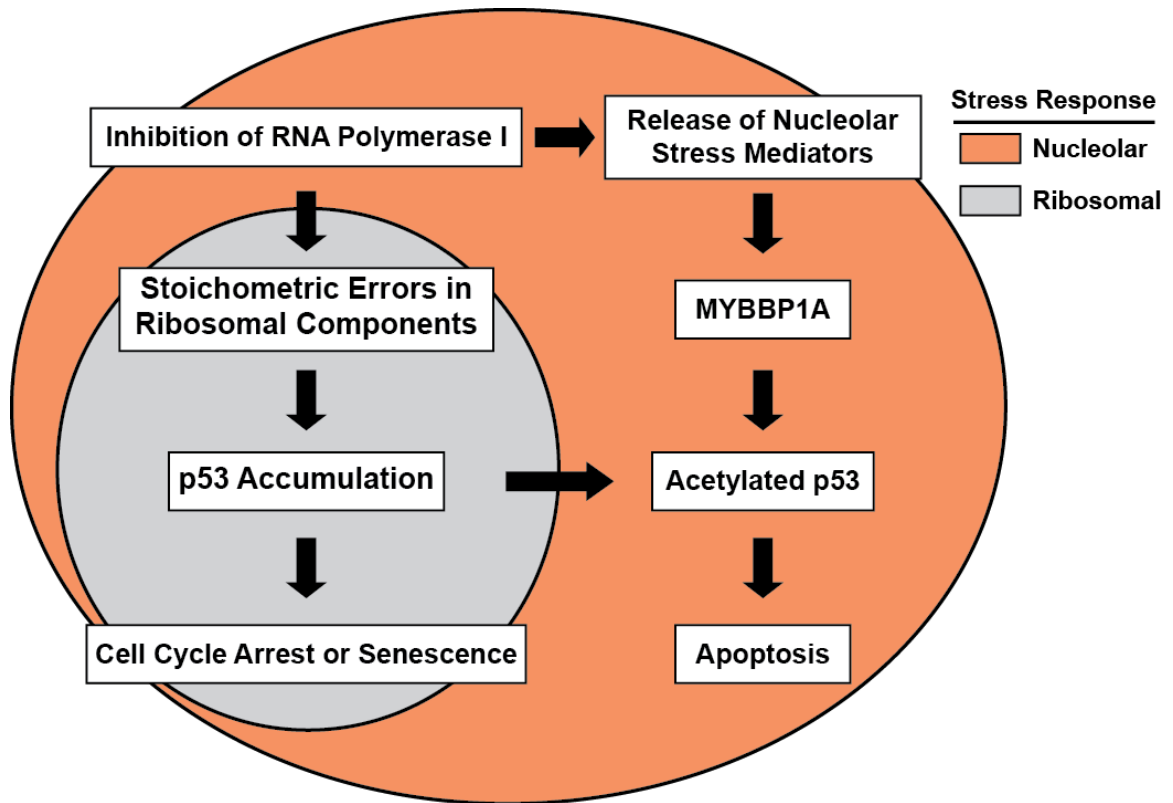


Figure 2. Overlap of the nucleolar and ribosomal stress responses.

The ribosomal stress response is depicted within the blue/gray oval. The nucleolar stress response is contained within the orange/tan oval. In many cell types, ribosomal stress without nucleolar disruption triggers a non-apoptotic accumulation of p53. Numerous stress mediators are sequestered inside the nucleolus, but are released following inhibition of RNA polymerase I. One such mediator, MYB binding protein 1A is known to acetylate p53 altering the spectrum of its target genes and switching the p53 response from cell cycle inhibition to apoptosis.

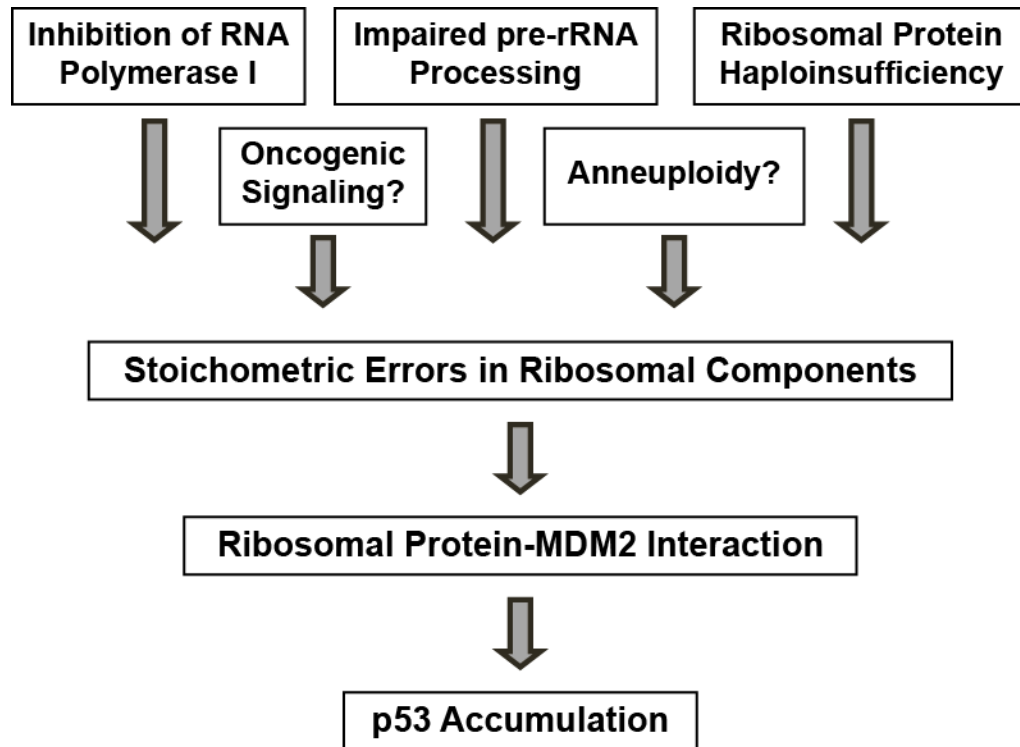


Figure 3. Potential inducers of ribosomal stress.

Multiple mechanisms for altering ribosomal stoichiometry are possible. The canonical model of nucleolar stress is centered on inhibition of RNA polymerase I, but impairment of pre-rRNA processing and haploinsufficiency of ribosomal protein genes are also well documented mechanisms for altering ribosomal stoichiometry. Preliminary evidence suggests oncogenic signaling also engages the ribosomal stress response. Lastly, aneuploidy has been hypothesized to alter the stoichiometry of ribosomal components, although direct evidence for such a phenomenon is still lacking.

Myc driven oncogenesis engages the ribosomal stress response in some manner. Thus, it is likely that oncogenic stimuli and Myc overexpression in particular, disproportionately stimulates production of certain ribosomal components relative to others. In support of this concept, a constitutively active variant of the oncogene Harvey Rat Sarcoma Viral Oncogene Homolog (HRAS), was shown to stimulate the production of 47S pre-rRNA in excess of the cells capacity for processing [187]. Such a condition induced p53 mediated senescence and was rescued by overexpression of several processing proteins. While it was not shown explicitly, this strongly suggests cells contained insufficient quantities of mature rRNA species relative to ribosomal proteins. A fourth possibility is aneuploidy [114]. To date, 14 ribosomal proteins are known to bind MDM2 and importantly they are dispersed throughout the genome [114]. Such a distribution may enable a ribosomal stress-mediated p53 response in aneuploid cells. Therefore, stoichiometric imbalances of ribosomal components can be generated by numerous mechanisms that are independent of RNA Polymerase I. Thus, nucleolar disruption is sufficient, but not necessary to induce ribosomal stress. Additionally, nucleolar stress may involve numerous stress mediators not seen with other inducers of ribosomal stress due to failure of nucleolar sequestration. For example, the protein Myb binding protein 1 A (MYBBP1A) exits the nucleolus during nuclear stress and acetylates p53 [125]. Interestingly, the nucleolar structure also excludes the entrance of some stress mediators as the tumor suppressor p19-alternate reading frame (ARF) enters the nucleolus upon nucleolar stress [225].

The mechanisms of ribosomal protein-MDM2-p53 interactions are only partially understood. While it appears that the 5S RNP is potentially the major mediator, many other ribosomal protein have been reported as MDM2 binding partners [24, 49, 63, 64].

As stated previously, 14 ribosomal proteins have been identified as binders of MDM2 and some have speculated that this number is an underestimate [114]. MDM2 ribosomal protein binding locations appear to be determined by acid base mediated protein-protein interactions [114]. Thus with 80 ribosomal proteins, the majority of which are highly basic, there is opportunity for more.

Some have questioned the physiological relevance of some ribosomal protein-MDM2 interactions [24]. Many of these interactions have been identified in overexpression studies and thus may represent experimental artifact [41, 146, 157, 264]. In support of this concept, two studies using different methods to impair ribosomal biogenesis both demonstrated that only RPL5 and RPL11 are absolutely necessary for p53 stabilization, suggesting that they alone are the master mediators[24, 64]. Moreover, Bursac et.al. (2012) demonstrated that following nucleolar disruption unbound ribosomal proteins are rapidly degraded by the proteasome [25]. In support of this finding, the Lamond laboratory has reported persistent proteasome-mediated degradation of excess ribosomal proteins under basal unstressed conditions[131]. Conversely, RPL5 and RPL11 escaped this degradation by co-dimerization [25]. Therefore, the buildup of “extra-ribosomal” ribosomal proteins other than RPL5 and RPL11 appears unlikely under physiological conditions. However on the other hand, three MDM2-interacting ribosomal proteins have been shown to produce differential effects on the transcription of p53 target genes [40]. Thus, the relevance of other ribosomal proteins is still under debate.

Interestingly, impairment of either the large- or the small subunit was shown to produce separate responses, which were additive upon co-occurrence [64]. In the case of

the large subunit, free RPL5 and RPL11 were generated by the expected stoichiometric imbalances of ribosomal proteins. Surprisingly, in the case of disrupted synthesis of the small subunit accumulation of p53 was achieved through increased translation of 5' terminal oligopyrimidine tract (5'TOP) mRNAs, which include RPL5 and RPL11 [64]. Such findings raise several interesting questions. First, are unbound ribosomal proteins always degraded by the proteasome or is this a feature specifically induced by nucleolar stress? If it is indeed nucleolar disruption specific, this increases the likelihood that additional ribosomal proteins are stress effectors. Second, if RPL5 and RPL11 levels can be increased in the absence of nucleolar disruption or large subunit impairment, could other stressors be converging on this mechanism? Third, given that RPL5 and RPL11 are necessary for p53 induction and the fact that other ribosomal protein-MDM interactions have produced differential effects on the expression of p53 target genes, could there be different outcomes of ribosomal stress dependent on which RPs are primary stress mediators [40, 64]?

The neuronal nucleolus plays major roles in cell growth, maintenance and repair. Thus in addition to the ribosomal stress response one must also consider loss of these functions as a possible contributor to neurodegeneration. Brain derived neurotrophic factor (BDNF) is a known inducer of both ribosomal biogenesis and neuronal growth. It has been shown that ongoing transcription of 47S pre-rRNA is necessary for BDNFs morphogenic effect on hippocampal neurons [72]. BDNF treatment was shown to significantly increase neurite length, neurite branching, and cell body volume and these increases were prevented after shRNA mediated knockdown of the RNA Polymerase I cofactor Transcription Initiation Factor 1A (TIF-1A). Importantly, these effects were not

due to p53 signaling or impairment of total cellular translation. Taken together, these observations suggest that the rate of ribosomal biogenesis regulates neuronal size.

Therefore, given the importance of ribosomal biogenesis for growth and maintenance of neurites suboptimal levels of ribosomal biogenesis may result in neuronal atrophy and synapse loss.

Similarly, increased rates of ribosomal biogenesis have been connected to a neuron's ability to regenerate after injury. In a series of related reports the Jones laboratory investigated nucleolar function in regeneration competent facial motor neurons and regeneration incompetent rubrospinal neurons [115, 116, 231]. Their data suggest that both classes of neurons experience stalled pre-rRNA processing and possibly increased RNA polymerase I activity following axotomy. In the case of regeneration competent neurons the inhibition of rRNA processing was transient and ribosome content increased acutely [115, 116]. Moreover, treatment with testosterone decreased the duration of processing impairment and further augmented recovery. Conversely, regeneration incompetent neurons did not experience any increases in ribosome content and testosterone had no effect on regeneration [231]. Thus, robust levels of ribosomal biogenesis are implicated in neuronal regeneration.

A substantial amount of evidence suggests that nucleolar dysfunction is present in human neurodegenerative diseases. In some cases one disease may engage multiple anti-nucleolar/anti-ribosomal mechanisms suggested while in other cases similar mechanism may compromise ribosomal biogenesis in multiple pathologies. In the sections which follow, I will review the literature that presents evidence of nucleolar involvement in the pathogenesis of various neurodegenerative diseases.

Parkinson's Disease

Nucleolar dysfunction is documented in both Parkinson's disease (PD) patients and animal models of PD. The size of the nucleolus is believed to be directly related to the amount of pre-rRNA stored within its borders[202]. Provided there is no impairment of processing, nucleolar size is a direct reflection of RNA Polymerase I activity. Decreased nucleolar volume has been documented in human PD tissue and two pharmacological PD models, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) [88, 156, 214]. In addition, increased frequency of dopaminergic neurons with dispersion of nucleolar nucleophosmin, (NPM1) was observed in substantia nigra from PD brains as compared to controls [214]. Moreover, decreased expression of nucleolin, a protein necessary for ribosomal biogenesis, has been detected in both PD brain and the rotenone pharmacological model [32]. Lastly, angiogenin, which has been suggested to be a disease modifier in PD, is known to promote RNA Polymerase I activity and its overexpression was shown to be protective in two out of three pharmacological PD models[229, 230].

Two genes commonly mutated in PD have direct connections to the nucleolus. Mutant DJ-1 aggregates have been shown to sequester TRAF and TNF Receptor Associated Protein (TTRAP) [247]. TTRAP is necessary for pre-rRNA processing and its entrapment in cytoplasmic aggregates prevents its nucleolar localization thus decreasing ribosomal biogenesis. Mutant Parkin is unable to bind the Parkin Interacting Substrate (PARIS) [107]. Unbound PARIS is then free to decrease rDNA transcription through interactions with the nucleolar protein MYB binding protein 1a (MYBBP1a), polymerase-associated factor 53 (PAF53), and the rDNA promoter.

In an attempt to determine the contribution of nucleolar stress to PD pathology Rieker et. al. generated a transgenic mouse model which contained floxed alleles for TIF-1A and crossed it with a mouse expressing tamoxifen inducible cre-recombinase under the control of the dopamine transporter promoter (TIF-IA^{DATCreERT2}) [214]. As TIF-1A is necessary for RNA Polymerase I activity, the authors could induce nucleolar disruption specifically in the dopaminergic neurons of adult mice.

Interestingly, TIF-IA^{DATCreERT2} mice developed a PD-like pathology upon deletion of TIF1A. With regards to cell signaling, both human PD and the TIF-IA^{DATCreERT2} mouse model have increased p53 signaling, decreased mTOR activity, and the accumulation of oxidative stress. With regards to pathology, the TIF-IA^{DATCreERT2} mouse presented with anatomical susceptibility similar to that of human PD, as dopaminergic neurons of the substantia nigra were considerably more sensitive than those contained in the ventral tegmental area. Moreover, TIF-IA^{DATCreERT2} mice experienced a prolonged chronic degeneration and reproduced motor deficits seen in other animal models of PD. Interestingly in the mouse model, adult dopaminergic neurons did not undergo acute apoptosis following loss of RNA Polymerase I activity and experienced a prolonged degeneration.

While the upstream mechanisms initiating PD are only partially understood, the general consensus is that the end result is death of dopaminergic neurons in the substantia nigra. Multiple pharmacological models, including dopamine, 6-OHDA, MPTP, and rotenone all strongly suggest p53 and caspase mediated apoptosis as the predominant source of neuronal death [139]. However, it should be noted that attempts to determine p53 mediated apoptosis in post mortem tissue have produced mixed results. Several

studies attempting to detect apoptosis by assessing DNA fragmentation in neurons have produced negative results [14, 101, 121]. However, two studies using more sensitive methods were successful at detecting apoptotic neurons [176, 241]. These struggles should not be surprising as neuronal death in PD occurs over decades, and typical apoptosis is initiated and executed over several hours or days. Thus, at any given time very few, if any, neurons are actually in the act of dying.

Molecular studies aimed at detecting apoptosis sensitizing proteins have been far more convincing, as significant increases have been detected for p53, its pro-apoptotic target gene, BCL2-associated X protein (BAX), and a pro-apoptotic active p38 mitogen-activated protein kinase (MAPK) [43, 85, 108, 177, 242]. Moreover, multiple studies have detected activation of apoptosis-associated caspases 3, 8, and 9 in PD compared to age matched controls [5, 84, 86]. Thus while it may be premature to say that apoptosis is major mode of neuronal loss in PD, there is considerable evidence that the pro-apoptotic arm of the p53 signaling is activated. Therefore, lack of apoptosis in TIF1A mouse model of PD suggests that it does not represent all pathological components of human PD.

Interestingly as part of the TIF-IA^{DATCreERT2} transgenic mice study, the authors stumbled upon an important link between nucleolar stress and oxidative stress. These two stressors appear to have a cyclical relationship as each one appears capable of inducing the other. TIF-IA^{DATCreERT2} transgenic mice had severely compromised mitochondrial function as determined by multiple parameters, including gene expression, enzymatic activity and markers of oxidative damage [214]. Expression of the transcription factor Yin-Yang 1 (YY1), which stimulates the transcription of multiple mitochondrial proteins, was decreased approximately 40% in mixed tissue samples.

Additionally, the YY1 target gene uncoupling protein 2 (UCP2) was also significantly reduced. Neuron specific measurement of cytochrome C oxidase activity was decreased approximately 40%. Additionally, 4 weeks after tamoxifen treatment the frequency of neurons which contained markers of oxidative damage, including tyrosine nitrosylation, 8-Oxo-2'-deoxyguanosine (8-OHdG), and neuroketals, were increased several fold. Taken together these data strongly suggest that nucleolar stress is sufficient to compromise mitochondrial function and induce oxidative stress. Interestingly, the oxidative stress based MPTP pharmacological model of PD also produced significant deficits in nucleolar function, as determined by decreased 47S pre-rRNA levels, increased NPM dispersion and decreased p-RPS6. Thus, nucleolar stress and oxidative stress appear to reciprocally regulate one another.

Poly Glutamine Diseases- Huntington's-Disease, Machado-Joseph's-Disease, and Several Spinal Cerebellar Ataxias

Lee et.al. (2011) demonstrated for the first time that pre-rRNA levels were significantly decreased in the R6/2 Huntington's Disease (HD) mouse model [135]. This finding would later be independently verified by two other laboratories [214, 245]. Additionally, they showed that protein levels of the RNA Polymerase I cofactor upstream binding factor (UBF) were also reduced in both R6/2 mice and human striatal tissue. Interestingly, UBF mRNA levels were not decreased suggesting that HD pathology may preferentially decrease its protein stability. Moreover, the ability of ectopically expressed UBF to stimulate transcription was reduced in cell lines expressing mutant huntingtin (htt)-Q111, but not htt-Q7 controls, suggesting that an upstream regulator of UBF was impaired in HD. Using both cell lines and animal models, the authors showed that CREB

binding protein (CBP) had decreased levels and a portion was also sequestered in htt aggregates. Moreover, they demonstrated that CBP mediated acetylation of UBF was necessary for RNA Pol I transcriptional activity and importantly, acetylation was significantly decreased in HD models. Taken together these findings support a model where HD aggregates sequester CBP preventing it from acetylating UBF which subsequently inhibits ribosomal biogenesis. Interestingly, in a follow up article this same group demonstrated that silencing of UBF through ESET mediated trimethylation is also up regulated in HD cell lines [94].

Tsoi et.al. (2012) focused almost exclusively on the CAG expansion in the Machado-Joseph's Disease gene (MJD), but their work also suggests that CAG expansion toxicity is "motif" specific and toxicity is independent of the gene where it is located [245]. This suggests MJD, HD, and CAG associated spinal cerebellar ataxias all contain the same pathological mechanism. Ribosomal biogenesis appears to be significantly reduced in MJD flies as both pre-rRNA levels were significantly reduced and rDNA promoter methylation, which is a biomarker for inactivity, was significantly increased. Moreover, RPL5, RPL11 and RPL23 were shown to be in complexes with MDM2. It is well documented that nucleolar stress activates p53 mediated apoptosis via ribosomal protein mediated inhibition of MDM2. Indeed, p53 mediated apoptosis was activated in response to CAG expansion in MJD flies, as determined by increased p53 expression, the appearance of cytosolic cytochrome C, and activated caspase 3.

The mechanism of MJD-associated ribosomal stress appears to be strictly RNA mediated and involves sequestration of the RNA Polymerase I cofactor nucleolin. This was determined in two ways. First, CAGCAA repeats which code for identical proteins

did not produce toxicity. Second, CAG expansion in the 3' untranslated region of a GFP vector still produced toxicity. Pull down experiments demonstrated that CAG RNAs were binding and sequestering nucleolin. In support of this concept, MJD cells displayed decreased binding of nucleolin to the rDNA gene upstream control element. Thus, if deficiencies in nucleolin activity were responsible for the pathology seen in MJD, overexpression would be expected to produce a significant rescue. Indeed, nucleolin overexpression reduced apoptosis and returned pre-rRNA to control levels. Interestingly, a similar mechanism has been implicated in chromosome 9 open reading frame 72 (C9ORF72) hexanucleotide expansion linked Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) [79].

Despite focusing primarily on the contribution of nucleolar disruption to HD pathology through the use of striatum specific induction of nucleolar stress, Kreiner et.al. (2013) still expanded upon the other studies claiming nucleolar stress in the R6/2 HD mouse model [122]. In addition to reproducing the pre-rRNA deficits seen by others, they demonstrated that the frequency of neurons with dispersed nucleophosmin was also significantly increased at time points occurring prior to the induction of neuronal death. Additionally at 13 weeks, total cellular 18S rRNA was decreased approximately 60% suggesting that cellular ribosome content was substantially reduced.

The authors generated a transgenic mouse line (TIF-1A^{D1RCre}) which contained genetic inactivation of TIF-1A in medium spiny neurons by crossing TIF-1A^{flox/flox} mice with Dopamine receptor D1 promoter driven Cre recombinase mice. The TIF-1A^{D1RCre} mouse model phenocopied many aspects of the R6/2 HD mouse. Abnormal posture and gait were evident at 40 weeks of age. As early as 16 weeks, more subtle differences such

as increased feet clasping behavior and decreased rotarod performance were evident. TIF-IA^{D1RCre} mice also experienced increased p53 and oxidative damage [122]. Gliosis occurred sometime between 9 and 13 weeks of age. As the TIF-IA^{D1RCre} mice modeled several functional and pathological elements of HD, large scale gene expression changes were also investigated. Microarray data from TIF-IA^{D1RCre} mice were compared against published mRNA profiles of R6/2 mice and early stage human HD. When considering the top 250 upregulated and downregulated genes, TIF-IA^{D1RCre} matched the molecular signature of human HD considerably better than the R6/2 mice.

Taken together these studies suggest nucleolar stress is a mediator of neurodegeneration in HD. While several neurodegenerative diseases contain evidence for insufficient ribosomal biogenesis, HD appears to contain several mechanisms capable of inhibiting RNA Polymerase I and inducing p53-mediated apoptosis. Moreover, both htt derived protein and RNA have been linked to toxicity. Therefore, nucleolar stress appears to be a major contributor to HD-associated neurodegeneration.

C9ORF72 Hexanucleotide repeat expansion - Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD)

Hexanucleotide repeat expansions in the C9ORF72 gene (C9) have been causally linked to both ALS and FTD, however the mechanisms are still under debate. C9ORF72 expansion is the most common source of both familial Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD). In the literature estimates for the percentage of ALS/FTD caused by C9 hexanucleotide expansion have been highly variable (20%-67%). The ALS association reports it accounts for approximately 40% of familial cases. Regarding familial FTD, C9 is believed to account for approximately

25% of cases. Interestingly, in very rare circumstances C9ORF72 expansion has also been linked to Parkinsonism [138].

Three major hypotheses exist regarding C9ORF72 pathology; 1.-protein based gain of function, 2.-RNA based gain of function, and 3.- loss of function of the C9ORF72 gene. It is important to note that these theories are not mutually exclusive, multiple mechanisms are possible, and evidence supporting all three can be found in the literature. However, there are no loss of function studies implicating nucleolar involvement. Contained within these 3 theories are 7 potentially toxic species, 5 dipeptide repeats (DPRs) and 2 long repetitive RNAs, both sense and anti-sense [252]. Long non-coding RNAs derived from highly repetitive sequences are believed to be translated by repeat associated non-ATG (RAN) translation [268]. The mechanisms of RAN translation are poorly understood, but the process is believed to be different from canonical AUG initiated CAP -dependent and -independent translation. As RAN translation can occur in either the sense or anti-sense direction and all reading frames are potentially possible, 5 different DPRs could theoretically arise from GGGGCC hexanucleotide repeats, poly-proline-arginine (PR), poly-glycine-arginine (GR), poly-glycine-alanine (GA), poly-glycine-proline (GP), and poly-proline-alanine (PA) [252]. Importantly, all 5 DPRs have been detected in post mortem ALS and FTD tissues.

Recent cell culture based studies suggest that the two arginine rich DPRs, poly-GR and poly-PR, are the major toxic species [129, 175, 240, 252]. Moreover, intricate *in vitro* studies have demonstrated that these two DPRs have a clear length dependence and dose response, as increasing plasmid dosage increased toxicity [252]. Multiple laboratories have suggested that these two peptides have a strong affinity for the

nucleolus, a feature which is not seen with the other three species. Tao et.al. (2015) showed that expression of poly-GR and poly-PR can produce significant reductions in 18S and 28S rRNA in just 48 hours [240]. Wen et.al. (2014) demonstrated that nucleolar aggregation preceded cell death [252]. In all cases neuronal death occurred 24 to 72 hours after arginine rich DPRs aggregated in the nucleolus and interestingly, cells with DPRs which remained diffuse did not die during the course of their study. However, it is important to note that some studies were not able to detect poly-GR in the nucleolus and DPR localization is not always consistent when comparing cell culture studies to post mortem tissue [219, 259]. Interestingly, one study, which did not report nucleolar localization of poly-GR, still documented nucleolar enlargement. Impairment of RNA processing bodies and altered RNA stress granule formation has also been reported after overexpression of poly-PR and/or poly-GR[252].

Kwon et al. (2014) has proposed that the two arginine containing dipeptide repeats essentially mimic the low complexity serine arginine (SR) domains which are contained in many pre-mRNA splicing factors such as, serine-arginine splicing factor 2 (SRSF2) [129]. Poly-SR, poly-GR, and poly-PR were all shown to bind heterogeneous nuclear ribonucleoprotein A2 (hnRNPA2) hydrogels, structures which display a high affinity for RNA granule components. However, SR domain proteins release binding following phosphorylation, a feature which cannot occur on the serine deficient poly-PR and poly-GR dipeptide repeats. They demonstrated that both 47S pre-rRNA and mRNAs had impairment of processing and aberrant splicing following treatment with recombinant DPRs. Similar findings have also been documented in human samples as the frequency of splicing errors is significantly higher in C9ORF72 patients compared to healthy

controls [37]. Interestingly, the frequency of errors correlates well with disease severity [37]. Such a phenotype could be potentially explained by arginine rich DPRs irreversibly binding RNAs and out competing mRNA splicing factors and pre-rRNA processing machinery. In support of this concept, PR and GR dipeptides localized to both nucleoli and nuclear puncta, which have been proposed to be sites of pre-mRNA splicing [252].

While several studies have demonstrated that C9ORF72 RNA species are toxic, only one has been linked to the nucleolus [79, 102, 252]. Haeusler et.al. (2014) proposed that the hexanucleotide repeat region of C9ORF72 forms both G-quadruplex structures and DNA-RNA hybrids which impede and ultimately stall RNA polymerase I generating “abortive transcripts” [79]. They demonstrated that these abortive transcripts had a significant affinity for both nucleolin, a fundamental nucleolar protein necessary for pre-rRNA processing, and the ribosome itself. Hexanucleotide repeat RNA transcripts localized to the nucleolus and in turn altered its morphology. Nucleoli were abnormally large and had a fragmented shape. Such morphology is not indicative of canonical nucleolar stress, but may suggest some degree of functional impairment.

Several studies, both DPR and RNA based, have described abnormally large fragmented nucleoli [79, 240, 252]. The size of the nucleolus is believed to be related to the amount of pre-rRNA stored within its borders, thus such a finding suggests two possible conditions. First, increased nucleolar activity could potentially be a compensatory response. Alternatively, decreased pre-rRNA processing could increase retention and allow for accumulation. The latter possibility appears most likely because, as previously mentioned; impairment of pre-rRNA processing has been detected after pharmacological treatment with recombinant poly-PR [129]. Importantly, while such a

condition is not indicative of canonical nucleolar stress, it may still represent impairment of ribosomal biogenesis. Nishimura et.al. (2015) showed that impairment of pre-rRNA processing can produce enlargement of the nucleolus and induce p53 through ribosomal proteins/MDM2 interactions [187]. Theoretically, inhibition of RNA Polymerase I and impairment of pre-rRNA processing should both produce stoichiometric errors in ribosomal components.

In conclusion, numerous studies depicting nucleolar involvement in response to C9 toxicity have been reported. However, none of these studies have unequivocally demonstrated the induction of nucleolar- or ribosomal stress. Therefore, the role of the nucleolus in C9 linked ALS and FTD appears to a promising topic for future studies.

Alzheimer's Disease

While nucleolar stress has not been directly implicated in Alzheimer's disease (AD), several studies have documented abnormalities in nucleolar volume and reduced ribosome content in Alzheimer's disease brain tissue. Over 30 years ago, Langstrom et al. (1998) initially documented decreased ribosome content in the frontal lobe of AD patients [132]. Moreover, the translation capacity per ribosome was also decreased. Importantly, such deficits were not seen in cerebellar tissues suggesting that impairments were specific to areas undergoing AD pathology. More recently, AD associated proteins synthesis deficits were confirmed by Ding et.al. (2005) using ribosomes purified from the inferior parietal lobe and superior middle temporal gyri [48].

Interestingly, there are conflicting reports about total ribosome content in AD. The aforementioned study by Ding et.al. (2005) concluded that total ribosome content

was unchanged in AD and MCI [48]. The authors were unable to identify decreases in ribosomal proteins or the 18S and 28S rRNA in patient tissues. However, they did report decreases in the 5S and 5.8S rRNAs. Ultimately, they concluded that total ribosome content was unchanged between AD and controls based on the constancy of the major RNA species and ribosomal proteins. Additionally, they documented significant increases in rRNA oxidization in both Alzheimer's disease and Mild Cognitive impairment. Such a condition could potentially explain the protein synthesis deficits seen in AD. They proposed that ribosomes were undergoing qualitative, rather than quantitative deficits. To paraphrase, they hypothesized that in regions of the brain which experience AD-associated pathology the pool of ribosomes was damaged and operating at suboptimal levels.

A year later this same group applied different methods for quantifying ribosomal content and found significant decreases in both the 18S and 28S rRNAs [47]. The Initial study utilized qPCR to measure total ribosome content, while the follow up study utilized spectrophotometric measurements after serial fractionation. Importantly, these deficits were found in both the mature 80S ribosomes fraction and precursor fractions. Interestingly, a recent report from the Ferrer laboratory was able to confirm decreased 28S rRNA in the CA1 region of the hippocampus in all stages of AD, but surprisingly 18S rRNA was unchanged [89]. They also reported decreases in some, but not all, ribosomal proteins. As ribosomes are large multicomponent complexes such stoichiometric imbalances suggest decreased ribosome content, despite the constancy of some components. In support of this concept, two groups have documented decreased

nissl staining intensity in both AD hippocampal neurons compared to controls [89, 150]. Nissl staining intensity is frequently used as a marker of cellular ribosome content [226].

Several reports also suggest impairment of RNA polymerase I. First, increased rDNA promoter methylation was demonstrated in both MCI and AD brain tissues [206]. Promoter methylation is used as a biomarker for recent transcriptional activity. Additionally, two very similar studies conducted almost a decade apart both identified decreased silver staining in the lymphocytes of AD patients [50, 200]. Such a phenotype is characteristic of decreased rRNA synthesis. Moreover, expression of the RNA Pol I cofactor Upstream binding factor and nucleophosmin 1 were significantly decreased in early AD [89]. Taken together these findings strongly suggest neuronal ribosome content is indeed decreased in AD.

Regardless, qualitative and quantitative deficits both suggest a failure of ribosomal biogenesis in AD. Homeostatic mechanisms would be expected to replace ribosomes which have incurred considerable amounts of oxidative damage. As ribosomal biogenesis is a highly dynamic processes which is known to be intricately regulated by numerous processes, both acute and chronic, it would be highly unexpected if cells did not up regulate ribosomal biogenesis in response to deficits in protein synthesis capacity. Therefore, both outcomes suggest a failure of homeostasis. In support of this concept, hypertrophic nucleoli have been identified in asymptomatic AD [95]. However, once patients became symptomatic (i.e. mild cognitive impairment and AD) nucleolar volume was significantly decreased [95]. Such findings suggest that neurons may indeed undergo an early homeostatic up regulation of ribosomal biogenesis, which subsequently fails in the later stages of AD.

It is important to consider that evidence for an AD-associated decrease in RNA pol I activity was derived from brain tissue, neurons, and peripheral lymphocytes. Numerous studies have documented decreased metabolic activity and hypoperfusion in the AD brain [22, 144]. As RNA Pol I is tightly regulated in response to metabolic stimuli, one could argue that such a phenotype occurs near the end of the disease cascade and is simply the result of hypoperfusion. However, the finding that peripheral lymphocytes also display decreased RNA Pol I activity suggests otherwise. Moreover, decreased UBF and NPM1 expression was shown to occur in the early stages of AD, well before neuronal death occurs [89]. As such, impairment of ribosomal biogenesis may indeed be an early feature of AD-associated neurodegeneration and not simply a downstream consequence of hypoperfusion or end-stage degeneration.

Neurodegeneration-associated Expansion of the rDNA locus

It is well accepted that highly repetitive DNA sequences can be notoriously unstable and this feature is especially true for ribosomal DNA [67, 97, 117, 119]. In 1972, Bernard Strehler proposed that neuronal rDNA loss was a feature of mammalian aging, although this hypothesis has subsequently been disproven [68, 69, 105, 205]. In some cases alterations in cellular rDNA content appear to be physiologically relevant responses the cell purposely undertakes. For example, it is known that amphibian oocytes have significantly amplified quantities of rDNA and this amplification is not maintained into adulthood [21, 174]. While the exact purpose of this amplification is not known, it seems logical that this feature could facilitate the rapid cell growth and expansion necessary during the early stages of development. Alternatively, loss of rDNA in yeast is believed to contribute to replicative senescence by triggering the DNA damage

response [118, 120]. The instability of rDNA is also evident in a number of pathologies including several forms of cancer, Bloom syndrome and ataxia telangiectasia [112, 234]. Interestingly, Werner syndrome has been associated not with changes in rDNA content, but instead with inversion of rDNA repeats [28]. The pathophysiological relevance of rDNA rearrangements is not known.

These findings and the AD associated ribosome abnormalities detailed in the prior section prompted our group to investigate rDNA gene content and promoter methylation in Alzheimer's disease and Dementia with Lewy Bodies. One potential explanation for an inadequate supply of ribosomes could be decreased synthesis of pre-rRNA. As stated previously, pre-rRNA synthesis is believed to be regulated in two ways first, by altering the number of actively transcribed copies and second, by altering the activity at each individual copy. Thus, by measuring promoter methylation, which is a mechanism for epigenetic rDNA silencing and a biomarker of recent activity, one can determine if rDNA copies were silenced. Moreover, loss of rDNA copies could also potentially explain ribosomal insufficiency.

In AD, rDNA promoter methylation was indeed increased, but unexpectedly rDNA content was also significantly increased [206]. In cell lines, increased rDNA promoter methylation was shown to resist rDNA instability[77]. The increased stability was demonstrated using cell lines transfected with shRNA directed against TTF interacting protein 5 (TIP5), which is a key component of the complex responsible for rDNA methylation. The authors demonstrated a loss of rDNA content in shTIP5 cells after ten passages. Such a mechanism raised an important question. Was the AD-associated increase in rDNA content the result of hyper methylation protecting against an

aging associated loss of rDNA or alternatively, was this feature a disease-associated amplification?

Despite the fact that Strehler's theory of aging related rDNA loss appeared to be thoroughly debunked in the 1970s, our results raised serious questions [68, 69]. Mechanistically, prevention of loss seemed more plausible than disease associated amplification. Therefore we revisited Strehler's hypotheses and probed rDNA content in both young and old tissue donors. Additionally, we chose to probe Dementia with Lewy Bodies tissue donors to determine if our prior results were specific to AD or if they were a general consequence of neurodegeneration. We found that there was no difference in rDNA content between young (2-25) and old (73-90) individuals as determined by qRT-PCR using two separate rDNA amplicons [81]. Additionally, we found that similar to AD, rDNA content was increased in the cortex of DLB patients [81]. However, that is where the similarities ended. rDNA methylation was unchanged in DLB and rDNA content was unexpectedly decreased in the pathology free cerebellum [81]. Such findings suggest that our prior AD results were not the prevention of rDNA loss and that DLB and AD may represent two separate phenomena.

Chemotherapy-associated neurological conditions

While not being neurodegenerative diseases per se, two neurological conditions, Chemotherapy-associated cognitive dysfunction and Chemotherapy induced peripheral neuropathy (CIPN) have the potential to involve nucleolar dysfunction. Relative to other diseases, very little is known about these two conditions. While CIPN is a well-documented phenomenon, it has historically been an afterthought for the research

community. Early cancer research focused heavily on effectiveness. Side effects were simply not a priority [54]. However, as success rates for chemotherapeutic treatment have increased dramatically over the last two decades, interest in CIPN and Chemotherapy associated cognitive dysfunction are now growing rapidly [26, 54, 190]. Additionally, the concept of Chemotherapy-associated cognitive dysfunction, or “chemobrain” as survivors refer to it, is only just beginning to be an accepted medical condition [190]. To date, direct evidence for nucleolar stress in either of these conditions has not appeared in the literature. In this section I will provide a general overview of both chemobrain and CIPN and summarize the evidence depicting nucleolar stress in response to many common chemotherapeutics. Lastly, I will present a working model in which nucleolar stress may indeed be a major source of chemotherapy associated neuronal dysfunction.

“Chemobrain” is a condition of cognitive impairment which is associated with chemotherapeutic cancer treatment. Patients commonly described it as a mental “fog” [190]. To date, very little is known about the source of chemobrain as it is only beginning to be accepted as a legitimate neurological condition. Additionally, some have proposed that this condition is not simply a side effect of certain chemotherapeutic drugs. In addition to pharmacological toxicity, stresses related to hormone therapy, surgery with general anesthesia, and radiation treatment have all been proposed to play a role in chemobrain [172].

Two decades ago chemobrain was a highly controversial topic and evidence for its existence was mostly anecdotal. More recently, numerous studies have validated its existence in patients who are actively undergoing chemotherapy [190]. However, the

severity and frequency of chemobrain varies wildly depending on the study. For example, moderate to severe cognitive deficits have been reported to occur in anywhere from 18% to 78% of breast cancer patients [103]. For perspective, 8% of the general population report having cognitive deficits [179]. Moreover, many patients present with fairly heterogeneous symptoms, however most display deficiencies in learning new information and accelerated forgetting of information [218]. While acute chemobrain is now well accepted, chronic forms are still debatable [190, 221]. Numerous studies both for and against the concept of a chronic post-treatment chemobrain have been reported. Such differences in findings may be due to factors such as individual heterogeneity, differences in choice of chemotherapeutic and differences in the duration of treatment [179].

It is important to consider that the mechanism of action for many chemotherapeutics is still fairly ambiguous. Moreover, many drugs may also be multi-mechanistic. A classic example of this phenomenon is 5-fluorouracil (5-FU). For years it was believed that 5-FU's primary mechanism of action was DNA damage [148, 197]. While 5-FU does indeed damage DNA by depleting the cell of dTTP and inducing the incorporation of dUTP into DNA, it also inhibits processing of pre-rRNA and mRNA splicing [237, 265]. Recent reports suggest the latter effect may actually be the major driver of 5-FU toxicity. Sun et.al. demonstrated that 5-FU impaired ribosomal biogenesis and induced p53 in RPL5 and RPL11 dependent manner [237]. Moreover, administration of thymidine which replenishes cellular pools of dTTP, thus preventing dUTP incorporation, produced only a minor rescue of cell survival following 5-FU treatment

[209]. Such results suggest that DNA damage is only a minor component of 5-FU toxicity.

Some have speculated that the nucleolus is the master regulator of the p53 response [23]. With regard to the DNA Damage response, Rubbi and Milner showed via highly focused UV radiation that nucleolar function must be altered before p53 would accumulate [217]. Severe DNA damage outside the nucleolus was unable to induce p53. It appears that cells are essentially sampling ribosomal DNA to approximate genome wide DNA damage. Moreover, it has been shown that p53 levels fluctuate inversely in response to relatively minor changes in the rate of ribosomal biogenesis, such as in response to cellular energy levels [126]. Thus, the nucleolus may indeed be a master regulator of p53.

Given the ambiguity detailed above and the emerging evidence suggesting the nucleolus as a master p53 regular, Burger et al. tested 36 common chemotherapeutics for an effect on ribosomal biogenesis [23]. The chosen drugs originated from 9 different classes. They showed that 16 common chemotherapeutics had large effects on either pre-rRNA synthesis, pre-rRNA processing, or induced the dispersion of NPM. Four others had modest effects. Sixteen drugs had no effect on ribosomal biogenesis or nucleolar morphology. Interestingly, while some classes of drugs produced similar effects, this was not always the case. For example, all intercalating agents tested (4/4) potentially inhibited RNA pol I, whereas none of the HDAC inhibitors (0/2) or mitosis inhibitors (0/2) produced potent effects. However, one mitosis inhibitor, Paclitaxel, did manage to produce some effect on late pre-rRNA processing. Conversely, alkylating agents were highly heterogeneous. Cisplatin and Oxaliplatin both strongly inhibited pre-rRNA

synthesis, while 6 others in this class had no effect and one had a weak effect. Similarly, antimetabolics were also highly heterogeneous, with 5-fluorouracil and methotrexate producing potent effects, while 5 others did not.

In contrast to chemobrain, chemotherapy-induced peripheral neuropathy is considerably better characterized. CIPN has a rate of incidence of approximately 30-40% and is typically a sensory neuron specific condition [257]. However, motor and autonomic functions have been reported to occur at very high doses [51, 61, 143, 254]. Symptoms typically include burning, tingling, hyper sensitive touch and cold sensation, loss of proprioception, loss of perception of vibration, loss of pinprick, and numbness [54]. Symptoms begin in a stocking and glove pattern [196]. This suggests that the largest neurons are preferentially susceptible and many speculate this condition is primarily an axonopathy [54]. While CIPN is not a critical condition, the patient's quality of life is often dramatically impacted. The time required for symptoms to resolve varies greatly and is believed to depend on the class of drug, duration of treatment, and total cumulative dose [34, 54]. Generally, most cases of CIPN resolve approximately 3-6 months following cessation of treatment [8]. However, in some cases it has been reported that CIPN can take up to 9-13 years to resolve [180, 232].

Four classes of chemotherapeutics are most frequently associated with CIPN. These classes include platinum containing agents, proteasome inhibitors, microtubule targeting agents, and angiogenesis inhibitors. Interestingly, the one chemotherapeutic agent most commonly associated with CIPN, oxaliplatin, is known to have a robust effect on nucleolar function. 86% of patients receiving oxaliplatin report immediate symptoms [7, 256]. Oxaliplatin is known to decrease nucleolar size in the dorsal root

ganglion of adult rats given pharmacologically relevant doses [99]. Moreover, in cell culture models oxaloplatin robustly inhibits rRNA transcription, disperses nucleophosmin, and induces fibrillar nucleolar caps [23]. In general, inhibition of rRNA polymerase I appear to be a common feature of platinum containing alkylating agents [70, 167]. Interestingly, cisplatin, another platinum containing alkylating agent, is associated with a delayed onset form of CIPN which peaks after cessation of treatment [19].

Several studies suggest that proteasome inhibitors, such as Bortezomib, affect nucleolar function. Similar to oxaloplatin, peripheral neuropathy is the number one dose limiting side effect of bortezomib treatment [192]. Bortezomib's primary mechanism of action is the reversible inhibition of the chymotrypsin like activity of the $\beta 5$ proteasome subunit [2]. Downstream effects are known to include endoplasmic reticulum stress, up regulation of pro-apoptotic factors, and inhibition of NF- κ B [165]. Interestingly, upon proteasome failure multiple proteasome targeted proteins have been shown to accumulate in the nucleolus [133]. Similarly, PML-containing nuclear bodies also translocate to the nucleolus under such conditions [192].

A recent report from the Lafarga laboratory documented increased nucleolar volume, increased number of nucleoli, enhanced cajal body assembly, and increased pre-rRNA synthesis in sensory ganglion neurons following bortezomib treatment [192]. They proposed that increased nucleolar activity was potentially a homeostatic mechanism to protect the neuron in response to proteotoxic stress. In support of this concept, expression of the transcription factor c-Myc was increased while p53 was decreased. Such findings strongly suggest this may indeed be a protective response. Moreover, the

authors thoroughly demonstrate a lack of RNA polymerase I inhibition. However, it is important to consider other potential mechanisms and outcomes in response to the hypertrophic nucleoli.

In contrast to the findings detailed above, Burger et al. has documented that both Bortezomib and MG-132 inhibit late pre-rRNA processing [23]. In cycling cells, such a condition is sufficient to produce hypertrophic nucleoli and induce the ribosomal stress response [187]. In addition to processing impairments, ribosomal stress associated hypertrophic nucleoli can also be triggered by oncogenic stimuli [187]. Such a condition could potentially follow significant increases in c-myc expression which interestingly was described by the Lafarga group. Therefore, more work is necessary to truly determine if bortezomib induced hypertrophic nucleoli are indeed a homeostatic response or alternatively, a biomarker of nucleolar stress.

Similar to bortezomib, the microtubule targeting agent Paclitaxel is also associated with both hypertrophic nucleoli and peripheral neuropathy [99, 100]. Interestingly, co-administration of paclitaxel with oxaloplatin prevents oxaloplatin induced reduction in nucleolar size. This phenotype was also associated with reduced neurotoxicity. Such a condition is perplexing. Oxaloplatin is a well-documented inducer of DNA damage and its ability to inhibit RNA polymerase I is most likely due to this feature. Increased DNA repair would be required for restoration of RNA Pol I activity under such conditions. It is unlikely that a cytotoxic agent such as paclitaxel would induce accelerated DNA repair. The more plausible hypothesis is that paclitaxel inhibits pre-rRNA processing similar to several other chemotherapeutics, such as 5-FU, bortezomib, and MG-132 [23]. However, to the best of my knowledge this has not been

reported in the literature. It will be important for future studies to address if inhibition of pre-rRNA processing is a common mechanism for inducing CIPN.

As detailed previously, I propose that ongoing ribosomal biogenesis is necessary for neuronal growth, maintenance, and repair. Given the well documented involvement of nucleolar stress in response to many common chemotherapeutics, I hypothesize that these agents induce a failure of neuronal maintenance. Under such conditions, chronic administration of chemotherapeutics would induce a gradual neuronal atrophy, ultimately leading to synapse loss and potentially a minor decrease in axonal length. Upon removal of chemotherapeutics normal function would be restored. Subsequently, symptoms would resolve following a period of regrowth and the generation of new synapses. Interestingly, the kinetics of such a model match well with the regenerative capacity of each class of neurons, as CIPN resolves faster than chemobrain in most cases. In support of this concept, CIPN is known to begin in a stocking and glove pattern [196]. Such a phenotype strongly suggests that the largest neurons are the most vulnerable [54]. Coincidentally, neuronal size strongly correlates with nucleolar size [130]. Robust nucleolar output is believed to be necessary to maintain such large sizes [91]. Thus, one would expect larger neurons to be disproportionately affected by a nucleolar loss of function.

In summary, the nucleolus is an integral component of neuronal function and its role is significantly larger than just ribosome production. The studies reviewed in this chapter clearly demonstrate several key points. First, ongoing ribosomal biogenesis is necessary for the growth of young neurons and maintenance of mature neurons. Second, the nucleolus contains free ribosomal protein that may activate stress response pathways

providing a mechanism to monitor the fidelity and effectiveness of ribosomal biogenesis. Third, there is ample evidence for nucleolar dysfunction in many neurodegenerative diseases. Moreover, in the case of poly glutamine diseases there is considerable evidence suggesting the engagement of the nucleolar stress response. However, it remains to be seen if the nucleolar and ribosomal stress mechanisms predominately described in yeast, cell lines and mouse liver are applicable to the mammalian nervous system. Therefore, we propose to investigate the role of the nucleolus, and specifically RPL11 in neurodegeneration and to investigate stability of rDNA loci in the brain.

CHAPTER II

NEURODEGENERATION ASSOCIATED INSTABILITY OF RIBOSOMAL DNA

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 [109, 117, 147]. DNA damage is well documented in neurodegeneration and in some cases it may be sufficient to cause disease [92, 109]. Moreover, significant increases in oxidative DNA damage are known to occur in PDAD, PD, and DLB [151, 158]. 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 [20, 30, 92, 109, 211]. Moreover, DNA damage is well known to induce cell death by a number of mechanisms including inhibition of nucleolar transcription and genomic instability [92, 106].

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 [181]. 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 [181]. Interestingly in yeast, the mechanisms for increasing ribosomal DNA content have been proposed to be homologous recombination with unequal sister chromatid exchange [119].

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 [140]. Conversely, a growing body of evidence suggests that homologous recombination can be a source of genomic instability in response to regulatory failure [31, 123, 222]. 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 [12]. 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 [35]. Moreover in yeast, mutation of the slow growth suppressor 1 (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. [258]. 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 AD and DLB [29, 127, 216, 243]. Conversely, increased telomere content has been documented in the hippocampus of AD patients [243]. Moreover, Pietrzak et al. (2011) have previously an increase in the ribosomal DNA content of parietal- and prefrontal- cortex of AD donor tissues [206]. 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 [112, 118, 234]. 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 [119]. 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 [105]. 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 [68, 205]. Moreover, mammalian cell culture based studies of replicative senescence did not document changes in ribosomal DNA content [80, 152].

Cellular growth and maintenance require enormous amounts of protein synthesis. The ribosome is the center of all cellular translation and as such ribosomal quantities are linked to the cell's protein synthesis capacity [76]. 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 [76]. Humans are estimated to have approximately 300 copies per haploid genome, which are organized in clusters of long tandem arrays on chromosomes 13, 14, 15, 21 and 22 [234]. Interestingly, in mature cells only about half of the ribosomal DNA copies are transcriptionally active while the other half is epigenetically silenced [169]. This excess number of rDNA copies suggests two possibilities. First, the cellular demand for protein synthesis may vary greatly throughout a lifespan. For example, such a large number 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 [10, 77, 97, 193, 195].

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 (ERCs) a ribosomal DNA cleavage product composed of 8 ribosomal DNA units [110]. 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 [67, 97]. 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 [117]. He went on to propose that such a function could monitor genomic integrity in a manner somewhat analogous to telomeres [117].

Previously Pietrzak et al. (2011) has documented an increase in ribosomal DNA copy number in the parietal cortex tissue of AD positive tissue donors using a qPCR based approach [206]. 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 [77]. These results suggest 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 genes. 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.4). 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 [263]. 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 [28]. 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 [28]. As Werner syndrome is known to be caused by a Req Q helicase mutation, this feature suggests homologous recombination is potentially 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 [233]. 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 [234]. 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 [112, 234]. Surprisingly, mutations in the Rec Q helicase WRN, which is implicated in Werner syndrome, did not alter the length of ribosomal DNA clusters [112]. 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 [77]. 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 [21, 141, 174]. Taken together these facts strongly suggest that ribosomal DNA content is unstable under both physiological and pathological circumstances in higher organisms.

Given the previously described increase in the 18S ribosomal DNA content in AD parietal cortex tissue and the increased ribosomal DNA promoter methylation which accompanied it, I investigated age related segmental changes in parietal cortex ribosomal DNA content. Thus I 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 AD or alternatively a general correlate of neurodegeneration, parietal cortex samples from 10 DLB tissue donors and 10 healthy

age matched controls were probed in an identical manner. Moreover, we acquired cerebellar tissue from DLB and control subjects to assess if the documented ribosomal DNA increases are confined to areas with pathology.

DLB is an age related neurodegenerative disease and is the second most common form of dementia in the elderly after AD. The major symptom of DLB is memory loss, but fluctuations in alertness, Parkinsonian motor deficits, and visual hallucinations are also common [83]. Moreover, DLB is associated with the loss of dopaminergic neurons and frequently contains reactive gliosis [55, 220]. Pathologically, DLB is similar to PD 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 [83]. Some have speculated that DLB and PD may be the same disease as they share significant symptomatic and pathological overlap. For example, the major diagnostic difference between DLB and PD dementia for clinicians is only the rate of dementia onset. DLB is associated with the widespread accumulation of Lewy Body pathology throughout the cortex, including the parietal- and prefrontal cortices which were probed in Pietrzak et al. (2011) [71, 206]. The cerebellum is predominately pathology free in DLB. Thus, DLB should serve as an ideal specificity control for AD. Moreover, synucleinopathies have been documented to contain increased amounts of oxidative stress and in particular increased amounts of oxidative DNA damage [151, 184]. 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 the 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 DLB studies, the donors were participants of the IRB-approved University of Kentucky AD Center cohort and were followed for at least 2 years before death [185]. 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 [1, 185]. All included DLB subjects met the clinical and histopathological criteria for diagnosis of DLB [186]. 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.4). 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 [206]. 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 [206] 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.

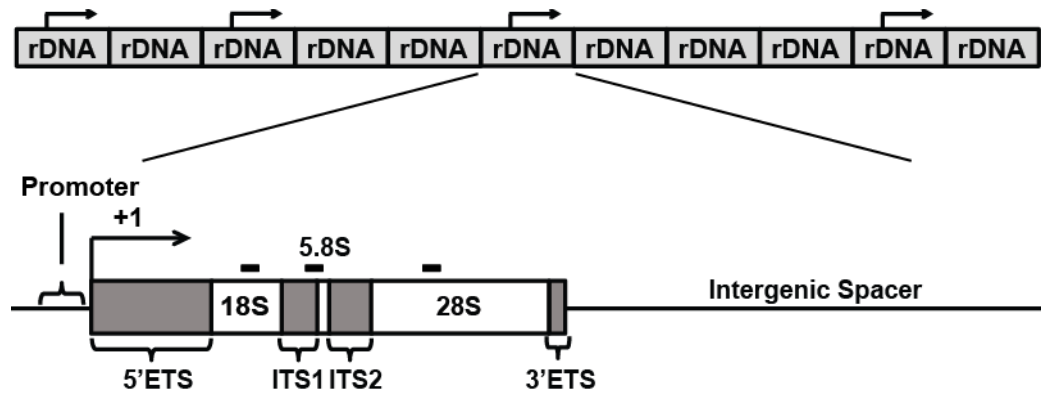


Figure 4. The qPCR-based assay to determine the genomic content of ribosomal DNA

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 PolI-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.

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 agctagctgcgttctcatc). 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 [206].

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
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	agcctgagaaaacggctacca
	Reverse	ggtcgggagtggttaattgc
5.8S	Forward	gaggcaacccctctctctt
	Reverse	gagccgagtgatccaccgta
28S	Forward	gacctcagatcagaggtggcga
	Reverse	ttaactcgccgttactgagggaaat
tRNA^{K-ctf}	Forward	ctagctcagtcggtagagcatg
	Reverse	ccaacgtggggctcgaac
ALB	Forward	cggcggcgggcggcggcgggctgggcggaaatgctgcacagaatcctg
	Reverse	gcccggcccggcggcccgtcccgggaaaagcatggtcgcctgtt
HPAII	Forward	gtatatctttcgtccgagtcg
	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.4). 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 [33] 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. 4). 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.5A, $a=0.7327$, $R^2=0.8103$). 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.5B, C). Usage of a coding region of the albumin gene (*ALB*) as an alternative genomic reference produced

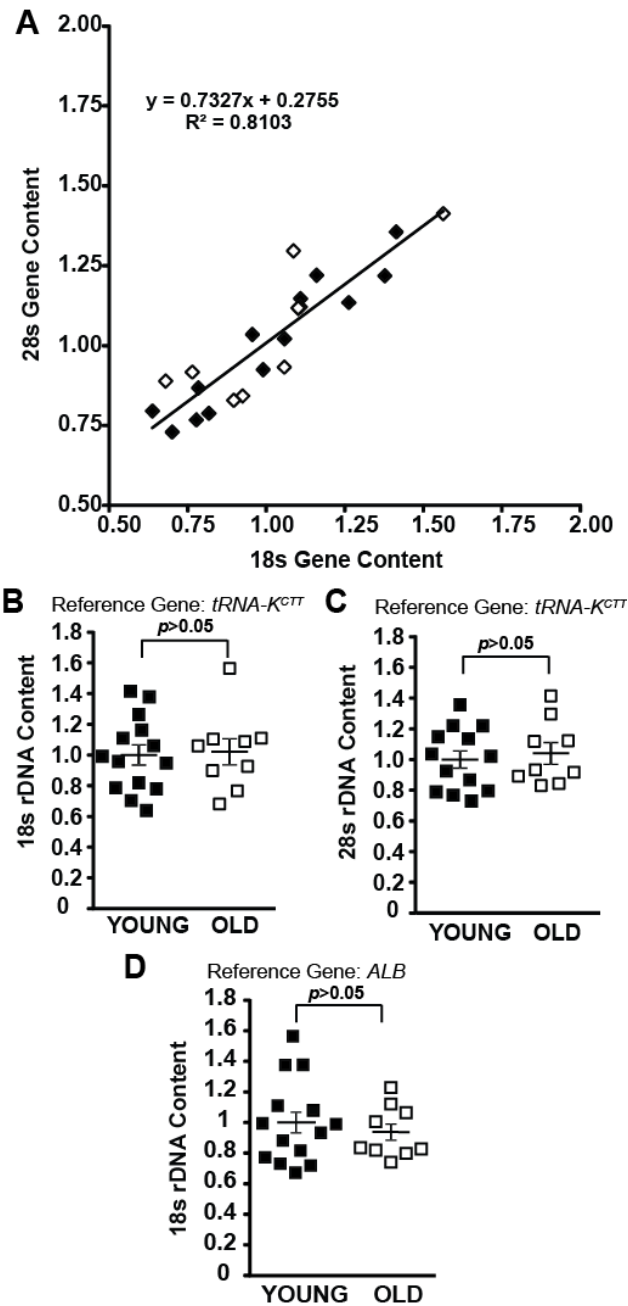


Figure 5. Effects of age on the cerebro-cortical content of ribosomal DNA

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 and 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.

similar results (Fig.5D). Therefore, in the cerebral cortex, genomic content of ribosomal DNA appears to be stable throughout the lifespan.

DLB-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 DLB 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 PD, DLB is a form of a synucleinopathy [168, 227]. Their common characteristic is the presence of intracytoplasmic α -synuclein-containing inclusions, the Lewy Bodies. In addition, DLB is associated with neuronal atrophy, neuronal death and reactive gliosis in the cerebral cortex including the parietal region [168]. Oxidative damage of macromolecules including DNA is observed in both conditions [3, 151]. Hence, DLB 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 (Fig.4). As expected for a tight physical linkage, the DLB- and the control group-derived individual values obtained with these probes correlated with each other fitting a linear model of direct correlation (Fig. 5A)(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 DLB. In this group, the 18S, 5.8S, and 28S probes

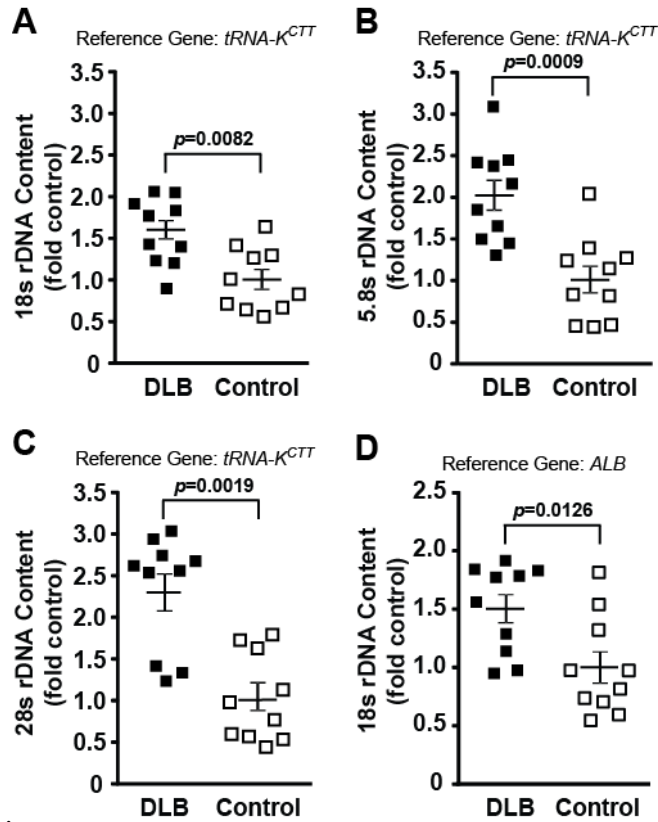


Figure 6. Increased ribosomal DNA content in the Dementia with Lewy Bodies parietal cortex

Genomic ribosomal DNA content was quantified in *post mortem* samples of the parietal cortex from DLB- 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.5A (see the Results section for more details). In the DLB 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

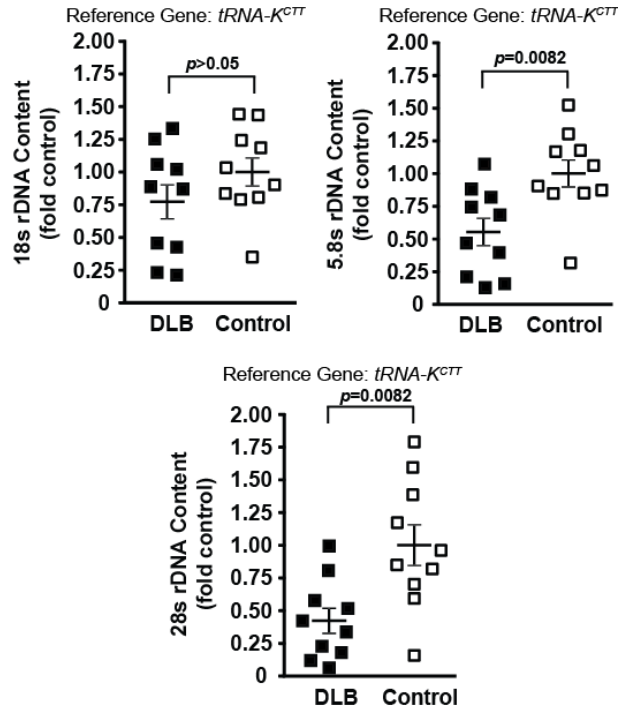


Figure 7. Decreased ribosomal DNA content in the Dementia with Lewy Bodies cerebellum.

Genomic ribosomal DNA content was quantified in post mortem samples of the cerebellum from 10 DLB- 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. 5A (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 DLB 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.

revealed ribosomal DNA content that was 1.6-, 2.0-, and 2.3-fold controls respectively (Kruskal-Wallis ANOVA, $p < 0.01$, Fig.6A-C). Similar results were obtained when *ALB* was used as an alternative genomic reference (Fig.6D). To determine whether this DLB-associated effect on ribosomal DNA content was directly related to pathological changes in the cortex, samples from the DLB 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 DLB 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.7). While the 18S probe did not detect significant differences between the two groups, the genomic 18S content also showed a downward trend in DLB (Fig.7). Therefore, DLB 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 [239]. 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 [169]. Moreover, epigenetic silencing of ribosomal DNA may stabilize the inactive genes preventing recombination; conversely, de-silencing may have an opposite effect [77, 117]. Thus, methylation of the ribosomal DNA promoter region was analyzed in the parietal cortex of the DLB- and control groups using the CpG

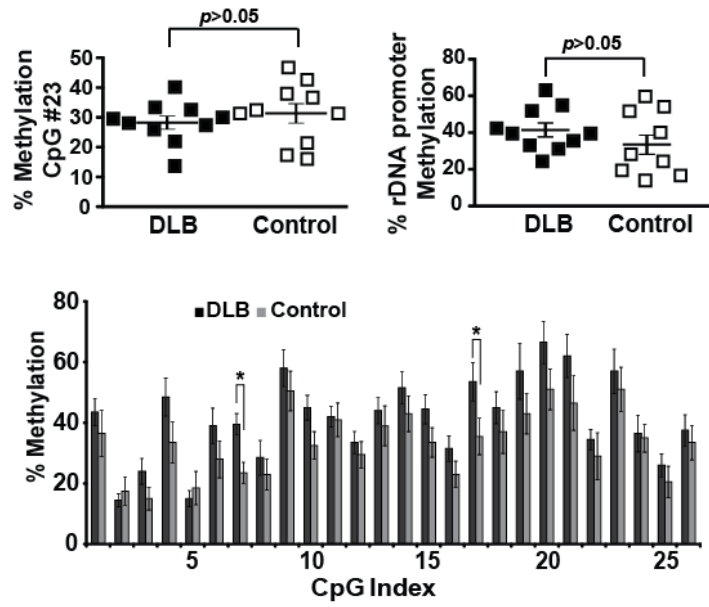


Figure 8. Similar methyl-cytosine content of the ribosomal DNA promoter region in the control- and Dementia with Lewy Bodies parietal cortex.

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 DLB on distribution of CpG methylation across the ribosomal DNA promoter. The data represent averages \pm SEM from 10 DLB- 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 hyper methylated in DLB, the overall trend of CpG methylation across the 26 ribosomal DNA promoter CpGs was not significantly affected by DLB (local regression analysis, data not shown).

methylation-sensitive restriction endonuclease HpaII or bisulfite sequencing.

Methylation of the ribosomal DNA promoter was similar in the DLB- and the control group (Fig.8). Therefore, in the DLB 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 DLB-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.9). 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 DLB samples.

Discussion and Conclusions

In this study, I probed for changes in ribosomal DNA content in the brain of old vs young subjects and DLB patients vs. controls using a qPCR based approach. No difference was detected in the parietal cortex ribosomal DNA content of young and old

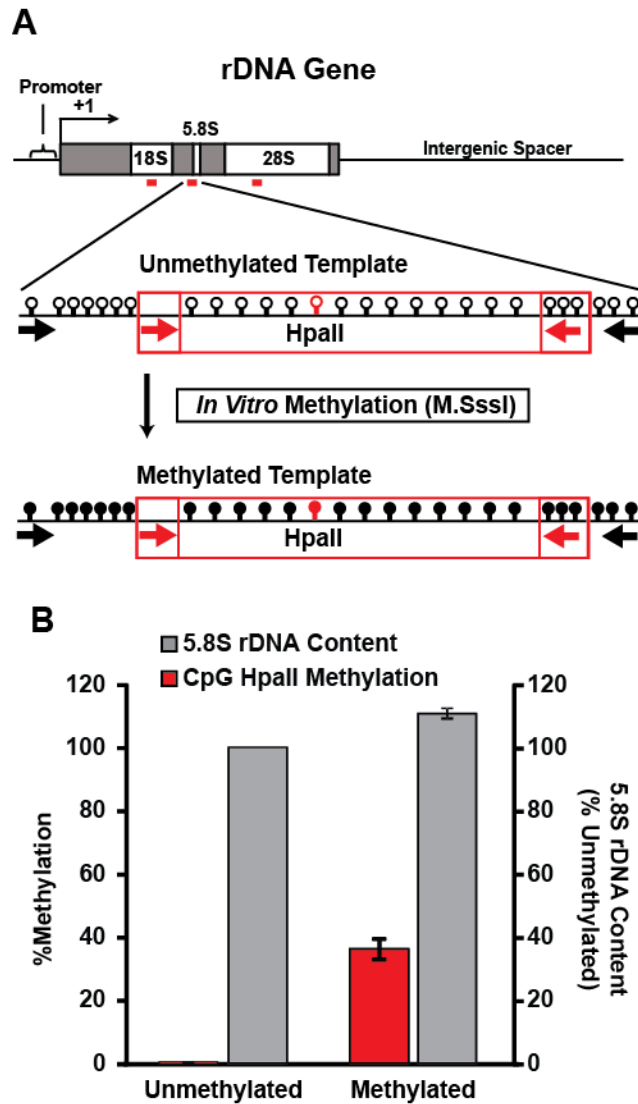


Figure 9. Effects of template methylation on qPCR efficiency

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 DLB (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 DLB-associated changes in ribosomal DNA content. Data represent two independent experiments; error bars are SDs.

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 DLB 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 DLB 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.

A prior AD study produced similar results for the parietal cortex as both DLB and AD contained elevated ribosomal DNA content [206]. Moreover, the magnitude of 18S increase, approximately 50%, was similar to what has been documented in mild cognitive impairment and late stage AD which contained increases of 50% and 69%, respectively. In contrast, the DLB' cerebellum contained a significant decrease in ribosomal DNA content, a finding which was not apparent in AD. 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 the prior AD study. The 18S region trended down in DLB but did not reach statistical significance. Thus, comparing the DLB and AD 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 AD and DLB telomere length was decreased in peripheral blood leukocytes, and, at least in the case of AD, increased in the hippocampus, a brain structure directly affected by AD pathology [29, 127, 243].

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. It is unclear which cell type or types are contributing to these findings. Homologous recombination is believed to be the major mechanism behind ribosomal DNA instability and readily occurs during both mitosis and meiosis. Therefore, such requirements suggests inappropriate activation of homologous recombination is occurring in cycling cycles [233].

Recent literature suggests homologous recombination may not be the only possibility for my findings. I propose three potential explanations. First, as stated previously, this phenomenon could be occurring in cycling cells, suggesting that the findings are the result of reactive gliosis, a condition which is involved in both AD and DLB [17, 215]. 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 [21, 141, 174].

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 AD pathology associated areas [27, 261, 262]. Proteins from all phases of the cell cycle have been shown to be increased in AD. Moreover, the frequency of aneuploidy has also been shown to be increased in AD [6]. 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 [65, 66]. 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 [52]. 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 [45, 194]. For example in the chicken study, embryos with 66% of the normal ribosomal DNA content grew and developed as expected [45]. 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 [195]. 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 [77]. 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 [193]. Furthermore, clusters of genes linked to both mitochondrial function and lipid metabolism were prominent in the population of altered genes [193]. Interestingly, alterations in both of these areas are common to both AD and DLB [18, 39, 184, 212].

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 [117]. Alternatively, increased ribosomal DNA

content could 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 (HSP70), MDM2, and the Von Hippel Lindau (VHL) 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 [10]. 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.10). I 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 instability 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.

I propose that genomic instability is induced by DNA damage. Additionally, the

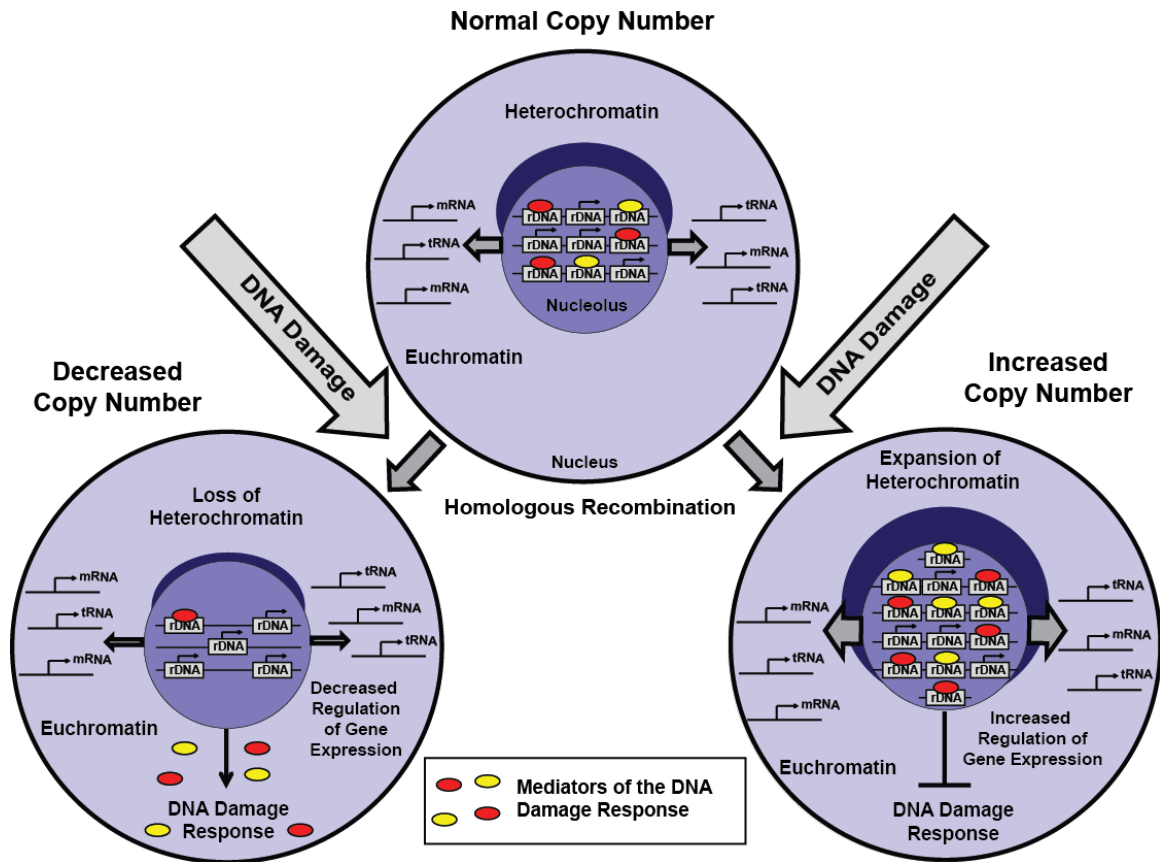


Figure 10. A hypothetical model summarizing possible causes and consequences of ribosomal DNA instability.

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 DLB- 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.

application of a selection pressure, such as the enhanced DNA damage which is common to the DLB and AD parietal cortex, would select for increased ribosomal DNA content and promote cell survival. Thus in the DLB 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 DLB 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, DLB, 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

CHAPTER III
PERSISTENCE OF BRAIN PRE-RRNA AND NUCLEOLAR PRESENCE OF
NEURONAL NUCLEOPHOSMIN AFTER DEATH

Introduction

It has been proposed that the neuronal nucleolus acts as a stress sensor and regulator of cell death [91, 162]. Dysfunction of the nucleolus and the subsequent activation of the ribosomal stress response have been implicated in multiple neurodegenerative and neurodevelopmental diseases. Such findings have prompted studies which probed nucleolar stress markers in post mortem human brain tissue [81, 166, 206, 214].

A major concern with these studies is the reliability of nucleolar stress markers in post mortem tissue. In response to practically all causes of death, the brain dies from nutrient and oxygen deprivation when the heart fails to supply adequate blood flow. It is well documented that nucleolar activity is directly related to overall cellular well-being and is highly responsive to factors including cellular energy levels, serum starvation, and hypoxia [52, 76, 126, 170, 182, 228, 260]. Thus, post mortem-associated brain ischemia may represent a significant source of nucleolar stress.

The stability of nucleolar stress markers in post mortem material has not been addressed in the literature. Several studies have probed both general protein stability and the stability of epigenetic markers in post mortem tissue, but importantly these studies did

not focus on the nucleolus. It appears that, as expected, increased protein degradation occurs with increased PostMortem Interval (PMI) and also during storage [56, 178]. However unexpectedly, these studies demonstrated that not all proteins degrade at the same rate. Moreover, a proteomics based study conducted a year later produced similar findings [38]. With regard to epigenetics, histone tail acetylation was found to be highly unstable while on the other hand DNA methylation was unchanged [15, 16]. However, the aforementioned methylation study did not investigate genes which are known to be regulated by energy status. Therefore, the specific set of genes investigated by Barrachina et.al. (2012) may be a poor predictor of the rDNA promoter. Lastly, the enzymatic activity of acetyltransferases and methyltransferases were not significantly altered in response to increasing PMI [178]. Thus, the mechanisms for epigenetic modification are potentially active for many hours after death.

The use of post mortem tissue is especially important for neurodegenerative disease research. Despite its flaws, post mortem tissue will in some cases be the best option for molecular studies. With regard to the vast majority of neurological conditions, non-post mortem human tissue samples are simply not available for research. In general, the only major exceptions to this rule are cancerous tumors, benign growths (such as neurofibromas) and temporal lobectomies [155]. The later condition is reserved for severe cases of epilepsy [253]. Moreover, for some neurodegenerative diseases the use of transgenic animals is severely limited and in such cases post mortem human tissue may be the only option for brain based molecular studies. For instance, some diseases, such as Dementia with Lewy Bodies, have not been linked to genetic mutations. Therefore, the generation of animal models can be problematic and in some cases

impossible. Additionally, some mutations appear to be specific to humans and have little to no phenotype in rodents. Such, a condition is evident for Ataxia Telangiectasia and Parkin mutations which are associated with familial Parkinson's disease [134, 203]. Lastly, one must also consider that familial neurodegenerative disease mutations may not be representative of sporadic disease and as such may only model discreet portions of disease cascades. Thus, despite the clear imperfections in post-mortem tissue- based studies they are still essential for the study of human neurodegenerative disease.

Therefore, I investigated the stability of rDNA promoter methylation, nucleophosmin localization, pre-rRNA content, and rDNA locus stability in rodent brain tissue in response to various experimentally controlled post mortem intervals. Additionally, we probed the constancy of pre-rRNA and nucleophosmin localization in response to energetic failure in living neurons.

Materials and Methods

Post Mortem Ischemia. All animal experiments complied with University of Louisville Institutional Animal Care and Use Committee (IACUC) protocols and National Institute of Health (NIH) guidelines. Six week old male Sprague Dawley rats (Harlan-Indianapolis, IN) were euthanized using CO₂. The carcasses were left at ambient temperature for various times before tissue collection. Cerebral cortex samples for RNA/DNA studies were frozen on dry ice and stored at -80C prior to nucleic acid isolation. For immunofluorescence whole brains were placed in 4% Paraformaldehyde overnight followed by storage in 0.12 M Millonig's phosphate buffer.

Nucleic acid isolation and PCR. For pre-rRNA quantification, total RNA was isolated using TRI Reagent (Invitrogen) according to manufacturer's protocol. Quantitative Reverse Transcriptase-PCR (qRT-PCR) that employed $\Delta\Delta C_t$ analysis was performed as described previously [106]. For assessment of rDNA locus stability genomic DNA was isolated using the DNeasy blood and tissue kit (Qiagen). Quantitative PCR (qPCR) that employed standard curve analysis was performed as described previously [81]. Primer nucleotide sequences are listed in Table 4.

Cell culture experiments. Primary cortical neurons were isolated from Sprague-Dawley rats (Harlan-Indianapolis, IN) on post-natal day 0 and cultured as described previously [78]. On day in vitro (DIV) 6-7, cells were treated with 10 mM Sodium Azide and 6 mM 2-deoxyglucose or 250 μ M glutamate which were dissolved in culture medium prior to use.

Immunofluorescence. Immunostaining was performed on paraformaldehyde-fixed primary cells or sections of paraformaldehyde-fixed brains as previously described [207] except the primary antibody was mouse monoclonal anti-b23 (Clone number FC82291, Sigma, dilution 1:1,500).

HpaII Restriction Endonuclease Methylation Analysis. HpaII methylation analysis was performed as described previously [81]. Briefly, genomic DNA was cleaved with the methyl-sensitive restriction endonuclease HpaII and analyzed by qPCR using the standard curve methodology. For normalization, an amplicon was designed in a region of the external transcribed spacer 1 (ETS1) which did not contain an HpaII cleavage site. Primer sequences are listed in Table 4.

Table 4. Chapter III primers

Experiment	Amplicon	Direction	Sequence (5'-3')
Pre-rRNA Quantification	45S pre-rRNA ETS1	Forward	TGGGGCAGCTTTATGACAAC
		Reverse	TAGACCAAACGGGAAAACC
	45S pre-rRNA ITS1-5.8S	Forward	CGCTCACACCTCAGATAACC
		Reverse	CTAGCTGCGTTCTTCATCG
rDNA Genomic Stability	18S rRNA	Forward	GTTGGTTTTCGGAACTGAGGC
		Reverse	GTCGGCATCGTTTATGGTCG
	28S rRNA Genomic	Forward	CCTCTGGCATGTTGGAACAATGTAGG
		Reverse	CCAGCCCTTAGAGCCAATCCTTAT
rDNA Methylation	tRNA-K ^{CTT} Genomic	Forward	CTAGCTCAGTCGGTAGAGCATG
		Reverse	CCAACGTGGGGCTCGAAC
	Promoter HPAII	Forward	GTGAGCACTCAGGGTTCTAAG
		Reverse	CTCCAGGTACAATAAGGACAG
rDNA Normalizer- No HPAII	28S HPAII	Forward	CCTCTGGCATGTTGGAACAATGTAGG
		Reverse	CCAGCCCTTAGAGCCAATCCTTAT
	rDNA Normalizer- No HPAII	Forward	TGTGTGTGTGGCAGTGTTAG
		Reverse	ATCCAACGGGAGCGAGA

Results

Given that pre-rRNA is rapidly processed and that RNA Polymerase I is highly sensitive to hypoxia and cellular energy content, we hypothesized that pre-rRNA levels would decline sharply following euthanasia [75, 170, 182]. To test this hypothesis qRT-PCR was used to probe pre-rRNA levels at 3- and 12 h post mortem. As I anticipated degradation, two separate regions of the transcript were probed. qRT-PCR amplicons probing both the 5' external transcribed spacer (5'ETS) and the internal transcribed spacer 1 (ITS1) regions of the pre-rRNA transcript were utilized. Indeed, pre-rRNA levels were significantly decreased in response to increasing PMI, however the rate of decline was unexpectedly low (Fig.11A, $p < 0.01$ for 5'ETS and $p = 0.01$ for ITS1, one-way ANOVA, factor PMI). Pre-rRNA was not significantly decreased at 3h PMI, although the ITS1 regions trended down. Amplicons in both the 5'ETS and ITS1 regions revealed pre-rRNA declines at 12 h (Fig.11A, $p < 0.05$ for 5'ETS and $p < 0.05$ for ITS1). However, the decreases were modest with levels approximately 70% of controls still present at this time. Therefore, pre-rRNA levels are not static after death, but the rate of decline does not phenocopy the sharp declines which are seen after treatment with RNA polymerase I inhibitors such as actinomycin-D (Act-D).

Re-localization of the nucleolar protein NPM into the nucleoplasm is a commonly used marker of nucleolar stress [235]. Therefore, the status of the nucleolar NPM was monitored in the cerebral cortex at various PMIs. Nucleolar NPM signal was visible in all cells at both 3- and 12 h PMIs. Such observations are consistent with moderate declines of pre-rRNA as NPM localization to the nucleolus is highly dependent on its interactions with pre-rRNA and its nucleolar stress-induced redistribution follows sharp

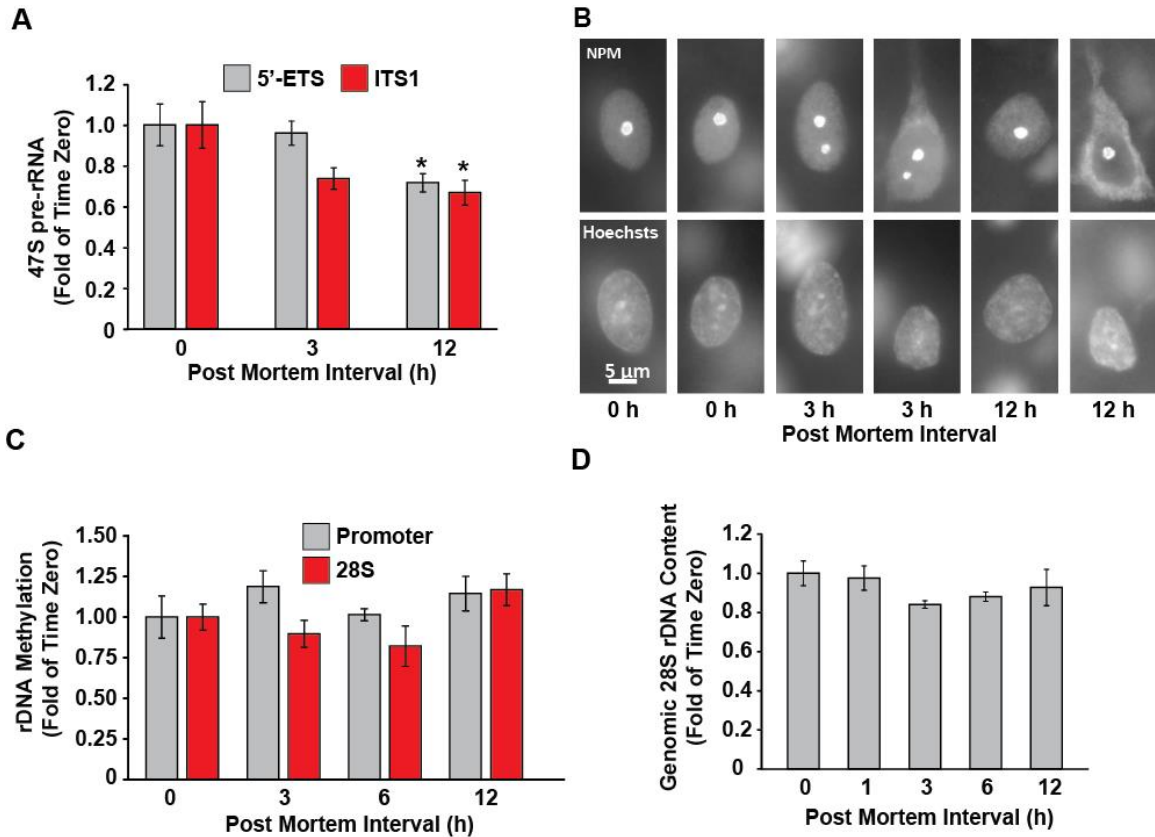


Figure 11. Effects of post mortem ischemia on brain cell nucleoli.

Rats were euthanized and left at ambient temperature for the indicated post mortem interval (PMI). **A,B**, In neocortex, pre-rRNA levels were determined using quantitative qRT-PCR that probed two amplicons including one from the 5' External Transcribed Spacer (5'-ETS) and one from the Internal Transcribed Spacer-1 (ITS1). 18S rRNA was used for normalization. Note moderate declines of pre-rRNA levels. Sample size is n=9 for each group. Error bars are SEM, p<0.05: *. **B**, Representative images of brain cells from neocortex that were stained for nucleolar Nucleophosmin(NPM). NPM-positive nucleoli are present at both 3- and 12 h PMI. A small fraction of neuron-like cells contained NPM not only in the nucleoli but also in perikarya and proximal neurites. **C**, HpaII restriction analysis of 2 rDNA CpG sites, one contained the promoter region and one contained within 28S in brain samples from neocortex. PMI had no significant effects on methylation of either CpG site. **D**, Quantitative PCR with Genomic DNA was used to determine the effect of increasing PMI on genomic rDNA content in neocortex. PMI had no significant effects on rDNA content.

declines of pre-rRNA [171]. Thus, in brain cells, post mortem ischemia does not present as canonical nucleolar stress.

Unexpectedly, NPM was also observed in the perikarya and in proximal dendrites of neuron-like cells at both 3- and 12 h PMIs (Fig.11B). In such cells, nucleolar NPM staining was still visible. However, most cells retained the entire NPM signal in the nucleoli/nuclei. These findings suggest that in most cells of the cerebral cortex, nucleolar NPM presence is relatively well preserved after death.

The density of DNA methylation and specifically gene promoters is believed to correlate with transcriptional activity [238]. As prior studies have shown that inactive rDNA copies contain increased cytosine methylation, we probed two cytosine-phosphate-guanosine (CpG) sites in the rDNA gene [169]. The first site was one of the 5 CpGs contained within the rat rDNA gene promoter, while the second was contained within the 28S rRNA region. Neither CpG displayed altered methylation levels at any time point during the 12 hour analysis (Fig.11C).

Dr. Pietrzak and I have documented abnormalities in rDNA gene content in neurodegenerative disease [81, 206]. While this feature is not linked to cellular energy status like the 3 prior stress markers, the multi-copy rDNA locus may still be sensitive to nuclease activity during the post mortem period. Therefore we probed for changes in rDNA content in response to increasing post mortem intervals. Importantly, genomic rDNA content did not change with increasing PMI at any time point during our analysis (Fig.11D).

Next it was investigated whether similar preservation of pre-rRNA and nucleolar NPM would be observed in primary rat cortical neurons in response to chemical ischemia. After a one hour exposure to sodium azide and 2-deoxyglucose, pre-rRNA levels were moderately reduced as revealed with the ITS1- but not the 5'ETS-amplicon (67% control, $p=0.001$, u-test, Fig.12A). Consistent with such a good preservation of pre-rRNA, chemical ischemia-exposed neurons still displayed nucleolar NPM (Fig.12B). However, in most cells a portion of NPM was also found in the perikarya and proximal dendrites.

The relatively modest impact of ischemia on pre-rRNA levels and nucleolar residence of NPM was unexpected given the high sensitivity of RNA Pol I to energy shortage. However, it is also possible that energy failure may mask the consequences of RNA Pol I inhibition by simultaneous disruption of pre-rRNA processing. To test such a possibility neurons were pre-treated with actinomycin D (Act-D) to prevent *de novo* synthesis of pre-rRNA. After 15 min, cells were exposed to chemical ischemia for one hour. Act-D treatment alone reduced pre-rRNA levels to 9% of control for both the 5'ETS and ITS1 probe. In neurons treated with Act-D and chemical ischemia declines of pre-rRNA were significantly attenuated as levels increased to 40% and 24% of controls for the 5'ETS- and ITS1 amplicons, respectively (Fig.12C, $p<0.01$, both amplicons u-test). Moreover, chemical ischemia attenuated nucleoplasmic release of NPM. However, the nucleolar NPM signal appeared reduced and perikaryal/dendritic NPM signal was also visible (Fig.12D)

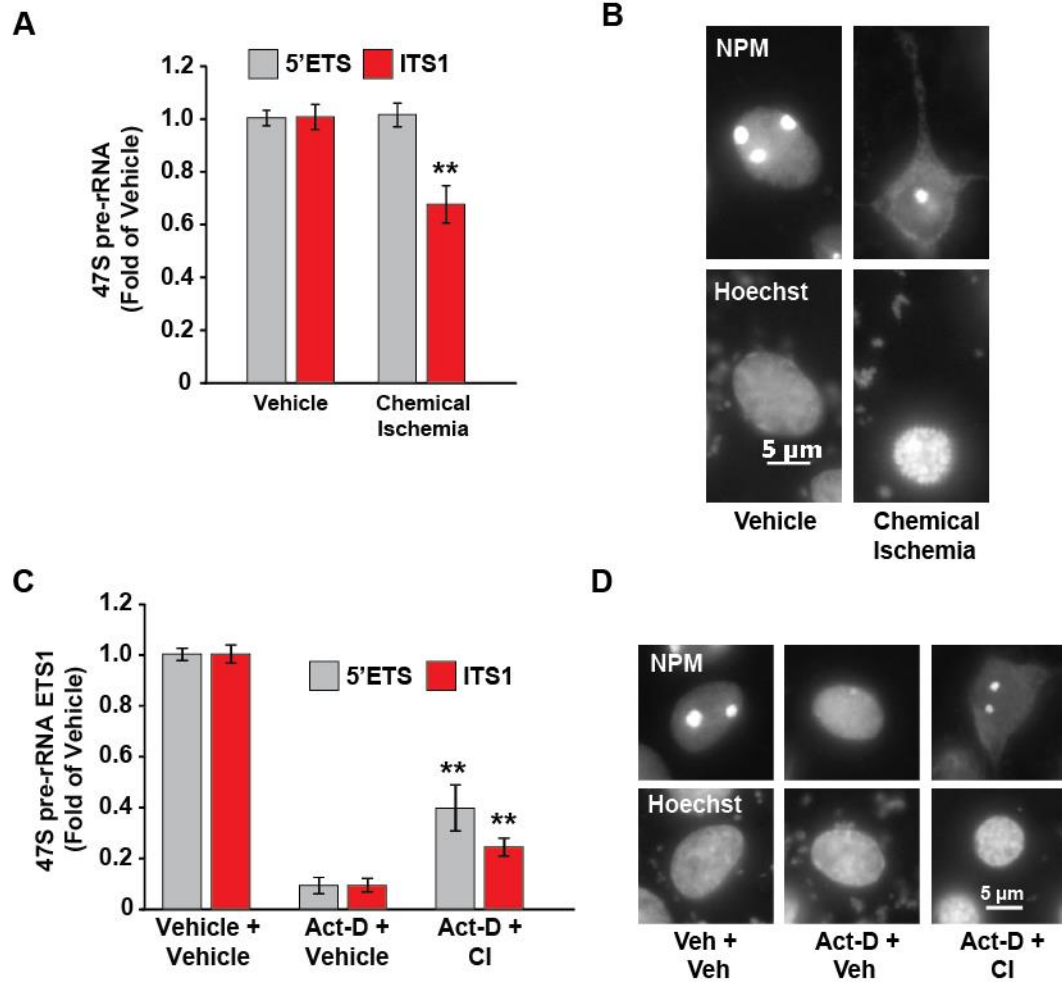


Figure 12. Effects of chemical ischemia on the neuronal nucleolus.

On DIV6 rat primary cortical neurons were treated with 10 mM sodium Azide and 6 mM deoxyglucose for 1 h to induce chemical ischemia (CI). **A**, Pre-rRNA levels were determined using quantitative qRT-PCR that probed two regions of the transcript, one from the 5' External Transcribed Spacer (5'-ETS) and one from the Internal Transcribed Spacer-1 (ITS1). Modest declines of pre-rRNA were detected in the ITS1 region. **B**, Representative images of chemical ischemia treated primary cortical neurons. Chemical ischemia was sufficient to induce extra-nuclear appearance of NPM, but nucleolar NPM was still present. **C and D**, Cells were treated with Actinomycin-D (act-D) for 15 minutes prior to chemical ischemia. **C**, Chemical ischemia attenuated declines of pre-rRNA in response to Act-D. Error bars are SEM, $p < 0.01$: **. **D**, Representative images of primary cortical neurons treated with Act-D and chemical ischemia. The Act-D-induced nucleoplasmic translocation of NPM was attenuated by chemical ischemia.

Discussion

Our results suggest that some frequently used parameters to probe the status of ribosomal biogenesis and/or the integrity of the nucleolus are remarkably stable for up to 12 h after death. These include the nucleolar localization of NPM, methylation of the rDNA promoter and the stability of the genomic rDNA locus. However, moderate declines of pre-rRNA which appear to increase with PMI were detected. Thus, caution should be used when attempting to measure this parameter in post mortem samples. However, given the slow rate of decline such studies may potentially be feasible if PMIs can be carefully matched.

Moderate losses of pre-rRNA after death may be the result of energy independent degradation rather than the canonical pre-rRNA processing. In support of this concept, in mice, decapitation was shown to deplete brain ATP levels within seconds or minutes depending upon the age of the animal [149]. Such a rapid rate of energy depletion argues strongly against energy dependent pre-rRNA processing occurring multiple hours after death. Moreover, we demonstrate that in primary neuronal culture energetic depletion increases the retention of pre-rRNA following pharmacological inhibition of RNA Polymerase I. Such findings are consistent with the demonstrated requirements for ATP in multiple steps of ribosomal biogenesis and rRNA processing, including ATP dependent DExH-box enzymes which facilitate RNA cleavage [124]. Given the ubiquitous nature of energy independent RNases and the energetic requirements for canonical pre-rRNA processing, I speculate that post mortem loss of pre-rRNA is the result of non-specific degradation rather than legitimate processing. On the other hand, it

is also conceivable that a portion of processing enzymes contain low levels of activity in the absence of ATP.

The ability of NPM to demarcate the nucleolus was unaltered by increasing PMI suggesting that it can be used to assess the presence of nucleolar stress in post mortem samples collected at least up to 12 h after death. The preservation of nucleolar NPM is presumably the result of the previously discussed pre-rRNA retention. While nucleolar assembly is not fully understood, the nucleolus is believed to form based on multiple proteins having a high binding affinity for pre-rRNA and/or other ribosomal proteins [191]. This feature has prompted some to refer to the nucleolus as an “organelle formed by the act of building a ribosome” [171]. In support of this hypothesis’, pre-rRNA was shown to be an essential component of nucleolar assembly and in its absence nucleoli cannot form [74]. Moreover, the three dimensional size of nucleoli is believed to directly related to the quantity of pre-rRNA contained within its borders [224]. Lastly, nucleolar presence of NPM is strictly dependent on pre-rRNA binding as its loss from the nucleoli after Pol1 inhibition is due to plunging pre-rRNA levels [93].

Of note, energy deprivation prevented the nucleoplasmic translocation of NPM after Pol1 inhibition in HeLa cells [57]. As in neurons, nucleolar retention of NPM was likely due to increased half-life of pre-rRNA. Hence, our observations on the effect of PMI on nucleolar NPM are likely applicable to studies of other organs besides brain.

Transport of NPM is not fully understood, but the process is known to be regulated by multiple localization signals, RAC1 and cellular ATP and GTP levels [36, 57, 58, 188]. Thus the accumulation of extra nuclear NPM could be a simple

mislocalization mediated by energy failure. However, such a mislocalization may have further consequences for cell survival. Interestingly, extranuclear NPM has been proposed to play a role in cell death. Surprisingly, NPM has been proposed to play contradictory roles depending on the context. In a rodent model of stroke, NPM has been implicated as a chaperone for the pro-apoptotic BCL2 Associated X Protein (BAX) [111, 249]. Conversely, in cultured rodent neurons, anti-excitotoxic effects were also demonstrated as NPM inhibited the Seven In Absentia Homolog 1 (SIAH1)-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) death cascade [136, 159].

rDNA methylation and gene content were both unchanged in response to increasing post mortem interval, suggesting these measurements are relatively free from post mortem artifact. Our methylation findings are in agreement with Barrachina et.al (2009) who demonstrated that methylation of neurodegenerative disease related genes was stable in the post mortem brain [15]. Importantly, our results support the validity of several prior studies which reported the effect of brain pathologies on rDNA status. Increased rDNA promoter methylation was found in the AD and suicide brain, but not in DLB [81, 166, 206]. Additionally, rDNA gene content was found to be increased in both AD and DLB [81, 206]. With regard to the PMIs in these studies, all donors in the AD and DLB reports had PMIs within the 12 h time frame of our experiments and most were under 4 h. Alternatively, the suicide study contained PMIs which averaged approximately 24 h. As this was double our latest time point, caution should be used if attempting to extrapolate our results to such high PMIs.

Separate from the methodological concerns addressed in this study is the larger concept that energetic failure can mask nucleolar stress. Such a phenotype suggests that

the ribosomal stress response could indeed be activated in conditions such as stroke or neurotrauma, despite the persistence of punctate nucleolar NPM localization. As the ribosomal stress response is centered on stoichiometric imbalances of ribosomal components, it's feasible that the dual inhibition of pre-rRNA synthesis and pre-rRNA processing could synergistically produce deficits in rRNA content [114]. The concept of ribosomal stress in the absence of NPM translocation should also be considered in situations other than energetic failure. Numerous neurodegenerative diseases contain evidence for nucleolar involvement despite the absence of reports demonstrating canonical nucleolar stress [47, 48, 122, 135, 214, 252]. Such a condition suggests that these diseases should be re-evaluated with additional stress markers as the dispersion of NPM appears to be sufficient, but importantly not necessary for the induction of ribosomal stress. Thus, these diseases could indeed contain induction of ribosomal stress while still maintaining punctate nucleolar NPM staining.

In summary, at least up to 12 h after death, brain cell nucleoli are relatively well preserved. Such preservation is likely due to ischemia-associated disruption of rRNA processing and the subsequent stabilization of pre-rRNA. Therefore, studies of post-mortem samples that were collected at PMIs lesser than 12 h should provide a relatively accurate insight into the status of the nucleolus in chronic brain pathologies. Conversely, in such acute conditions as stroke and neurotrauma, energy failure may mask signs of disrupted ribosomal biogenesis. Hence, there is a need to identify novel parameters of ribosomal biogenesis/nucleolar activity that will be unaffected by brain cell energy failure.

CHAPTER IV
THE ROLE OF RIBOSOMAL PROTEIN L11 IN NEURONAL DEATH

Introduction

The nucleolus is a vital component of neuronal function. While it is well known that the nucleolus is the center of ribosomal biogenesis, its overall role in neuronal well-being is considerably larger. Nucleolar activity has been linked to neuronal growth capacity, maintenance of size upon reaching maturity, and regeneration after injury [72, 115, 130, 231]. Therefore, adequate nucleolar activity appears to be necessary to prevent neuronal atrophy and synapse loss [198]. Moreover, cells have evolved ribosomal protein-mediated stress responses which monitor the fidelity of ribosomal biogenesis.

Ribosomal protein mediated stress responses appear to have evolved for two reasons. First, ribosomal biogenesis is the largest consumer of cellular energy in dividing cells [124]. Thus, error prone ribosome synthesis has the potential to waste considerable amounts of cellular resources. Second, in multicellular organisms ribosomal stress appears to have evolved into a tumor suppressive pathway. While more work is needed to clearly demonstrate ribosomal stress as a bona fide tumor suppressive mechanism, current reports suggest that it does indeed inhibit myc-driven oncogenesis [114, 153]. The precise mechanisms for such an effect are unclear; however ribosomal stress is known to be centered on stoichiometric imbalances of ribosomal components. Therefore, it is likely that oncogenes differentially stimulate the production of certain ribosomal components relative to others. In support of this concept, a constitutively

active HRAS mutant was shown to induce ribosomal stress by stimulating pre-rRNA transcription beyond the cells capacity for processing [187].

Nucleolar Stress and ribosomal stress appear to be separate pathways which result in distinct outcomes in many cell types. Traditionally, these terms have been used interchangeably, but current reports suggest that they are not identical, despite the fact these pathways partially overlap. Classically, nucleolar stress has been considered to be an inhibition of RNA polymerase I which releases several nucleolar proteins, including nucleophosmin and nucleostemin into the nucleoplasm and induces apoptosis in most cell types [11, 25, 106]. Alternatively, ribosomal stress is the result of stoichiometric imbalances in ribosomal components and in most cell types induces p53 mediated cell cycle arrest [63, 64, 114, 187]. Nucleolar stress is sufficient, but not necessary for the induction of ribosomal stress as multiple mechanisms for impairing the stoichiometry of ribosomal components are possible. For example, loss of a single ribosomal protein will induce ribosomal stress while having no effect on nucleolar morphology [63]. Essentially, nucleolar stress appears to encompass the entire ribosomal stress response, but also includes the release of a second layer of stress mediators not seen with simple stoichiometric imbalances of ribosomal components. The pro-apoptotic protein Myb binding protein 1a (MYBBP1A) is one such mediator [125, 126].

Nucleolar stress is known to induce apoptosis in immature neurons [106, 198]. Interestingly, this response is developmentally restricted as mature neurons do not undergo apoptosis under such conditions [198, 207]. They do however still accumulate p53 [214]. The effect of ribosomal stress on neuronal cell death has not been investigated. Such a condition may be of greater relevance to neurological disease as

evidence for catastrophic nucleolar failure is rare, but numerous studies have demonstrated more subtle impairments of ribosomal biogenesis in neurodegenerative disease [47, 48, 79, 129]. Moreover, two neurodevelopmental diseases involving mental retardation and microcephaly, Bowen Conradi Syndrome and Alopecia, Neurological Deficits and Endocrinopathy (ANE) Syndrome have been linked to genes with documented roles in ribosomal subunit assembly [9, 189]. Such diseases would be expected to engage the ribosomal stress response.

Perhaps the best described mediator of nucleolar- and ribosomal stress in non-neuronal cells is RPL11. Multiple reports suggest the 5S ribonucleoprotein particle (5S RNP), a subcomponent of the large ribosomal subunit composed RPL11, RPL5, and the 5S rRNA has been suggested to be the major stress effector [25, 49, 63, 64]. The current model suggests that the 5S RNP translocates from the nucleolus and binds the p53 inhibiting protein MDM2 allowing for induction of p53-mediated cell cycle arrest or apoptosis [183]. Moreover, RPL11 has been demonstrated to have stress related “extra” ribosomal functions independent of its assembly in the 5S RNP and MDM2. Such functions include binding the promoter of p53 target genes and directly binding p73 [266, 267].

Thus, there is a growing body of evidence suggesting the impairment of ribosomal biogenesis in both neurodegenerative- and neurodevelopmental disease. Moreover, RPL11 has been demonstrated to be a major regulator of both nucleolar- and ribosomal stress in non-neuronal cells. Therefore we investigated RPL11 as a mediator of apoptosis in response to nucleolar stress, ribosomal stress, and other pro-apoptotic stimuli in primary cortical neurons.

Materials and Methods

Antibodies. The following antibodies were used in this study; anti-RPL11 (Proteintech, dilution 1:1,000), anti-NeuN (Millipore, dilution 1:500), anti-p53 (Santa Cruz dilution 1:500), anti-phospho-Ser15-p53 (Cell signaling technologies, dilution 1:1,000), anti-cleaved caspase 3 (Cell signaling technologies, dilution 1:1,000), Anti-Beta Actin (Sigma, dilution 1:2,000), anti-B23 (Sigma, dilution 1:1,500), chicken anti-beta-galactosidase (Abcam, dilution 1:1,00) and goat anti-chicken 594 Alexa Fluor conjugated (Jackson ImmunoResearch Laboratories, dilution 1:400),

Plasmids

Previously described plasmids. The following previously described plasmids were used in this study; β -Gal (EF1 α LacZ β -galactosidase)[98], p53 luciferase reporter gene (PG13-Luc) [53], Temperature Sensitive p53 (p53val135) [173], dominant negative p53 (pCMV-p53-DD)[223]

Novel plasmids- RPL11-GFP (pEGFP-N1-RPL11) was cloned from cDNA obtained from a rat primary cortical neuron culture using primers containing EcoRI (forward) and BamHI (reverse) restriction sites. RPL11 PCR product was inserted into the pEGFP-N1 vector between the EcoRI and BamHI restriction sites in the multiple cloning site.

pSUPER shRNA plasmids. All shRNA plasmids were produced using the pSUPER RNAi system according to manufacturer's protocol. shRNA sequences were designed using shRNA or RNAi design tools available online from Invitrogen and genelink.com. Nucleotide sequences are contained in Table IV.

Drug treatment. Camptothecin (CPT) and etoposide (Etopo) stock solutions were dissolved in DMSO and diluted to a 100X working solution with basal medium eagle (BME) prior to addition to culture media. The final concentration of DMSO was 0.04% or less in all experiments. Stock solutions of 5-fluorouracil (5-FU), ethynyl uridine (EU), and fluorodeoxyuridine (FdU) were dissolved in water and diluted with BME prior to addition to the culture media.

Cell Culture and transfection. Primary cortical neurons were cultured from Sprague Dawley rats (Harlan, Indianapolis IN) on post-natal day zero as previously described [78]. Culture medium consisted of BME supplemented with 10% heat-inactivated bovine calf serum (Hyclone, Logan, UT) 35 mM glucose, 1 mM L-glutamine, 100 U/mL penicillin and 0.1 mg/mL streptomycin. For immunofluorescence studies, 0.5×10^6 neurons were seeded on poly-D lysine coated plastic cover slips contained in 24 well plates. For quantitative RT-PCR studies, 2×10^6 neurons were seeded in poly-D lysine coated 35 mm cell culture dishes. 2.5 μ M cytosine arabinoside was added on day in vitro (DIV) 2 to prevent growth of non-neuronal cells. All transfections used Lipofectamine 2000 and occurred on DIV 4.

Luciferase Reporter Gene and Beta Galactosidase Assays. Luciferase and beta galactosidase enzymatic activities were determined using standard kits obtained from Promega (Madison, WI).

Immunoblotting. For western blotting primary cortical neurons were washed twice with PBS and lysed in SDS Page sample buffer. Western blotting was performed as

previously described [106]. Antibodies and dilutions are listed in the antibodies portion of the methods section.

Isolation of Nuclear fraction. RPL11 immunoblots were conducted using nuclear lysates. To isolate the nuclear fraction, neurons were lysed by a 10 minute 4°C incubation with NTEN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris pH=8.0, 0.5% NP-40) supplemented with Protease Inhibitor Cocktail Set III (calbiochem used at manufacturer's recommended dilution) and 1 mM PMSF. Lysates were centrifuged at 13,000 RPMs for 20 minutes at 4°C to separate the cytoplasmic fraction. Supernatant was removed and the pellet was resuspended in standard western blot SDS-sample buffer and boiled for 10 minutes prior to use.

Apoptotic Counts. Beta galactosidase positivity was used as a marker of transfection. Nuclei were stained with Hoechst 22358 to visualize morphology. Apoptosis was defined as condensed chromatin and significantly degenerated processes. In most cases only cell bodies remained. Cells counted as surviving contained large round uncondensed nuclei and no clear degeneration of processes.

Northern Blot. Northern blots were performed as previously described [137]. Nucleotide sequences are listed in Table IV. 5.8S probe was previously described [60].

Quantitative RT-PCR. Quantitative RT-PCR was performed as previously described [106]. Primer sequences are listed in Table IV

Ethynyl uridine incorporation. Neurons were treated with 5-FU or vehicle for 2 hours prior to 50 minute incubation with 1mM EU. After fixation cells were permeablized with 0.5% triton in PBS for 30 minutes. After washing, neurons were incubated with click

reagent (100 mM Tris, 1 mM CuSO₄, 100 mM ascorbic acid, 50 μM Oregon green azide dye-Invitrogen) for 1 hour. After washing, cells were then stained with Hoechst 33258 to visualize nuclei.

MTT Assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed as previously described [90]

Table 5. Chapter IV Nucleotide Sequences

Experiment	Amplicon/Target	Direction	Sequence (5'-3')
Pre-rRNA	45S pre-rRNA ETS1	Forward	TGGGGCAGCTTTATGACAAC
		Reverse	TAGACCAAACGGGAAAACC
	45S pre-rRNA ITS1-5.8S	Forward	CGCTCACACCTCAGATAACC
		Reverse	CTAGCTGCGTTCTTCATCG
	18S rRNA	Forward	GTTGGTTTTCGGAACTGAGGC
		Reverse	GTCGGCATCGTTTATGGTCG
Northern Blot	5' ETS	Reverse	TAGACCAAACGGGAAAACC
	ITS1	Reverse	GGTTATCTGAGGTGTGAGCG
	5.8S	Reverse	CTAAGAGTGGTACGAGGTCG
shRNAs	shRPL11-1	Forward	GGTGCGAGAGTACGAATTA
	shRPL11-2	Forward	CTTCTCAGATACCGGCAAC
	shRPL4-1	Forward	CTTTGTTACACCAACTTG
	shRPL4-2	Forward	GGCCTTCAGAAACATCCCT
	shRPS6-1	Forward	GATGATGTCCGCCAGTATG
	shRPS6-2	Forward	GATTCAGCGTCTTGTTACT

Results

First we tested the ability of RPL11 to modulate p53-mediated transcription relative to empty vector and RPL4 controls using a luciferase p53 reporter. We employed a temperature sensitive p53 construct which at temperatures greater than 32° C acts as a dominant negative. Thus, we could quench p53 activity and allow RPL11 to accumulate for 24 h prior to an 18 h incubation at 30°C. Over expression of RPL11, but not RPL4, was able to increase p53 transcriptional activity under such conditions (Fig.13B). Moreover, in cortical neurons that were treated with the nucleolar stress inducer CPT, nuclear content of RPL11 did not change (Fig.13C). Therefore, RPL11 is a good candidate for a mediator of the nucleolar stress response including p53-induced neuronal apoptosis.

To further evaluate this possibility, shRNAs were developed to knock down RPL11 (Fig.14A,B). As an additional control, validated shRNAs against L4 were also generated (Fig.14C). We previously reported that shRNA mediated knockdown of RNA Polymerase 1 cofactor Transcription Initiation Factor 1A (shTIF) induces nucleolar stress and p53-mediated neuronal apoptosis [106]. Therefore, shTIF1A was used to examine the role of RPL11 in mediating the nucleolar stress. First, RPL11 knockdown did not affect shTIF1A-induced nucleolar stress and did not induce nucleolar stress by itself as determined by NPM immunofluorescence (Fig.13D). Thus, the NPM fluorescent signal was reduced in the nucleolus and increased in the nucleoplasm in shTIF1A-transfected neurons and was not modified by RPL11 knockdown (Fig.14D). However, RPL11 knockdown attenuated shTIF1a-induced apoptosis (Fig.14E,F). The anti-apoptotic effects were observed with single shRNAs targeting RPL11 or with their equimolar mix. No

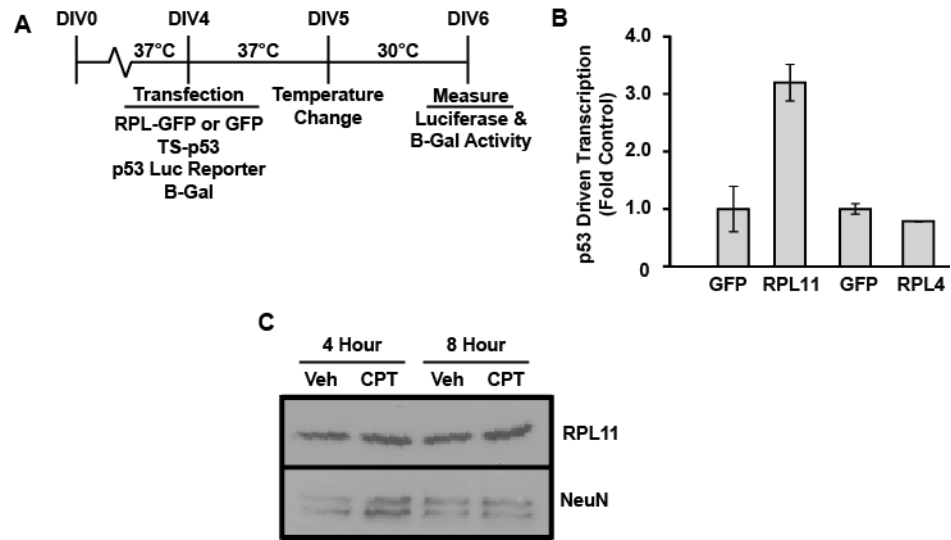


Figure 13. Overexpression of RPL11 increases p53-mediated transcription.

A, General design of the temperature sensitive p53 (TS-p53) experiment. **B**, On DIV 4, cells were transfected with PG13-Luc (p53 reporter gene), TS-p53, β -galactosidase for normalization, and RPL11-GFP or RPL4-GFP or empty vector. One representative experiment is shown. 24 hours after transfection, the temperature was lowered to 30°C for 18 hours thus allowing conformational change and activation of TS-p53. Overexpression of RPL11 but not RPL4 was sufficient to increase p53 mediated transcription. **C**, Western blot analysis demonstrating that RPL11 remains in the nucleus following treatment with 5 μ M camptothecin.

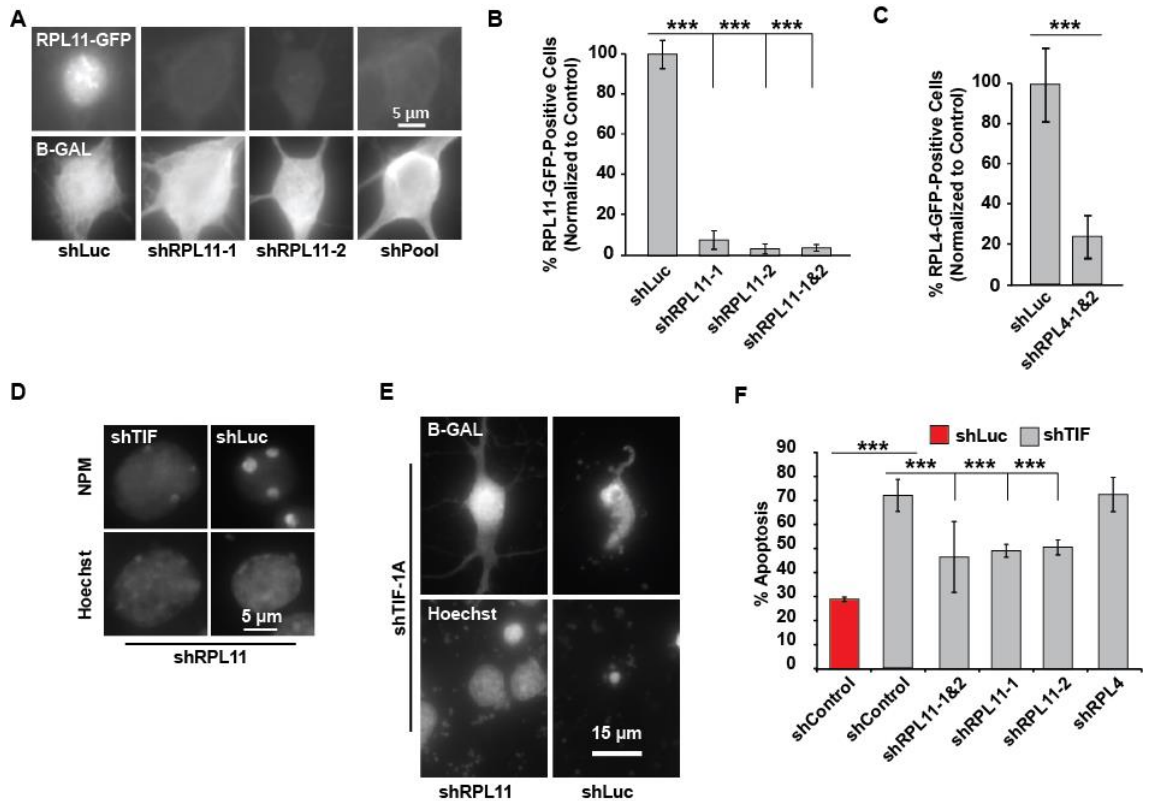


Figure 14. Knockdown of RPL11 increases neuronal survival following shRNA induced nucleolar stress.

A, Immunofluorescence validation of RPL11 shRNA constructs. **B**, Quantification of shRPL11 efficiency. Data represent the percentage of neurons which were double positive for RPL11-GFP and the β -Galactosidase marker of transfection. **C**, Quantification of shRPL4 efficiency. **D**, Immunofluorescence demonstrating that shRPL11 does not prevent nucleolar disruption induced by shRNA mediated knockdown of Transcription Initiation Factor 1A (shTIF). **E**, Representative images depicting the criteria for quantification of neuronal apoptosis. **F**, Quantification of neuronal apoptosis following shTIF induced nucleolar stress. Knockdown of RPL11 significantly increased neuronal survival. For post hoc comparisons $p < 0.001$ is denoted by ***.

rescue was observed with shL4. Therefore, RPL11 contributes to the activation of neuronal apoptosis in response to shTIF1A-associated nucleolar stress.

Previous work demonstrated that the DNA single strand break inducing chemotherapeutic CPT is an inducer of both nucleolar stress and apoptosis [106]. To strengthen the results described above, CPT was used as an additional inducer of nucleolar stress. Similar to shTIF1A, CPT induced the translocation of NPM from the nucleolus to the nucleoplasm as determined by NPM immunofluorescence and was not modified by RPL11 knock down (Fig.15A). RPL11 knockdown attenuated CPT induced apoptosis (Fig.15B,C) Therefore, our results suggest that RPL11 is a genuine mediator of nucleolar stress-induced neuronal death as its knockdown attenuated apoptosis in two mechanistically different models.

After establishing the role of RPL11 in the neuronal nucleolar stress response, we next sought to determine its contribution to ribosomal stress, using two models which importantly do not disrupt the nucleolus. In non-neuronal systems, the RNA precursor 5-FU induces such a state by perturbing the processing of pre-rRNA without interfering with RNA polymerase I activity [23]. We confirm that in rat cortical neurons 5-FU impairs the processing of pre-rRNA as determined by northern blot analysis (Fig.16A). First, 5-FU appeared to destabilize the initial 1kb region of the 5'ETS as both the full length 47S precursor and the 5'ETS-derived smaller fragment were sharply down-regulated when a 5'ETS probe was used. These changes were also observed with qRT-PCR (Fig.16B). Processing defects were also indicated by the disappearance or reduction of more advanced intermediates including 12S, 20S, and 36S rRNAs (Fig.16A). 5-FU did not inhibit RNA Pol I as indicated by unaffected nucleolar incorporation of the RNA

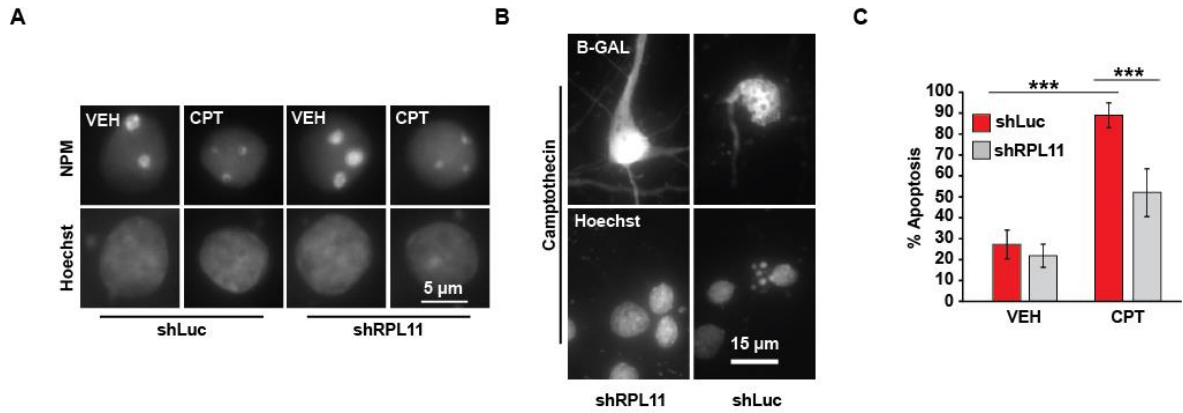


Figure 15. Knockdown of RPL11 increases neuronal survival following camptothecin induced nucleolar stress.

A, Immunofluorescence demonstrating that shRPL11 does not prevent nucleolar disruption induced by 24 hour 2 μ M camptothecin treatment. **B**, Representative images depicting neuronal apoptosis. **C**, Quantification of neuronal apoptosis following 24 hour 1 μ M camptothecin induced nucleolar stress. Knockdown of RPL11 significantly increases neuronal survival. For post hoc comparisons p<0.001 is denoted by ***.

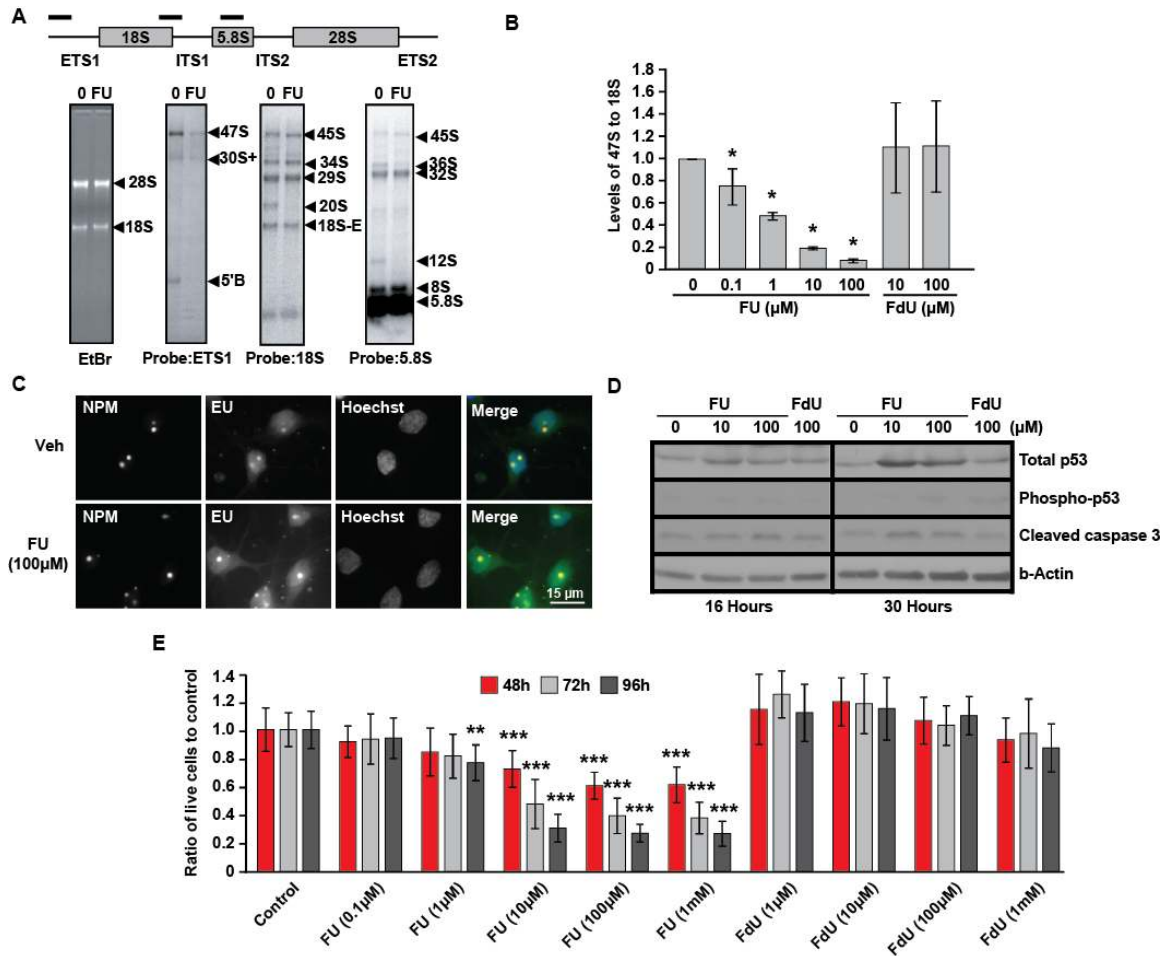


Figure 16. Characterization of 5-fluorouracil induced neuronal death.

A, Northern blot analysis depicting rRNA processing following 4 hours 5-fluorouracil (5-FU) treatment. Multiple rRNA species which are generated during pre-rRNA processing are no longer apparent following 5-FU treatment. **B**, 5-FU dose response depicting increasing inhibition of pre-rRNA levels as determined by quantitative rt-PCR measuring the 5'external transcribed spacers region against total 18S rRNA. **C**, Primary cortical neurons were treated with 100 μM 5-FU or vehicle for 2 hours prior to a 1 hour hour incubation with 1mM ethynyl uridine. Pre-treatment with 5-FU did not inhibit pre-rRNA synthesis as determined by immunofluorescence utilizing click chemistry to visualize EU incorporation into de novo synthesized RNA. **D**, Western blot analysis of apoptosis related proteins following 5-FU treatment. 30 hours of 5-FU treatment, but not 16 hour, increased total p53 and cleaved caspase 3 levels. Interestingly, phosho-p53 was not induced at either time point. **F**, MTT assay depicting the dose response and time course of 5-FU induced neuronal death. For post hocs $p < 0.05$:*, $p < 0.01$:**, $p < 0.001$:***. Data for panel A was generated by Dr. L. Slomnicki and Dr. S. Ellis. Data for panels B-E generated by Dr. A. Vashishta.

precursor ethynyl uridine (EU) (Fig.16C). Moreover, there was no evidence of NPM dispersion (Fig.16C). Such a ribosomal but not nucleolar stress was accompanied by accumulation of p53 (Fig.16D). Interestingly, 5-FU did not increase p53 phosphorylation at Serine 15. However, it did activate apoptosis as indicated by increased levels of caspase-3 and reduced neuronal survival (Fig.16D,E). Importantly, similar responses were not seen in control experiments which utilized 5-FU's DNA precursor variant FdU. Lastly, 5-FU dosages of 10 μ M and higher significantly reduced neuronal death 48 hours after treatment as determined by MTT assay (Fig.16E).

As the results detailed above suggest that 5-FU induces ribosomal stress in primary cortical neurons, we tested if knockdown of RPL11 would attenuate neuronal death. Indeed, knockdown of RPL11 was able to completely rescue neuronal survival in response to 36 hours of 5-FU treatment (Fig.17C). After RPL11 knockdown the rate of apoptosis dropped approximately 50% to below 30% which was equal to control levels (Fig 17C). Interestingly, while 5-FU did not induce translocation of NPM, it did alter the nucleolar morphology. The shape of nucleoli became increasingly irregular and less circular following 5-FU treatment (Fig. 17A).

It has been reported that knockdown of RPS6 induces ribosomal stress in the absence of nucleolar disruption in both mouse liver and cell lines [63]. Therefore, we sought to confirm similar results in primary cortical neurons and to determine if such a response is RPL11 mediated. Nucleolar morphology was unaltered by shRNA mediated knockdown of RPS6 (shRPS6) as there was no evidence of NPM dispersion (Fig.17D). Moreover, nucleoli appear to have unaltered circularity, unlike what was seen with 5-FU.

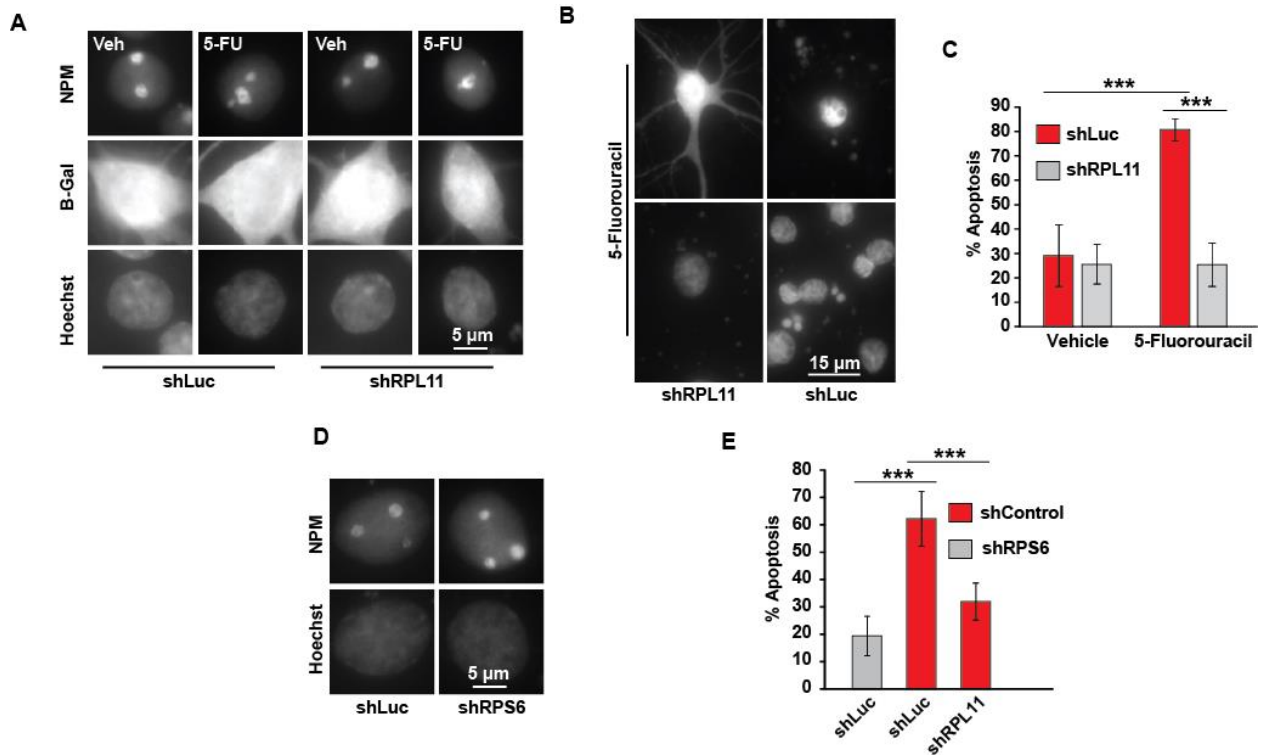


Figure 17. Knockdown of RPL11 increases neuronal survival following ribosomal stress induced apoptosis.

A, Immunofluorescence demonstrating that 10 μ M 5-FU does not induce nucleolar stress, but does alter nucleolar shape as they become less circular. **B**, Representative images depicting the criteria for quantification of neuronal apoptosis. **C**, Quantification of neuronal apoptosis following 36 hours of 10 μ M 5-FU treatment induced ribosomal stress. Knockdown of RPL11 significantly increased neuronal survival. **F**, Immunofluorescence demonstrating that shRS6 does not induce nucleolar disruption. **E**, Quantification of neuronal apoptosis following 48 hours of ribosomal stress induced by shRNA mediated knockdown of RPS6. Knockdown of RPL11 significantly increased neuronal survival. For post hoc comparisons $p < 0.001$ is denoted by ***.

shRPS6 induced significant neuronal apoptosis 48 hours after transfection and importantly apoptosis was attenuated by co-depletion of RPL11 (Fig. 17E).

Lastly, we tested the ability of RPL11 to rescue cell survival in response to the double strand break inducing chemotherapeutic Etopo. Initially, Etopo was intended to be a specificity control as it is an inducer of p53-mediated apoptosis and is not known to induce nucleolar or ribosomal stress at low concentrations. First, we verified by immunofluorescence that the dose chosen for rescue experiments, 1 μ M, did not induce dispersion of NPM. Indeed, the nucleolar localization of NPM was not altered by a 24 h treatment of 1 μ M Etopo (Fig.18A). Unexpectedly, knockdown of RPL11 attenuated apoptosis following Etopo treatment (Fig.18B,C). Such a result suggested that Etopo could be impairing ribosomal biogenesis in the absence of nucleolar disruption. Interestingly, Etopo dose response revealed biphasic effects on pre-rRNA levels (Fig.18D). In cortical neurons, the threshold for Etopo-induced nucleolar disruption and decrease in pre-rRNA has been confirmed to be between 10-25 μ M. However, pre-rRNA levels were up at 0.3 and 1.0 μ M (Fig.18D). Finally, western blot confirmed that RPL11 was present in the nucleus after low dose Etoposide treatment (Fig.18E).

Discussion

Our results suggest that RPL11 functions as a bona fide neuronal death mediator. Nucleolar disruption is a well characterized inducer of apoptosis in young neurons [106, 198]. We demonstrate that knockdown of RPL11 is sufficient to increase survival following nucleolar stress induced by both genetic silencing of RNA Pol I and pharmacological treatment with CPT. Moreover, we show that the effect was not due to impairment of the large ribosomal subunit assembly as knock down of RPL4 did not

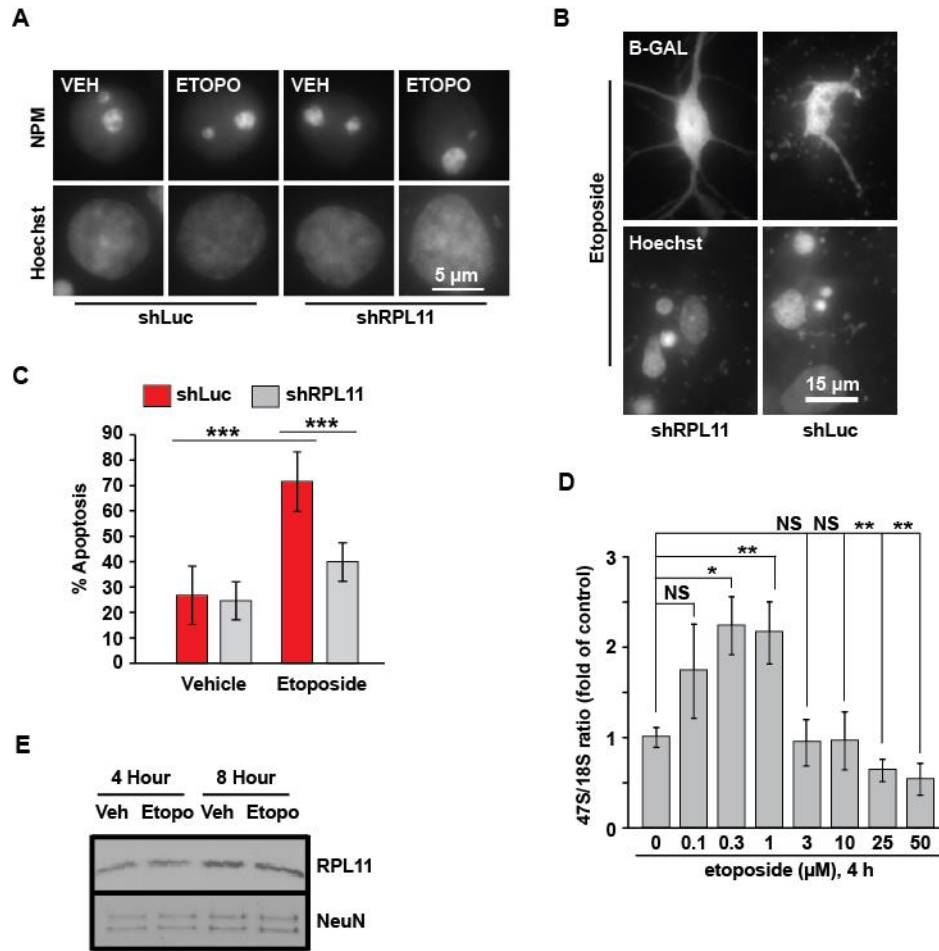


Figure 18. Knockdown of RPL11 attenuates etoposide-induced apoptosis of cortical neurons.

A, Immunofluorescence demonstrating that 1 μM etoposide does not induce nucleolar stress. *B*, Representative images depicting the criteria for quantification of neuronal apoptosis. *C*, Quantification of neuronal apoptosis following 24 hours of 1 μM etoposide treatment. Knockdown of RPL11 significantly increased neuronal survival. *D*, Dose response depicting the effect of etoposide on pre-rRNA levels. *E*, Western blot analysis demonstrating that RPL11 remains in the nucleus following treatment with 1 μM etoposide. For post hoc comparisons $p < 0.001$ is denoted by ***

rescue neuronal survival following inhibition of RNA Pol I. Additionally, stoichiometric errors in ribosome assembly, produced by both knockdown of RPS6 and 5-FU treatment, were sufficient to induce apoptosis in the absence of nucleolar disruption. Importantly, knockdown of RPL11 increased survival in both instances and in the case of 5-FU treatment produced a complete rescue of survival.

Unexpectedly, knockdown of RPL11 also increased survival following treatment with the double strand break inducing chemotherapeutic etoposide. Surprisingly, pre-rRNA levels increased in response to low dose etoposide treatment. Taken together such findings suggest that concentrations of Etopo below the threshold for nucleolar disruption still impair ribosomal biogenesis. It is tempting to speculate that Etopo-induced increases in pre-rRNA content are the result of inhibited pre-rRNA processing. Interestingly, multiple proteins involved in ribosomal biogenesis have been described as putative substrates for the double strand break responsive protein, ataxia telangiectasia mutated [161]. Such interactions could underlie the effects of Etopo on pre-rRNA content. Alternatively, it is also plausible that RPL11 may be a more general mediator of cell death than originally anticipated. In support of such a concept, Fumagelli et.al. (2012) demonstrated that increased translation of 5' Terminal Oligopyrimidine Tract (5'TOP) mRNAs is sufficient to impair the stoichiometry of ribosomal components and induce ribosomal stress [64]. Therefore, a mechanism for inducing ribosomal stress without directly effecting RNA polymerase I or pre-rRNA processing does indeed exist.

The ability of ribosomal stress to induce apoptosis has not been documented in prior reports and may be a property unique to post mitotic neurons. These results are in contrast to two prior studies which demonstrated ribosomal protein induced cell cycle

arrest and non-apoptotic accumulation of p53 [64, 125]. Using cell lines Fumagelli (2012) et.al. demonstrated that loss of an individual ribosomal protein other than RPL11 or RPL5 induced p53 mediated cell cycle arrest [64]. Importantly, they found no evidence for apoptosis during their experiments. Moreover, Kumazawa et.al. (2015) utilized a different approach, siRNA mediated knockdown of several pre-rRNA processing proteins, but came to similar conclusions. They demonstrated that in the absence of RNA Pol I inhibition, impairment of processing was sufficient to induce accumulation of p53. Interestingly, p53 remained in the unacetylated form and apoptosis did not occur [125]. They proceeded to show that once RNA Pol I was inhibited, the nucleolar protein MYBBP1A was released from the nucleolus and free to acetylate p53 and induce apoptosis. It is tempting to speculate that the pool of neuronal p53 is being modulated by an additional signaling pathway not active in cell lines. Interestingly, nucleolar disruption induced apoptosis is developmentally restricted and occurs only in immature neurons [106, 198, 207]. It will be important for future studies to determine if the ribosomal stress apoptotic response is subject to similar restrictions.

Importantly, our results demonstrate that catastrophic nucleolar failure is not necessary for impaired ribosomal biogenesis to induce neuronal death. Many neurodegenerative diseases have been proposed to involve nucleolar dysfunction, yet evidence suggesting full blown nucleolar stress is minimal or not existent. AD is a prototypical example of such a condition. Post mortem AD brain tissue has been reported to contain decreased ribosome content, increased ribosomal oxidative damage, and decreased protein synthesis capacity [47, 48]. Oxidative stress is a well characterized feature of Alzheimer's disease, thus increased ribosomal damage and some amount of

decreased protein synthesis capacity is not entirely unexpected [42]. However, cells should contain homeostatic mechanisms for increasing ribosomal biogenesis under such conditions. Our laboratory has identified increased methylation of the rDNA promoter in post mortem AD brain tissue [206]. Such a phenotype is believed to be a biomarker of decreased transcriptional activity, and suggests decreased ribosomal biogenesis during a time when cellular demands are expected to be high [238]. When considered together the trio of decreased total ribosome content, increased ribosomal damage and increased rDNA promoter methylation suggest an AD-associated homeostatic failure of ribosomal biogenesis. The finding that RPL11 functions as a stress mediator in the absence of nucleolar disruption suggests that it may indeed play a role in AD-associated neurodegeneration.

The translational relevance of RPL11-induced neuronal death may be of importance for C9ORF72 hexanucleotide repeat expansion associated ALS/FTD (C9) and poly glutamine diseases (PolyQ), such as Huntington's- and Machado-Joseph's-Disease. These diseases have been proposed to contain RNA and protein species which have a direct impact on nucleolar function [79, 129, 135, 244, 245]. Interestingly, they may be associated with different stress responses. PolyQ derived RNAs and proteins appear to induce a primarily nucleolar stress as inhibition of RNA Pol I has been demonstrated by multiple mechanisms [135, 244, 245]. Alternatively, C9 toxicity has been proposed to involve inhibited pre-rRNA processing, which suggests a predominantly ribosomal stress. Interestingly, the morphology of C9 nucleoli appears highly irregular and bulbous similar to 5-FU treatment [79]. Our findings suggest that RPL11 may be a highly desirable target for future PolyQ and C9 studies.

It will be important for future studies to address the possibility of other ribosomal proteins as stress mediators. Fourteen ribosomal proteins have been documented to bind MDM2 to date and experts predict this number will grow [114]. The physiological relevance of many of these has been debated given that free ribosomal proteins are rapidly degraded by the proteasome [25, 131]. Although, several key points argue for their relevance. First, MDM2-Ribosomal protein interactions happen at several discrete locations. Such specificity argues against these interactions being non-physiological. Moreover, three of these ribosomal proteins have been documented to modulate p53 mediated transcription towards different target genes [40]. Thus, the functions of these 14 MDM2 binding ribosomal proteins may be highly relevant for stress responses. Lastly, 11 ribosomal proteins were identified as potential tumor suppressors in a zebrafish-based screen [4]. Therefore, a wealth of additional ribosomal proteins are potential targets for future investigations.

In conclusion, our results demonstrate that RPL11 is bona fide neuronal death mediator in response to both nucleolar stress and ribosomal stress. In addition, we demonstrate that catastrophic nucleolar failure is not necessary for ribosomal protein mediated apoptosis. Lastly, we provide preliminary evidence suggesting that etoposide may be a novel inhibitor of pre-rRNA processing. Hence, RPL11 is an intriguing target for therapeutic intervention in several neurological conditions.

CHAPTER V

DISCUSSION

Restatement of Key Findings

These studies focused on elucidating the role of nucleolar dysfunction, including both canonical nucleolar stress and atypical forms of dysfunction, in neuronal death and degeneration.

In Chapter II, I report that genomic rDNA content is unstable in the Dementia with Lewy Bodies (DLB) brain and is not the result of altered rDNA promoter methylation. These studies were necessitated by prior Hetman laboratory reports demonstrating increased rDNA promoter methylation and gene content in the Alzheimer's disease (AD) brain [206]. Such findings raised two possibilities. First, AD could potentially involve a disease-associated amplification of rDNA genes or second, an AD-associated increase in promoter methylation could be reducing the aging-associated loss of rDNA genes. Loss of rDNA is a well-accepted source of senescence in yeast and in the 1970s was proposed to occur in the mammalian brain by Bernard Strehler [105]. However, subsequent studies did not confirm such a phenomenon [68, 205]. Despite a general consensus that Strehler's hypothesis was sufficiently debunked; the co-incidental occurrence of increased methylation and increased copy number in elderly samples was highly intriguing. Thus, I deemed it necessary to revisit Strehler's hypothesis with more modern technology as a means to discriminate between these two potential hypotheses. Therefore, I probed rDNA content in post mortem parietal cortex tissue from both young

and old individuals. No aging-associated loss of rDNA content was found. Next, I addressed if increased rDNA content was specific to AD or a general feature of neurodegeneration by probing rDNA content in the DLB parietal cortex using genomic qPCR. Indeed, DLB samples contained more rDNA copies than elderly controls. Lastly, I probed the DLB rDNA promoter for increases in methylation and found it to be unchanged. Thus, in the brain, increased genomic rDNA content is not unique to AD as it was also found in DLB. Conversely, changes in promoter methylation appear to be an AD specific condition.

In Chapter III, I assessed the integrity of several markers of nucleolar dysfunction in response to increasing PMI. I report relatively modest decreases in pre-rRNA for up to 12 h. Transcription of pre-rRNA is believed to be sensitive to cellular energy levels and the supply of available nutrients [76, 182, 260]. Thus, significant declines in 47S pre-rRNA content would be expected under ischemic conditions. Such a modest effect suggested the simultaneous inhibition of both transcription and processing. Indeed, I demonstrated that following pharmacological inhibition of pre-rRNA synthesis, energy deprivation prolongs the half-life of pre-rRNA. Therefore, my results suggest that the dramatic declines in pre-rRNA which would be expected to occur during ischemia-induced energetic failure are blunted by the simultaneous inhibition of processing. Additionally, I report that punctate nucleolar localization of nucleophosmin was detected in all cells though PMIs of 12 h, despite a percentage of cells having accumulation of nucleophosmin in the perikaryon and proximal neurites. Lastly, rDNA promoter methylation and genomic rDNA content were unaffected through PMIs of up to 12 h

In Chapter IV, I report the ability of RPL11 to function as a bona fide death mediator in primary cortical neurons. Overexpression of RPL11 was sufficient to augment p53-mediated transcription. Knock down of RPL11 significantly increased neuronal survival in response to nucleolar stress which was induced by both genetic manipulation and pharmacological means. Moreover, I demonstrated that the chemotherapeutic agent 5-FU is an inducer of ribosomal stress in primary cortical neurons. Such a conclusion is based on its ability to significantly impair pre-rRNA processing and induce p53 in the absence of RNA polymerase I inhibition. Importantly, canonical nucleolar stress was not necessary for RPL11 induced death as its knockdown increased survival in response to ribosomal stress induced by knockdown of RPS6 and treatment with 5-FU. Lastly, RPL11 knock down unexpectedly increased neuronal survival in response to etoposide treatment and preliminary data suggests that etoposide may increase pre-rRNA levels at low doses. Taken together, these findings suggest that low dose etoposide may be a previously unreported inducer of ribosomal stress

Impact

In chapter II, I demonstrated genomic instability in the dementia with Lewy bodies brain. Genomic instability in the mature, non-neoplastic mammalian brain is a newly emerging research topic with only a small number of documented cases. To the best of my knowledge, evidence for such a phenomenon is limited to three specific research areas, activation of Line-1 (L1) retrotransposons, neurodegenerative disease-associated telomere shortening, and the rDNA studies of myself and Dr. Pietrzak. Thus, while I am not the first to document such a phenomenon, my work still provides

significant weight of evidence support for genomic instability playing a role in human neurodegenerative diseases.

Additionally, the pro-neurodegenerative potential for nucleolar stress must also be considered. While I speculate in chapter II that expansion of rDNA gene content could potentially blunt the DNA damage response, it is important to consider that the ribosomal stress response is centered on stoichiometric imbalances. However, given the intricate regulation of RNA polymerase I, expansion of rDNA content alone is presumably not sufficient to induce stoichiometric errors in ribosomal components. Speculation aside, I have, at the very least, demonstrated a significant disease-associated alteration of a genomic region which has been proposed by some to be a major regulator of p53 [23].

In chapter III, I assessed the stability of nucleolar stress mediators in post mortem tissue. While post mortem ischemia altered the localization of a minor portion of NPM, 100% of the cells examined contained punctate NPM staining. Thus, at least for animal models, binary assessment of nucleolar morphology appears to be highly reliable in post mortem brain tissue. Such results strengthen the findings of others who have quantified nucleolar disruption in post mortem specimens of brain tissue to uncover PD-, HD- or AD-associated nucleolar stress.

While not common in recent years, studies utilizing an NPM localization index can be found in the literature. As interest in nucleolar stress is currently on the rise, it would not be surprising to see this method re-emerge in the near future. Similar to the NPM localization index, Kwon et.al. has claimed nucleolar stress in cell culture models despite the presence of punctate nucleoli [129]. Such a claim was based on a portion of

NPM being released into the nuclear compartment, despite the retention of punctate nucleoli. While such a criterion of nucleolar stress may work in cell culture models, my results suggest that it may not be reliable in human tissue due to significant effect of post mortem interval. .

Additionally, I assessed the post mortem stability of three other nucleolar stress markers, 47S pre-rRNA content, rDNA content, and rDNA promoter methylation. Importantly, I found that rDNA content and rDNA methylation were not significantly altered during the 12 hour analysis. Such findings help to strength the results of myself and Dr. Pietrzak who have quantified these parameters in post mortem tissue [81, 206]. This finding strongly suggests that the effect of post mortem interval on our results is relatively minimal. On the other hand, 47S pre-rRNA content was significantly decreased by 12 hours, despite the magnitude of decline being surprisingly low. Interestingly, select regions of the transcript appeared to have increased stability. Therefore, I propose that 47S pre-rRNA measurements are potentially feasible in post mortem tissue given the slow rate of decline, but such measurements would require tightly grouped PMIs and carefully designed amplicons to minimize the impact of PMI.

While this particular study focused on post mortem ischemia inducing energetic failure in brain tissues, a much broader conclusion can also be drawn from my studies. Namely, impairment of pre-rRNA processing can mask the inhibition of RNA polymerase I. This implies that stoichiometric imbalances in ribosomal components and induction of the ribosomal stress response can occur regardless of changes in nucleolar morphology. Such a condition is of great importance as there is considerable evidence for abnormal nucleolar function in many neurodegenerative conditions, despite the

persistence of NPM staining in the nucleolus. Therefore, activation of the ribosomal stress response cannot be excluded solely based on the staining of morphological markers.

In chapter IV, I demonstrated that RPL11 is a bona fide mediator of nucleolar stress, ribosomal stress and most importantly neuronal death in primary cortical neurons. While similar results have been described in other cell types such as immortalized cell lines and liver tissue from in vivo models, I am the first to validate RPL11 as a death mediator in the nervous system. Elucidation of stress mediators is essential for assessing the contribution of nucleolar- and ribosomal stress to human neurodegenerative disease. Thus, this is a fundamental discovery that myself and others can build upon in future studies.

Next, we demonstrated that the DNA double strand break-inducing chemotherapeutic etoposide may harness ribosomal stress to activate p53-mediated apoptosis. Interestingly, we identified a biphasic response with increased 47S pre-rRNA and no nucleolar stress at etoposide doses below 1 μ M at which apoptosis was attenuated by depletion RPL11. When considered together these findings suggests that low frequency DNA double strand breaks engage the ribosomal stress response independently of the RNA polymerase I inhibition which is seen at significantly higher doses of etoposide.

While evidence for full blown nucleolar stress is rare, there is considerable evidence suggesting perturbation of nucleolar function, nucleolar insufficiency, and nucleolar hypertrophy in many neurodegenerative and neurodevelopmental diseases.

Importantly, I demonstrated that knockdown of RPL11 rescues neuronal survival in response to numerous apoptotic insults, several of which do not disperse NPM or inhibit RNA polymerase I. This strongly suggests that nucleolar stress is sufficient, but, importantly, is not necessary for RPL11-mediated neuronal death. Therefore, pro-apoptotic ribosomal stress may indeed be active in conditions in which nucleoli appear unaffected.

Limitations and Alternative Interpretations

In Chapter II, the biggest limitation for drawing conclusions is the descriptive nature of the study design. While I speculate on the potential consequences of increased rDNA content in this chapter, the true biological relevance is unknown. To address this situation, transgenic mice with variable rDNA content would have to be generated. Such mice could be utilized to test the working model which speculated that increased rDNA content increased the resistance to DNA damage induced cell death.

The lack of information regarding the responsible cell type is also a major limitation to the interpretation of this study. Importantly, such information could allow for the generation of more accurate hypotheses and better shape future studies. For instance, if this is a glial phenomenon I would hypothesize that glial-specific rDNA expansion is a mechanism for the rapid expansion of these cells which occurs during gliosis. On the other hand, if this is a neuron-specific rDNA instability, I hypothesize that neuron specific rDNA expansion is a mechanism to increase cellular reliance when faced with increasing amounts of DNA damage. Similarly, knowledge regarding the cell cycle status of affected cells, i.e. mitotic vs post-mitotic, would be of considerable value when attempting to determine the mechanism of rDNA amplification. Thus, the lack of

an identified cell type severely impairs the ability to assess the biological significance of rDNA amplification.

Some critics may have a strong preference for southern blot and would claim that the choice of qPCR is a limitation to this study. While I agree that a southern blot analysis would provide weight of evidence support to strengthen my claims, I would argue that it is simply not necessary. I conducted numerous quality control measures to verify the accuracy of my results. First, as the gene specific stability of DNA in post mortem tissue is unknown I employed two separate reference genes for normalization, one multi-copy and one single copy. Importantly, both normalizers produced highly similar results. Therefore, my results do not appear to be skewed by instability of the reference gene. Second, I measured the rDNA unit at three separate locations. Importantly, all three amplicons produced comparable results. Third, given that all three PCR amplicons are physically linked in the same rDNA unit, there should a high degree of correlation when comparing rDNA content from various amplicons of the same individual if measurements are accurate and indeed, the regression analysis for 18S vs 28S was $R^2=0.81$. Fourth, I demonstrated that DNA template methylation levels do not skew qPCR results. Thus, while a southern blot analysis would complement my results and provide weight of evidence support it is unnecessary as my numerous quality control measures suggest this method is indeed sufficient.

In this chapter II also assessed changes in methylation of the rDNA promoter using two separate methods, bisulfite clonal sequencing and qPCR utilizing genomic DNA cleaved by the CpG methyl sensitive restriction endonuclease HpaII (HpaII). Neither method is perfect as both contain limitations, but importantly they complement

each other well. Bisulfite sequencing provides information about every CpG in the promoter and can show trends contained within the same clone, but the analysis is limited to finite a number of clones. On the other hand, HpaII assesses only one CpG and is applicable to only a fraction of CpGs. However, the major benefit is that this method theoretically probes 100% of loci contained within a sample. These two methods in combination strongly suggest that the rDNA promoter does not contain increased methylation in the DLB brain. Regarding the biological significance of promoter methylation, this epigenetic modification is merely a biomarker of recent RNA Pol I activity and not a direct measurement of RNA Pol I activity. Thus, changes in methylation do not guarantee changes in pre-rRNA synthesis. To further characterize RNA Pol I activity in post mortem tissue chromatin immunoprecipitation (CHIP) could be performed to assess levels of rDNA promoter occupancy. Moreover, the data contained in chapter III suggests that pre-rRNA levels could also be used as a marker of RNA polymerase I activity.

In Chapter III, I assessed the stability of nucleolar stress markers in response to increasing PMI. All findings are based on experiments conducted in adult rats or rat primary cortical neurons in culture. Despite the fact that I utilized both *in vivo* and *in vitro* methods, one major limitation to this study is the lack of assessments made in human tissue. It is not guaranteed that my findings will translate to the human brain. For example, I conclude that large quantities of NPM are retained in the nucleolus through PMIs up to 12 h. Conversely, a study conducted by Rieker et.al. demonstrated that some percentage of neurons in post mortem control tissues did not contain visible punctate nucleoli [214]. However, it is important to consider that this study was restricted to an

elderly population, thus the lack of punctate nucleoli in some neurons may be related to senescence and not post mortem degradation [214].

Two other conclusions in this chapter are based solely on qRT-PCR measurements. First, I conclude that pre-rRNA is unstable in response to increasing PMI. Second, I conclude that pre-rRNA processing is inhibited by energy deprivation. The validity of both of these claims could be greatly strengthened by including a northern blot analysis. Moreover, this method would also provide in depth information regarding the stability of specific 47S pre-rRNA fragments in response to increasing PMI and acute energy failure. Such information could allow for the design of qRT-PCR amplicons which display optimum stability in post-mortem tissue. Thus, the use of additional methods could not only strengthen this conclusion, but also provide practical information to improve the design of future experiments.

Additionally, we speculate that the loss of pre-rRNA at later PMIs is due to non-specific energy independent RNases. Such speculation is based on the ubiquitous nature of energy independent RNases and the rapid decline of ATP which is known to occur in post mortem tissue. However, such a condition cannot be determined by only qRT-PCR. An alternative explanation is that some amount of pre-rRNA processing enzymes retain a portion of their activity in the absence of ATP. A northern blot analysis could address this issue as the retention of specific fragments would suggest cleavage by the canonical processing pathway, while the absence of specific fragments and a smear pattern on these blots would suggest non-specific degradation.

In chapter IV, I begin by proposing that nucleolar stress and ribosomal stress are separate but partially overlapping stress pathways. Nucleolar stress is sufficient but not necessary to induce ribosomal stress. The two major conclusions in this chapter are 1- RPL11 is a mediator of both the nucleolar stress response and the ribosomal and, 2- Ribosomal stress induces apoptosis in immature cortical neurons. Interestingly, the latter result differs from other studies which suggest ribosomal stress induces cell cycle arrest, premature induction of senescence and in general a non-apoptotic accumulation of p53 (Kumazawa et al. 2015; Fumagalli et al. 2012; K. Nishimura et al. 2015; Fumagalli et al. 2009).

One important consideration to explain such a discrepancy is the choice of models utilized in our studies. The Kimura laboratory utilized several cell lines including, MCF-7, WI-38, and MEFs (K. Nishimura et al. 2015; Kumazawa et al. 2015). The Thomas laboratory utilized the livers of transgenic RPS6 knock out mice along with the A549 and U2-OS cell lines (Fumagalli et al. 2012; Fumagalli et al. 2009). I utilized rat primary cortical neurons. The most likely explanation for the difference in findings is that apoptotic outcome of ribosomal stress is cell type specific. It may also be dependent on maturation stage as adult neurons do not undergo apoptosis in response to nucleolar stress. Thus, there is already evidence that neurons are indeed unique with regard to ribosomal protein-mediated death. However, for scientific stringency, the limitations of the model should also be considered.

The finding that ribosomal stress induces apoptosis is based on experiments conducted exclusively in rat primary cortical neurons. Ribosomal stress was induced using shRNA-mediated knockdown of RPS6 and treatment with 5-FU. Moreover, data

generated by myself and Dr. Smith suggest that low dose etoposide is a previously unidentified inducer of ribosomal stress. Thus, if we are indeed correct about etoposide, we used 3 separate inducers of ribosomal stress. Importantly, all 3 inducers are mechanistically very different. However, each method does have drawbacks. For example, etoposide is a well-documented DNA damaging agent. Thus, even at low doses which do not inhibit RNA polymerase I the resulting apoptosis is most likely a combinatorial effect of DNA damage and possibly pre-rRNA processing impairment. Moreover, 5-FU is also multi-mechanisms, suggesting that this approach may also include combinatorial effects [23]. Lastly, all experiments utilized shRNA-mediated knockdown of RPL11 to rescue survival.

As all key experiments of Chapter IV relied on shRNAs, it is important to consider the drawbacks of this approach. For example, the act of expressing shRNAs may be toxic due to activation of cell intrinsic anti-viral response. Primary neurons appear to be disproportionately sensitive to shRNA-mediated toxicity relative to other cell types [160, 164]. In support of this concept, when comparing shRNA transfected neurons to those which are untransfected or transfected with a plasmid expressing a non-toxic protein such as green fluorescent protein (GFP) or td-tomato, considerably lower dosages of DNA damaging agents are required to produce equivalent levels of cell death (unpublished observations). Moreover, the effect of shRNA toxicity in primary neurons is evident when considering the kinetics of 5-FU induced apoptosis. Dr. Vashishta characterized the model of 5-FU induced death in primary cortical neurons and demonstrated that 5-FU (10 μ M) induced death is not detectable at 48 hours. At 72 hours approximately 80% death was detected. Surprisingly, in shRPL11-mediated rescue

experiments the same dose of 5-FU produced approximately 80% death by 36 hours. Thus, the mere presence of control shRNAs approximately doubled the rate of 5-FU induced death. Therefore, at least in the case of primary cortical neurons, the contribution of shRNA-associated stress may synergize with ribosomal stress to kill. Such a condition should not affect the finding that RPL11 is a bona fide stress mediator; however, it is possible that the induction of apoptosis represents the sum of two separate stressors, ribosomal stress and shRNA-induced toxicity. I will further address this possibility in the Future Directions section of this chapter.

Lastly, the *in vitro* approach utilized in chapter IV is a potential limitation. It will be important for future studies to validate if the major conclusions generated in this chapter translate to whole animal studies. Potential models for pursuing *in vivo* studies will be described in the future directions section.

Future Directions

Validate the claim of ribosomal stress induced neuronal apoptosis using alternative approaches and *in vivo* models.

As described in the limitations section of this chapter, shRNA-mediated induction of ribosomal stress has been successfully used in other cell types, but the potential exists for confounding toxicities in neuronal models. As I am specifically, interested in cell death pathways, such a condition could be problematic. Therefore, with scientific stringency in mind, I propose to build upon my initial findings by further testing my hypothesis in several additional models, both *in vitro* and *in vivo*.

A number of potential approaches exist to induce ribosomal stress in primary cortical neurons without using shRNA-mediated knock downs. Nishimura et.al. (2015) demonstrated that loss of individual pre-rRNA processing proteins is sufficient to induce ribosomal stress. While they used shRNA-mediated knock down of Ribosome Biogenesis Regulator Homolog (RRS1), Dyskeratosis Congenita 1 (DKC1), and Ribosomal Processing Protein 5 (RRP5), a similar phenotype can be obtained by expressing dominant negative (DN) mutants of other pre-rRNA processing proteins. Importantly dominant DN mutants have been described in the literature for both Pescadillo Ribosomal Biogenesis Factor 1 (PES1) and Block Of Proliferation 1 (BOP1) [73, 204]. Together along with WD Repeat Domain 12(WDR12) these three proteins form the PeBoW complex which is essential for pre-rRNA processing. Thus, through the use of dominant negative variants I can easily induce ribosomal stress in primary neurons without using shRNAs or concern for off-target pharmacology. Moreover, as two components of this complex are already validated in their dominant negative form, this controls for the possibility of promiscuous protein-protein interactions as both DN-BOP1 and DN-PES1 would be expected to produce the same phenotype.

Additionally, there are several other potential approaches for inducing ribosomal stress without shRNAs in the unexpected event that DN-BOP1 and DN-PES1 do not produce ribosomal stress in primary cortical neurons. In other cell types, overexpression of TIF-1a is sufficient to increase rRNA transcription, nucleolar rRNA content, and the accumulation of p53 and p21 [187]. Thus, this may be another viable alternative. Additionally, mutant RPS19^{R62W} has been proposed to be a dominant negative ribosomal protein [46]. Lastly, a constitutively active variant of the Harvey Rat Sarcoma Viral

Oncogene Homolog (HRAS^{G12V}) is known to induce ribosomal stress [187]. However, it is important to consider that HRAS^{G12V} would alter cell signaling well upstream of ribosomal stress and would be expected to have multiple effects on cell signaling. In summary, there are many additional options for further *in vitro* validation of ribosomal stress as an inducer of neuronal apoptosis.

Another major consideration is that my findings are limited to only *in vitro* experiments. To address this deficit, I propose the generation of a tamoxifen-inducible ribosomal stress mouse model. Nucleolar stress has been successfully modeled using a similar approach. The Schutz laboratory has taken a transgenic mouse containing floxed TIF-1a alleles and crossed it with tamoxifen inducible cre-recombinase mice under the control of several different neuron specific promoters [122, 214]. This approach could be adapted for ribosomal stress by substituting a single ribosomal protein for TIF-1a. The Thomas laboratory has generated a transgenic mouse with floxed RPS6 alleles [64]. This mouse could be crossed with CaMKCreERT2 mice to generate an inducible neuron-specific ribosomal-stress-associated transgenic mouse model.

Such a transgenic mouse model could be used to address a number of concerns. First, it could be used as another mechanism to validate the claim of ribosomal stress induced neuronal apoptosis. Second, it is important to determine if my results translate to *in vivo* conditions. Such a deficit greatly limits the impact of my findings. Lastly, this model could also be utilized to test any potential pharmacological interventions.

Determine in greater depth the mechanisms of RPL11-mediated neuronal apoptosis

Many questions remain unanswered regarding the mechanisms behind RPL11-mediated neuronal apoptosis. The next logical assessment would be to determine if neuronal ribosomal stress induced apoptosis is p53-mediated. This outcome is highly expected, as neuronal nucleolar stress is p53-mediated as is ribosomal stress in cell lines, but still requires verification.

If neuronal ribosomal stress is indeed p53-mediated, the next step would be to determine if the response requires MDM2. The involvement of MDM2 in neuronal nucleolar/ribosomal stress can be assessed in several ways, both *in vivo* and *in vitro*. First, if RPL11's effects are indeed MDM2-mediated, 5-FU and CPT would be expected to induce RPL11-MDM2 protein-protein interactions which could be verified by co-immunoprecipitation.

Second, the protein Proline Rich AKT Substrate (PRAS40) is a negative regulator of 5S RNP-MDM2 interactions and as such, its overexpression would be expected to decrease 5-FU induced apoptosis [87]. Third, the effects could be verified *in vivo* with the MDM2-C305F transgenic mouse line. The C305F mutation is located at the first amino acid in the zinc finger just outside the central acid region and disrupts the binding of both RPL11 and RPL5 [114, 142]. Interestingly these mice retain the p53-mediated response to DNA damage but not Act-D-induced nucleolar stress. Therefore, if the neuronal ribosomal stress response is RPL11-MDM2 mediated it should be inactivated in these mice.

Third, another important consideration will be to determine if RPL11 is functioning independently or in conjunction with other ribosomal proteins. RPL11 and

RPL5 are known to co-localize with the 5S rRNA in the 5S ribonucleoprotein particle (5S RNP), a subcomponent of the large ribosomal subunit. Despite the fact that at least 14 ribosomal proteins possess the ability to bind MDM2, the 5S RNP appears to be the major stress effector in other cell types as several papers have demonstrated that its three components are essential for p53 induction [25, 49, 64]. Thus, it is tempting to speculate that the entire 5S RNP may potentially be the effector molecule in neurons. Under such conditions knockdown of RPL5 or inhibition of RNA polymerase III would be expected to phenocopy the effect of RPL11 knockdown.

Lastly, if RPL11's effects are independent of the 5S RNP, MDM2 or p53 several other mechanisms are potentially possible. For instance, RPL11 has been shown to bind the promoters of p53 target genes and MDMX [154]. Thus, RPL11 can still potentially modulate p53 in the absence of MDM2 interaction. Additionally, while RPL11 does not directly bind p53, it has been reported to bind TAp73, suggesting this could be a potential alternative mechanism [266]. Moreover, RPL11 is also known to interact with NEDD8 [154]. NEDD8 presents an especially interesting possibility as it has been linked to non-p53-mediated apoptosis, p53-mediated senescence, and pre-rRNA processing [13, 248]. RPL11 is also known to have interactions with MYBBP1A and the tumor suppressor Alternate Reading Frame (ARF), although in both cases it's believed to be assembled in the 5S RNP [128, 225]. Therefore, there are a number of potential mechanisms if the canonical pathway is not active in neurons.

Investigate the role of other ribosomal proteins in neuronal nucleolar and ribosomal stress.

It will be important for future studies to address the potential role of other ribosomal proteins in response to nucleolar and ribosomal stress. Ribosomal proteins other than RPL5 and RPL11 present an interesting scenario. On one hand, the translation of ribosomal proteins is believed to exceed the production of other ribosomal components, and as such excess ribosomal proteins are rapidly degraded by the proteasome [131]. Moreover, studies on nucleolar stress have reiterated these early findings and suggest that RPL5 and RPL11 escape degradation by dimerizing to one another [25]. Thus, this has led some to question the physiological relevance of free ribosomal proteins. In opposition to this hypothesis, numerous extra-ribosomal functions of ribosomal proteins have been described in the literature [251]. Thus, the numerous reports of extra-ribosomal functions strongly suggest that some quantity of free ribosomal proteins must escape degradation. Additionally, 12 other ribosomal proteins in addition to RPL5 and RPL11 have been documented to bind and inhibit MDM2 [114]. Moreover, these 12 ribosomal proteins do not all bind in the same location on MDM2. Such specificity suggests that they cannot be disregarded as simple acid-base interactions. Intriguingly, overexpression of three of these ribosomal proteins, RPL37, RPS15 and RPS20, produced differential effects on the expression of several p53 target genes [40]. Given such results it would be tempting to explore the combinatorial effect of certain ribosomal proteins. Thus, more work is needed to determine the legitimacy of other ribosomal protein-mediated mechanisms.

Lastly, 11 ribosomal proteins have been linked to peripheral nerve sheath tumor formation in zebra fish. As a majority of tumor suppressor genes are recessive lethal, the authors screened several hundred lines of zebrafish which were heterozygous for a

recessive embryonic lethal mutations [4]. Interestingly of the 12 total hits, they identified 11 which were ribosomal proteins. Importantly, only two of these ribosomal proteins identified in this screen are known binders of MDM2. This suggests that the other 9 ribosomal proteins may produce their effects by an alternative mechanism.

Determine the role of nucleolar- and ribosomal-stress in CIPN and Chemobrain.

After determining the basic mechanisms of neuronal ribosomal stress in rat primary cortical neurons, and then validating these mechanisms in an in vivo model, the next logical step would be to determine the contribution of ribosomal stress to disease relevant models. In chapter I, I summarized the evidence which suggests nucleolar stress, via direct inhibition of RNA Polymerase I, or ribosomal stress, via inhibition of 47S pre-rRNA processing, are potential mechanisms for both CIPN and Chemobrain.

Admittedly, the evidence for such a condition is disjointed. There is a significant amount of evidence demonstrating that numerous chemotherapeutics negatively alter nucleolar function. There is also a significant amount of evidence suggesting that robust nucleolar output is necessary for such fundamental processes as neuronal growth, maintenance and recovery after injury [72, 115, 116, 226, 231]. It is also curious, that in the case of CIPN the largest neurons appear to be disproportionally affected as this population requires the greatest rates of ribosomal biogenesis. Therefore, I propose that many common chemotherapeutics induce CIPN and chemobrain by induction of nucleolar stress and/or ribosomal stress.

Such a hypothesis could be tested in several ways. First, wild type rodents could be given chronic administration of pharmacologically relevant doses of several

chemotherapeutics associated with CIPN and/or chemobrain. The presence of canonical nucleolar and/or ribosomal stress markers could then be quantified at various time points throughout the course of administration. Moreover, the induction of these stress markers could then be correlated against the onset and severity of symptoms, including those associated with learning and memory deficits, behavior, electrophysiology, and histological changes. Second, for proof of principle, cre-lox technology could be used to generate inducible sensory neuron specific RPS6 knock out mice. Such mice could then be tested for sensory deficits which phenocopy human and rodent CIPN. Lastly, the Zhang laboratory has generated a transgenic mouse containing a mutation in MDM2 which prevents its interaction with ribosomal proteins. If my hypothesis is correct, such a mouse should have significantly reduced symptoms or possibly even be immune to CIPN. However, it is important to consider that while these mice would be expected to have a blunted stress response, they would still have decreased ribosomal biogenesis. It is currently unclear how much each component (i.e. stress response vs ribosomal deficits) contributes to the overall phenotype.

Assess the role of ribosomal stress in Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD).

The results generated by my co-workers and I suggest that the impairment of pre-rRNA processing is an inducer of ribosomal stress, ribosomal stress is an inducer of apoptosis in primary neurons, and that RPL11 mediates this apoptotic response. While many neurodegenerative diseases have evidence for nucleolar abnormalities, in some cases it is unclear if such a phenotype is a cause or alternatively, an effect of neurodegeneration. However in the case of C9ORF72 hexanucleotide expansion-linked

ALS and FTD, there are numerous reports suggesting that nucleolar and ribosomal stress occur early in the disease process. Thus, C9ORF72-linked ALS and FTD appear to be the best option for future studies centered on neurodegenerative disease.

Hexanucleotide repeat expansions in the C9ORF72 gene are the most frequent cause of both ALS and FTD [246]. Recent reports describe a mechanism whereby RNAs originating from this repeat expansion region are transcribed by RAN translation generating several types of dipeptide repeats (DPRs) [252]. The two arginine rich DPRs are believed to mimic the binding properties of many RNA processing proteins and have been proposed to irreversibly bind RNA processing bodies [129, 252]. This concept is supported by evidence demonstrating impairment of pre-rRNA process and mRNA splicing [129, 210]. I speculate that 5-FU induced impairment of pre-rRNA processing and DPR-induced impairment pre-rRNA processing converge at the same endpoint, stimulation of the ribosomal stress response. Thus, as shRPL11 produced a complete rescue of neuronal survival in response to 5-FU, RPL11 may also be a significant mediator of C9 pathology. Interestingly, others have proposed that C9 RNAs inhibit RNA Polymerase I through inhibition of nucleolin. Thus, it appears that C9 may inhibit ribosomal biogenesis at multiple points.

First, I propose to better characterize ribosomal stress in C9. Despite several studies depicting rRNA processing deficits in C9, there are no reports legitimately characterizing impairments of ribosome synthesis or activation of the ribosomal stress response in post mortem tissue, transgenic animals, or *in vitro* models. Moreover, the data demonstrating mRNA splicing deficits is considerably stronger than those depicting impaired pre-rRNA processing [129, 210]. Thus, it will be important to determine if post

mortem tissue , animal models, or cell culture models contain common markers of aberrant ribosomal biogenesis such as increased p53, decreased ribosome content, decreased protein synthesis and decreased mTOR signaling, RP-MDM2 interaction, etc. [214]. Moreover, a northern blot based analysis of pre-rRNA processing using RNA from a C9 animal model or possibly an iPSC derived neuronal culture is necessary to truly substantiate the claims of impaired pre-rRNA processing.

If indeed C9 is found to involve dysfunctional ribosomal biogenesis and induction of ribosomal stress, I propose crossing C9 transgenic mice with the MDM2-C305F mouse line. As I have demonstrated that RPL11 is a mediator of ribosomal stress, the inability of RPL11 to bind MDM2 would be expected to significantly reduce a large portion of C9 related pathology. However, the one caveat to this approach is that to date, MDM2's involvement in the neuron specific ribosomal stress response has not been verified.

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