New roles for the nucleolus in health and disease

Lorena Núñez Villacís[1], Mei S. Wong[1, 2, 3], Laura L. Ferguson[1], Nadine Hein[1], *Amee J. George[1,4,5] and *Katherine M Hannan[1,6].

[1]ACRF Department of Cancer Biology and Therapeutics, John Curtin School of Medical Research, The Australian National University, Acton 2601, Australia; [2]Oncogenic Signalling and Growth Control Program, Peter MacCallum Cancer Centre, Melbourne 3000, Australia; [3]Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville 3010, Australia; [4]School of Biomedical Sciences, University of Queensland, St Lucia 4067, Australia; [5]Department of Pathology, The University of Melbourne, Parkville 3010, Australia; [6]Department of Biochemistry, The University of Melbourne, Parkville 3010, Australia

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Corresponding author:

Lorena Núñez Villacís

E-mail: lorena.nunez@anu.edu.au

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ABSTRACT

Over the last decade, our appreciation of the importance of the nucleolus for cellular function has progressed from the ordinary to the extraordinary. We no longer think of the nucleolus as simply the site of ribosome production, or a dynamic subnuclear body noted by pathologists for its changes in size and shape with malignancy. Instead, the nucleolus has emerged as a key controller of many cellular processes that are fundamental to normal cell homeostasis and the target for dysregulation in many human diseases; in some cases, independent of its functions in ribosome biogenesis. These extra-nucleolar or new functions, which we term 'non-canonical' to distinguish them from the more traditional role of the nucleolus in ribosome synthesis, are the focus of this review. In particular, we explore how these non-canonical functions may provide novel insights into human disease and in some cases new targets for therapeutic development.

1. The nucleolus.

The nucleolus is a dynamic, non-membrane bound compartment within the nucleus, and is the hub for ribosomal RNA (rRNA) synthesis and assembly with ribosomal proteins (rproteins) to generate the ribosome subunits[1-6]. The nucleolus is surrounded by the peri-nucleolar heterochromatin (PH) and consists of three distinct structural regions defined by their appearance and the presence of specific components required for ribosome synthesis: i) the fibrillar center (FC); ii) dense fibrillar component (DFC); and iii) granular component (GC) (**Figure 1**)[4,5,7].

Nucleoli form around actively transcribed clusters of the 200-400 rRNA genes, which are typically arranged as tandem-repeats[5], and as a consequence occupy the same three dimensional nuclear space. In non-transformed human cells these genes localize to the short arms of five acrocentric chromosomes (13,14,15,21,22), forming the nucleolar organizer regions (NORs). Nucleoli assemble in the early G1 phase of the cell cycle, first as a number of smaller units, which later coalesce into 1-2 larger functional nucleoli containing multiple NORs. At this stage, the nucleoli are associated with PH derived from DNA close to the NORs. The rate of ribosomal gene (rDNA) transcription does not peak until S phase, and remains elevated in G2 until the nucleoli disassemble at the end of mitosis. Interestingly, a number of nucleolar

proteins remain associated with the rDNA throughout mitosis, eg., upstream binding transcription factor (UBTF, also called UBF). Disassembly in M phase is triggered, at least in part, by cyclin dependent kinases (CDK), specifically CDK1-cyclin B phosphorylation of RNA Polymerase (Pol) I components which shuts down rDNA transcription[2,3,6]. The size and number of nucleoli per cell can vary considerably, for example during differentiation or malignancy. However, broadly speaking, the "larger the nucleoli, the faster the cell divides," which correlates with higher rates of ribosome biogenesis and growth.

1.1 The nucleolus and ribosome biogenesis

The majority of ribosome biogenesis occurs in the nucleolus[2] and requires all three RNA polymerases; Pol I and Pol III generate the rRNA's (28S, 18S, 5.8S, 5S), and Pol II the various rproteins and obligatory processing/modulatory factors (**Figure 2**). To achieve the required coordination, every step is tightly regulated via signaling pathways that respond to growth/proliferation, differentiation and stress cues[2,8-10]. Transcription of rDNA by Pol I generates a 47S rRNA precursor commencing with the regulated formation of a pre-initiation complex (PIC) at the promoter and recruitment of RRN3, toposiomerase IIα and the Pol I complex[11,12,]. PIC formation is regulated at multiple levels, including competitive binding by other proteins, post-translational and/or epigenetic modifications[3,6,13]. While the rate of transcription is dynamically regulated[14-16], it remains controversial as to which step is rate-limiting, traditionally this was considered to be initiation, however recent evidence supports elongation and/or processing of the rRNA precursor[14-16]. In reality, it is likely that all steps require modulation in unison to achieve the dynamic range of rRNA synthesis observed in growing cells. Consistent with this, master regulators have evolved such as the oncoprotein and transcription factor MYC which can directly and indirectly regulate all three Pol's (**Figure 2)**, thus ensuring coordination of all components required for ribosome biogenesis[9,10].

The rRNA also requires extensive processing, which is only beginning to be understood in eukaryotes[17-19], followed by a series of conformational changes to allow rproteins to cooperatively bind. Factors, such as snoRNPs and assembly complexes, facilitate the assembly and maturation of the pre-40S and pre-60S subunits, before

export to the cytoplasm for the final maturation steps to a functional ribosome [18,20-22]. Intriguingly, domains that are essential for ribosome function, such as the peptidyl-transferase domain, tend to form during the latter stages of assembly, perhaps as a means to prevent any translation from immature ribosomes[1,22,23].

1.2 Insight from the nucleolar proteome

In 2002, seminal nucleolar proteomic studies were published by Andersen[24] and Scherl[25] and collaborators, which provided strong, but somewhat unexpected evidence that the nucleolus was not just the site of ribosome biogenesis, but also a reservoir of 'non-ribosomal' proteins. Andersen identified 271 proteins, of which 11 relocated to the nucleolus when all three Pol's were inhibited by the transcription inhibitor Actinomycin D[24], while the Scherl study[25] identified 213 proteins. When combined, these studies identified a total of ~350 nucleolar proteins, although due to assay limitations, some of the 'expected' proteins were missing (e.g. RRN3). Recent studies using technically improved mass spectrometry have expanded this list to over 4500 proteins which resulted in the curation of a Nucleolar proteome database[26] and the Human Protein Atlas (www.proteinatlas.org).

Perhaps even more surprising than the sheer number of nucleolar proteins identified, was the diversity of their functions. Indeed over 30% had seemingly no connection to ribosome biogenesis. Scherl[25] and Andersen's[24] initial work identified six major ontological categories (chromatin structure, mRNA metabolism, translation, chaperones, fibrous proteins, others) which has now been expanded to include control of cell cycle and proliferation, cell death, telomere metabolism, RNA post-translational modification, energy production, and DNA replication, recombination or repair[26-28]. The identification of these "non-ribosome biogenesis associated proteins" opens up a number of possibilities, perhaps they have a nucleolar function, localize there to be modified or are sequestered and released as a means of regulation. There is emerging literature supporting such possibilities. For example, the poly(A)-specific ribonuclease PARN and non-canonical poly(A) RNA polymerase PAPD5 localize to the nucleolus, while their accepted role is to process mRNAs in the nucleoplasm. However, recent studies demonstrated that nucleolar PARN and PAPD5 can mediate

post-translational modification and maturation of small nucleolar RNAs (snoRNAs) which in turn modulate rRNA, tRNA and perhaps mRNA[29,30].

The first reported, and now accepted, non-canonical role for the nucleolus is its ability to act as a "stress sensor"[7,31,32]. That is the nucleolus responds to a range of stresses by facilitating the accumulation of the tumor suppressor protein p53. This has been termed the 'nucleolar stress response' (NSR), but a more accurate description might be the 'nucleolar surveillance pathway' (NSP), since the pathway seems to function constitutively as a cellular rheostat to monitor ribosome production and fidelity, and to titrate p53 levels accordingly. With this change in thinking, it is possible to argue that NSP is an evolved canonical function of the nucleolus, linked to ribosome synthesis. However for this review we will discuss it as a non-canonical function of the nucleolus to distinguish it from the core functions of the nucleolus, the synthesise and assembly of the 40S and 60S ribosomal subunits.

2. The non-canonical roles of the nucleolus

2.1 The nucleolus as a site of protein sequestration and release

Studies have reported that proteins flux in and out of the nucleolus in response to various stressors (e.g. UV irradiation, hypoxia, serum starvation) or drug treatments (e.g. Etoposide, Actinomycin D)[7,31-33]. In general, these studies reported that acute stress mediates a net efflux of proteins, irrespective of their function, and proposed that protein sequestration in the nucleolus is largely constitutive (passive), whereas their release was dynamic (regulated). There are however some notable exceptions, including RelA (p65 subunit of NF-kB[34]) and the promyelocytic leukemia tumour suppressor (PML)[35], which are actively sequestered to the nucleolus during stress and mediate apoptosis. Another example is the heat shock protein 70 which enters the nucleolus in response to stress (including viral infection), perhaps to facilitate restoration of nucleolar function[36]. Other proteins such as nucleolin (NCL), and nucleophosmin (NPM1) are described as scaffolds, anchors or "nucleolar hubs" that facilitate nucleolar localization of proteins[37]. For example, NPM1 interacts and shuttles proteins between cellular compartments, but can also bind nucleic acids with a preference for G-quadruplex sequences, common to the rDNA. Thus NPM1 has a dual role in the nucleolus, as a protein chaperone and an anchor[37]. Other proteins,

such as nucleostemin (NS) interact with numerous proteins, using a nucleolar localization sequence to target them to the nucleolus[38]. Interestingly, nucleolar protein localization can also be regulated by environmental changes, for example, altered cellular pH mediates stabilization of the von Hippel-Lindau protein, mediating nucleolar accumulation[28].

Nucleolar protein sequestration and release provides a convenient mechanism to modulate cellular processes. For example, by retaining critical cell cycle proteins (e.g. cdc14, MDM2) in the nucleolus, the cell has a means of both temporally and spatially regulating the rate of cell division and coupling it to growth through the role of the nucleolus in ribosome synthesis and assembly[39,40]. Similarly nucleolar retention of DNA repair proteins (e.g. DDB1, PARP1, PNKP, XRCC1), would facilitate simultaneous release into the nucleoplasm, and thus a coordinated response to stress stimuli[7]. The non-canonical apurinic/apyrimidinic endonuclease 1 (APE1), a major cellular scavenger of damaged DNA, was also identified in the nucleolar proteome. APE1 not only interacts with NPM1 and nucleolar rDNA[41], but functions within the nucleolus to provide quality control of the highly transcribed rRNA, which is prone to error[28]. Thus, APE1 may be a conduit between the DNA repair processes and RNA metabolism.

Proteins associated with other DNA repair mechanisms (e.g. homologous recombination (HR): RAD50 & 51, WRN, XRCC5, DNA-PK; non-homologous end joining (NHEJ): PARP1, PNKP, XRCC1) also localise within the nucleolus[7]. Interestingly, the mechanism by which the rDNA is repaired is still controversial with publications implicating both HR[42] and NHEJ[43], though the data of McStay and colleagues on HR associated repair appears particularly compelling. Clearly, a significant body of work is required to understand the varied mechanisms and extent to which localisation/release of nucleolar proteins controls cellular homeostasis. What is clear however is that insults to the cell, that perturb the normal functions of the nucleolus, would have profound consequences for any sequestration/release processes. We speculate this is likely to contribute to the therapeutic effect of targeted Pol I inhibitors in the treatment of cancer (described below).

2.2. The p53-dependent nucleolar surveillance pathway (p53NSP)

The canonical p53NSP, describes a signalling pathway in which p53 is activated in response to a broad range of cellular stresses[44]. Specifically, these stresses perturb ribosome biogenesis and function, resulting in p53-dependent cell cycle arrest, and in extreme cases, apoptotic cell death. Intrinsic stresses that induce NSP include, mutations in rproteins or other components of the ribosome biogenesis pathway (eg: ribosomopathy diseases[31,45]), mutations in components of Pol II, DNA damage, extreme environmental conditions, hyperproliferative signals, oxidative stress, nutrient deprivation, hypoxia, osmotic stress, viral infections, energy deprivation, and oncogenic stress[46]. As described above, rather than being an acute response to stress, it is more likely that the p53NSP functions constitutively in proliferating cells as a balancing mechanism matching cellular proliferative capacity "fitness" with the rate of ribosome biogenesis.

Specifically the p53NSP mediates the accumulation of excess free rproteins, which are not required for ribosome synthesis. In particular the RPL5/L11/5S ribonucleolar protein (5SRNP) complex which is no longer being incorporated into the ribosome binds and sequesters the p53 E3 ubiquitin ligase MDM2, resulting in accumulation of p53[13,31]. Alternatively during conditions of high demand for ribosome biogenesis, the 5SRNP is efficiently incorporated into newly assembled ribosomes and thus MDM2 is free to target p53 for degradation, thereby reducing p53 protein abundance (**Figure 3**). This model was perhaps best described by Rubbi and Milner[44] who proposed that "the nucleolus as a stress sensor is analogous to the 'dead man's foot' safety system: a p53 response will occur unless the nucleolus is constantly capable of promoting its degradation".

Interestingly, a number of other rproteins have been postulated to mediate the release of MDM2 from p53, however it is now more widely accepted that the 5SRNP complex is the predominant culprit[47,48,49,50]. Our unbiased functional RNAi screen supports this observation as depletion of RPL5 or RPL11, but none of the other tested rproteins, prevented p53 stabilization (unpublished observations). It is clear that other rproteins mediate effects on p53, but these are likely to be independent of the interaction with MDM2. For example, RPL26 binds the 5' untranslated region of p53 mRNA and promotes its translation[51], where as RPS26 binds and directly influences

p53 transcriptional activity[52]. The relative importance, of these mechanisms modulating p53 abundance and activity remains unclear.

2.2.1 Other p53-dependent, but rprotein-independent, regulators of NSP

Recent studies revealed that p53NSP is more convoluted than previously thought, with proteins not part of this complex identified as interactors with, and impacting on, MDM2 mediated degradation of p53 also in response to nucleolar stress. For example, Xie and colleagues[53] demonstrated that stress caused translocation of the nucleolar protein cellular senescence-inhibited gene (CSIG) to the nucleoplasm, which bound MDM2 and inhibited its function, thus p53 accumulated. Intriguingly, CSIG is structurally similar to rproteins harbouring an N-terminal ribosomal L1 domain which mediates its binding to MDM2[53]. Other nucleolar proteins, including ARF, NS and NPM1, also use a similar mechanism of modulating p53 abundance[31,53,54]. As an additional layer of complexity, when perturbed, nucleolar proteins such as the rRNA processing protein RRP15, which is required for pre-40S/SSU and pre-60S/LSU ribosomal subunit formation and rDNA transcription, can promote 5SRNP excess which then outcompetes p53 for its interaction with MDM2[55].

Interestingly CSIG and ARF also mediate a p53–independent NSP[31]. Specifically, CSIG in the absence of p53 activates the ATR-Chk1-γH2AX DNA replication/damage checkpoint, mediating a delay in S-G2 which culminates in cell death. ARF, rather than binding MDM2, interacts and antagonizes MYC and E2F, mediating cell cycle arrest. In fact, there is now a growing list of proteins that so far have only been associated with a p53-independent NSP which are discussed below.

2.3 p53-independent mechanisms of sensing nucleolar stress

Depletion of the largest subunit of Pol I in p53-silenced cells inhibits rDNA transcription and initiates the NSP, mediating a G1/S phase cell cycle arrest. This occurs via phosphorylation of retinoblastoma protein and reduced expression of E2F1-regulated genes, especially those critical for S phase[56]. Intriguingly, even in the absence of p53, the binding of "free" RPL11 (i.e not incorporated in a ribosomal subunit) to MDM2 is still able to mediate cell cycle arrest but via proteosomal degradation of E2F1[56]. Induction of the NSP by the nucleic acid synthesis inhibitor 5-

Fluorouracil (5FU) leads to G1 cell cycle arrest and apoptosis, and this process was mediated by RPL3-directed elevation of p21 expression. Specifically ERK phosphorylation enhanced RPL3 and Sp1 binding to the p21 promoter increasing transcription at the expense of Sp1-dependent cystathione-beta-synthase (CBS) transcription and free RPL3 triggered translocation of CBS to the mitochondria inducing apoptosis[57].

Not surprisingly, the characterisation of the nucleolar proteome has led to the identification of a collection of novel NSP mediators. Peter Pan (PPAN) is one such protein; while identified in the nucleolus, its canonical function is as a responder to Wnt signaling with an anti-apoptotic role[58]. In the nucleolus PPAN promotes rRNA maturation after it has been incorporated in the pre-60S. PPAN is presumably retained in the nucleolus via its binding to UBF or NPM1, and in response to stress it exits and is cleaved by caspases. While this pathway does not require p53, altered ribosome biogenesis by PPAN does correlate with accumulation of p53[31].

Another is the chaperone and guanine nucleotide exchange factor SmgGDS1 (or RAP1GDS1), which when cytoplasmic binds GTPases and promotes plasma membrane trafficking, alternatively in the nucleolus it is sequestered by UBF binding. The role of nucleolar SmgGDS1 is unclear although its depletion leads to reduced expression of ~600 genes, most of which are associated with G1 cell cycle arrest or nucleolar disruption, and are transcribed by the dimerization partner, Rb-like, E2F and multi-vuval class B (DREAM) transcription complex[59]. SmgGDS1 is overexpressed in cancers, thus may suppress unwanted NSP activation and promote malignancy[59]. In summary, the list of nucleolar proteins that are regulated by or are components of the p53-dependent and -independent NSP is ever growing. The challenge will be to identify under what conditions they are biologically important, and if they represent new mechanisms or targets that might be useful to treat diseases associated with nucleolar dysfunction.

2.4. The nucleolus and genomic stability

It has been suggested that the nucleolus can impact on genomic stability in a number of ways; as the rDNA is highly repetitive it is prone to HR, this would negatively

impact on genome stability. The PH around the nucleolus is tightly packed, and as such, may provide a barrier to prevent HR occurring between the rDNA repeats. Not surprisingly then, disruption of the PH has been associated with genomic instability, premature aging and age-related neurodegenerative diseases[7,60]. The PH also assists in the preservation of nucleolar architecture; the silencing of the rDNA via epigenetic mediated repressive marks and the organization of nucleosome structures. As an extension to this, since rDNA is located on five separate chromosomes, it is also possible that the PH mediates interactions, both short and long range, between specific genes thus may regulate their accessibility and transcription. This is based on recent publications utilising circular chromosomal conformation capture (3 or 4C) techniques[7,60]. While we have accepted that the nucleolus is a hub for sensing stress, we are only just beginning to understand how it might also be a hub for genome maintenance, and Pol II /III gene regulation.

3. The nucleolus and disease

For over a century, pathologists have used changes in nucleolar morphology and number as a marker of disease. Abnormal nucleoli and increased rDNA transcription are accepted as common pathological features of many cancers[8], and targeting rDNA transcription can be therapeutically used to treat cancer[6,32,61]. In addition, mutations in ribosomal components mediating reduced ribosome biogenesis and/or abnormal nucleoli have been attributed to the genetic diseases known collectively as ribosomopathies. While the involvement of classical ribosome biogenesis components have been assessed with respect to their impact on these disease, new roles for the nucleolus and the nucleolar proteomic studies may provide novel druggable targets and therapies.

3.1 Ribosomopathies

Ribosomopathies are diseases characterized broadly by the presence of mutations or deletions of genes related to ribosome biogenesis; i) genes encoding rproteins for Diamond Blackfan Anaemia (DBA) and 5q minus (5q-) syndrome; ii) genes associated with the synthesis or modification of the rRNA, for Treachers-Collins Syndrome (TCS), Dyskeratosis congenita (DC) and Cartilage hair hypoplasia (CHH);

iii) genes encoding proteins (not rproteins) associated with the ribosome, for Shwachman-Diamond syndrome (SDS). Each of these diseases have similar and varying phenotypes which are summarized in **Table 1**[62-64].

While intriguing in nature, one interesting observation is that many of the ribosomopathy phenotypes tend to affect specific tissues rather than all. This could be due to either quantitative (translation capacity) and/or qualitative (targeted translation by specialized ribosomes) differences or dependent on the tissue[62-64].

For example loss of one rprotein gene (haploinsufficency) allele may reduce its expression below a critical level for maintaining ribosome biogenesis in one cell type but not in another, thus leading to heterogeneous phenotypes and pathologies. That being said, this group of rare diseases represent a huge unmet need in the clinic, as in almost all cases treatments are palliative rather than curative. Importantly until we understand the mechanism(s) of action underlying these diseases, this is unlikely to change.

DBA: DBA is associated with mutations in rproteins, however only 17 of the ~80 possible have been identified, and in 25% of cases the mutations were in RPS19. This might be explained by the loss of certain rproteins only mildly perturbing ribosome biogenesis, not enough to activate p53 or, the phenotype is too severe for survival. While the classical p53NSP has been linked to the phenotype of DBA, so have p53-independent mechanism(s)[63-65].

Intriguingly, mutations in two non-rproteins also mediate a DBA-like phenotype, the putative RPS26 chaperone TSR2 and the haematopoetic transcription factor GATA-1[66,67]. Mutations in *TSR2*, reduced RPS26 transport to the nucleolus, thus phenocopying loss-of-function RPS26 mutations identified in DBA patients[68]. Mutations in *GATA-1* reduced synthesis of the long form of GATA-1, a critical transcription factor in erythroid differentiation, thus altering the translational landscape and mediating a DBA phenotype. Interestingly, when the transcription profile of rprotein- verse GATA1-mediated DBA were compared there were overlaps and critical differences at the mRNA level[69]. OBrien[69] proposed a model by which GATA-1 mutations reduced the expression of proteins required for ribosome synthesis, placing it upstream of rproteins, yet the end result for both is reduced

translation. There are also examples where rprotein mutations reduce the translation of GATA-1 in DBA[68]. Together, these examples support the hypothesis that dysregulation of ribosome biogenesis and translation drives DBA pathology.

5q-syndrome: 5q-syndrome (a bone marrow disorder also called myelodysplastic syndrome) is caused by a deletion in the long arm of chromosome 5[65] an area that includes ~40 genes. This inclused three rproteins (RPS14, RPL7, RPLP1), two microRNAs (miR-145, miR146a)[65,70] and other genes which mediate varying degrees of disease presentation (*CSNK1A1*[71], *SPARC, CTNNA1*[7,2] *EGR1*[73]). Thus far, research has focussed on the *RPS14* deletion in human CD34+ cells which was sufficient to recapitulate the 5q-erythroid defect and reversed by deleting p53[74]. Interestingly, a recent study suggestes this process is more complicated, describing an early p53-independent erythroid defect followed by a p53-dependent defect[75]. To further complicate things, loss of the most recent 5q-associated gene, *LARP1*, which is known to stabilize 5'TOP mRNAs, reduced ribosome biogenesis and stabilized p53[76,77].

SDS: SDS is an autosomal recessive disease with 90% of cases arising from mutations in the Shwachman-Bodian Diamond syndrome (*SBDS*) gene[78]. While SBDS has been implicated in a cellular stress response[78,79] there is still confusion over the role of p53 in SDS[78,80,81]. Current studies demonstrate that SBDS is a cofactor for the elongation factor-like GTPase 1 (EFL1), which facilitates removal of eukaryotic initiation factor 6 (eIF6) from the 60S during the final stage of maturation[82]. Not surprisingly, recently *EFL1* mutations mediated clinical symptoms similar to those observed for SDS[83]. Mutations in *DNAJC21,* which is also required for the maturation of 60S and can bind rRNA, have also been identified in SDS patients[84,85].

TCS: Intriguingly, mutations in genes associated with rRNA synthesis cause TCS rather than DBA or 5q-syndrome. TCS is an autosomal dominant disorder mediated by *TCOF1* or *POLR1D* mutations, or recessive if the mutation is in *POLR1C*[86-88]. *TCOF1* encodes the nucleolar phosphoprotein Treacle, which regulates rDNA transcription and pre-RNA methylation[88-90], whereas *POLR1D* and *POLR1C* encode subunits of Pol I and III respectively. Currently, at least for *TCOF1* mutations,

haploinsufficiency reduces rRNA synthesis and ribosome biogenesis/capacity thus induces p53NSP and apoptosis, predominantly of neural crest cells during embryonic development[88-90].

DC: DC at a glance might be considered more akin to diseases associated with dysregulated telomerase function rather than ribosomopathies. To date, *DKC1* (20-25% of sporadic cases), *TERT* and *TERC* mutations occur in ~64% of cases, with numerous other gene mutations identified in the rest[91-95]. Collectively, these mutations mediate telomere shortening and DNA damage, resulting in p53 stabilization and cell death[94]. However, some of these genes have other functions which links them to ribosomopathies. For example, *DKC1* encodes the protein dyskerin which associates with numerous H/ACA small RNAs to form RNP complexes, these pseudouridylate small nucleolar (sno) RNAs and rRNAs[95], thus when DKC1 is mutated translation is impaired[96], although some of the specifics are still controversial. Alternatively mutations in *TERT* or *TERC* alter telomere function and stability. Telomerase reverse transcriptase, along with telomerase RNA component forms an RNP, known as telomerase. Telomerase, like the H/ACA snoRNPs, also contains dyskerin, however in this case dyskerin promotes complex stability rather than pseudouridylation. Thus, all three genes are linked so perhaps there are subtypes of DC, some of which are ribosomopathies and others not.

CHH: CHH remains the sole ribosomopathy not linked to p53. It is caused by mutations in *RMRP*, which encodes a long non-coding RNA (liRMPR) that is a component of the mitochondrial RNA processing complex (RNase MRP enzyme). Nucleolar RNAse MRP enzyme cleaves the pre-rRNA and process the 5.8S rRNA[97,98]. When *RMRP* is mutated, liRMPR becomes unstable and is unable to bind the RNAse MRP complex thus impairing processing. The liRMPR is also then cleaved into two siRNA's (RMRP-S1 and S2) that have the potential to silence genes. Any of these liRMPR functions could contribute to the clinical symptoms of CHH[99]. An earlier study suggested liRMPR interacts with TERT to form a RNA-dependent RNA polymerase that produced double stranded RNAs, which were processed to siRNAs in a DICER-dependent manner, again capable of silencing genes[100]. Clearly

there is considerable work required to understand the pathology of CHH and the contribution of rRNA defects to the pathology of the disease.

Others: Other putative ribosomopathies have been identified, such as Bowen-Conradi syndrome and North American Indian childhood cirrhosis syndrome (NAIC), which are extremely rare, and as a result, there is limited information about the mechanism underlying these diseases. Interestingly, both are mediated, at least in part, by mutations in genes critical for 18S rRNA processing. Bowen-Conradi syndrome is mediated by mutations in the gene essential for mitotic growth 1 (*EMG1*), a pseudouridine methyltransferase, and NAIC is caused by mutations in the *CIRH1A* (*hUTP4/Cirhin)*[101] gene which both encode factors that are components of the ribosomal small subunit (SSU) processome[102]. Undoubtedly this is not a completed list of ribosomopathies and as sequencing capabilities improve additional ribosomopathies associate with novel ribosomal gene related mutations will be identify.

3.2 Cancer

There is a long history and a well-accepted correlation between hyperactivated ribosome biogenesis, abnormal nucleoli and cancer. However, the exact mechanism by which elevated ribosome biogenesis contributes to cancer is less clear. Highly proliferative cancer cells require elevated translational capacity, an increased number of ribosomes per cell, which is often driven through oncogenes or loss of tumor suppressor pathways and the enhancement of ribosome biogenesis. Interestingly, not all tumour cells are highly proliferative, nor does the efficacy of targeting a nucleolar-function necessarily correlate with proliferation rate (personal observation). One reason why tumor cells may have developed robust mechanisms to drive ribosome biogenesis, and promote nucleolar formation is a means to suppress the NSP and thus repress p53-dependent and -independent mechanisms that block cellular proliferation. Consistent with this, targeting the NSP with small molecule inhibitors that selectively inhibit Pol I transcription and rRNA synthesis have shown promise in the treatment of cancer[6,32]. However, targeting the NSP to treat tumors is perhaps not as novel as we thought, retrospective studies reported that the mechanism of action for numerous

chemotherapeutic drugs, at least in part, target the nucleolus, inhibit ribosome biogenesis, and mediate cell death via activation of p53, i.e. they activate the NSP[103]. While such drugs typically disrupt the nucleolar structure[103], their other mechanism of action often cause unfavourable toxicities to normal cells. In contrast selective small molecule inhibitors of Pol I transcription (eg., CX-5461) are a new class of drug mediating NSP activation of p53 in the absence of global DNA damage[61,104]. In particular CX-5461 has opened up a new avenue for cancer therapy and spurred an intensive effort to develop new drugs that cause nucleolar stress without generic DNA damage; including an acridine derivative known as CID-765471[105], BMH-21[106] and Inauzhin[107]. All three drugs activate the NSP in the absence of global DNA damage; while CID-765471 and BMH-21 do so by selectively degrading the RPA194 subunit of Pol I and disrupting rDNA transcription, Inauzhin inhibits SIRT1 and inosine monophosphate dehydrogenase 2 (IMPDH2), reducing nucleostemin expression and decreasing rRNA processing[107]. At this stage only CX-5461 is in clinical trial including a phase I trial for hematologic cancers (ACTRN12613001061729-Australia) and a phase I/II for triple negative breast cancer (NCT02719977-Canada).

3.3 Parkinson, Huntington's, Alzheimer's disease and Multiple Sclerosis

Interestingly, as in the case for many cancers, changes in the nucleoli are common in a number of age-related neurological disorders including Parkinson's (PD), Huntington's (HD) and Alzheimer's disease (AD). There is increasing evidence for dysregulated rDNA transcription, nucleolar dysfunction and induction of the p53NSP contributing to these diseases, as well as the normal aging process[108]. The possible contribution of other non-canonical roles for the nucleolus in neurological diseases and aging is unknown, but clearly warrants investigation.

PD: Five genes have been associated with familial PD; α-synuclein (*SNCA*), parkin (*PARK2*), PTEN-induced putative kinase 1 (*PINK1*), *DJ-1* (*PARK7*), and Leucine-rich repeat kinase 2 (*LRRK2)*. Mutations in at least two of these (*PARK7*, *PARK2)* are associated with nucleolar defects and altered rDNA synthesis. *PARK7* mutations cause protein misfolding and is associated with autosomal recessive early-onset PD[109], while mutations in *PARK2,* which encodes an E3 ubiquitin ligase (Parkin), are frequently identified in early-onset familial PD[110]. A number of these disease-

associated genes also encode proteins that can modulate ribosome function, specifically translation[111]. While ribosome biogenesis and function is dysregulated in PD, the details are unclear, thus more research to understand their full impact is required to uncover possible therapeutic targets.

HD: HD is associated with mutations in only one gene, *Huntingtin*, which, when mutated, results in protein misfolding and aggregation. One consequence of cytoplasmic aggregation is the disruption of global protein trafficking, which may negatively impact on the nucleolus, especially its roles in sequestrating proteins. Interestingly, nuclear aggregates of huntingtin interact with NPM1, which may act as a chaperone and shield the aggregate[112]. More directly, with respect to altering nucleolar functions, HD was linked to disruption of CBP-acetylation of UBF and increased methylation by SETDB1, the end result being reduced rDNA transcription[113]. What is less clear, is how causative these impacts on the nucleolus are on HD pathology, especially given the broad range of other consequences nuclear huntingtin aggregates are likely to have.

AD: AD is a progressive chronic disorder characterized by β-amyloid plaques, Tau (Tubulin associated unit) pathology, neuronal cell death, and inflammatory responses. Recent studies have linked AD to dysregulated ribosome biogenesis and nucleolar function. Specifically reduced mRNA, and in some cases also protein expression, of a range of nucleolar chaperones and/or regulators of rDNA transcription (e.g. NCL, NPM1, and UBF) were identified in AD patients[114]. Decreased rDNA transcription was also reported, which correlated with hypermethylation (silencing) of the rDNA promoter, and disease progression[115]. An established marker for AD is aggregation of Tau, which is not only cytoplasmic but also localizes to the NORs, nucleus and nucleolus DFC. Thus Tau may modulate the formation of the nucleolar PH, regulate rDNA transcription and ribosome assembly, and has also been implicated in the NSP[116]. Moreover, reduced nucleolar expression of PARP1 and fibrillarin were reported in the hippocampal cells of AD patients[117], however the link to pathology is yet to be confirmed.

Multiple Sclerosis (MS): Benign MS is a sub-group of non-active MS patients who are protected from long-term disability despite ongoing disease progression. Recently, high throughput microarray analysis identified a signature characterising benign MS[118], which included reduced expression of the Pol I transcription pathway, down regulation of NFkB and upregulated p53-dependent apoptosis. Intriguingly, a second generation derivative of CX-5461 (RAM-589.555) was tested in models of experimental autoimmune encephalomyelitis and mediated selective induction of apoptosis in inflammatory cells. It is hoped that this drug may transform the active MS disease to the preferable benign subtype[119].

4. CONCLUSION

Recent studies have revealed that the central role played by the nucleolus in cellular homeostasis beyond its canonical functions in synthesising ribosomes. It is clear that the nucleolus is no longer a bystander in the regulation of crucial cellular processes but a fully contributing player. These newly discovered functions range from detecting cellular stress through to maintaining genomic stability and even aging. The molecular mechanisms underlying these novel functions are beginning to be teased apart. In particular the discovery of multiple pathways and genes modulating the p53-dependent and -independent NSP are providing new targets and approaches to treat cancers, ribosomopathies and neurodegenerative diseases associated with ribosome dysfunction. Indeed although the nucleolus was the first identified sub-nuclear body, it is only now some 180 years later that are we beginning to unlock the secrets to its plurifunctionality.

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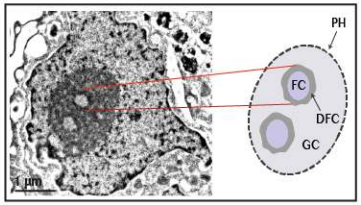
Disease	Description of disease	Clinical Features	Haematologic characteristics	Genetic Lesions	Cancer Risk	Rare Disease	p53 implicated
Diamond-Blackfan anaemia (DBA)	• **bone marrow failure syndrome**	• **craniofacial & thumb abnormalities** • **cleft lip/palate** • pallor • lethargy • short stature • heart defects • urogenital defects	• **normochromic macroytic anaemia** • reticulocytopenia • absence of erythroid precursors • normocellular bone marrow	• rprotein mutations: *RPS19, RPS7, RPS10, RPS17, RPS24, RPS26, RPS27, RPS29, RPS28, RPL5, RPL11, RPL15, RPL26, RPL27, RPL35A* • Other mutations: *GATA-1, TSR2*	High risk of AML & solid carcinomas	7 cases per million live births	Yes
5q minus-syndrome (5q-syndrome)	• independent subtype of myelodysplastic syndrome	• pallor • fatigue	• **macrocytic anaemia** • decreased erythroid progenitors • thrombocytosis • hypolobulated micromegakaryocyte	• deletion of the long arm of chromosome 5 • Genes in this deleted region include *RPS14, RPL7, RPLP1, miR-145, miR-146a, CSNK1A1, SPARC, CTNNA1, EGR1, LARP1*	Risk of progression to AML	1 case per million live births	Yes
Shwachman-Diamond syndrome (SDS)	• autosomal recessive disorder • **bone marrow failure** • exocrine pancreatic dysfunction	• short stature • skeletal defects	• **anaemia** • depression of myeloid lineages • chronic neutropenia • thrombocytopenia	• *SBDS* mutation • *EFL1* and *DNAJC21* mutations	High risk of developing leukaemia	1 case in 20,000 or 200,000 live births	Yes
Treacher-Collins syndrome (TCS)	• autosomal dominant or recessive disorder	• **craniofacial defects** • abnormal brain development • airway, swallowing, hearing issues	• none	• *TCOF1, POLR1D* and *POLR1C* mutations	None reported	1 in 50,000 live births	Yes
Dyskeratosis congenital (DC)	• **bone marrow failure syndrome** • a disorder of telomere dysfunction	• triad diagnostic criteria: -reticular skin pigmentation -nail dystrophy -mucosal leukoplakia • premature aging	• **anaemia**	• *DKC1, TERT* and *TERC*, mutations • *ACD, NHP2, RTEL1, TINF2, NOP10, TCAB1, PARN, WRAP53* mutations	High risk of developing leukaemia, and solid tumours	1 case per million live births	Yes
Cartilage hair hypoplasia (CHH)	• inherited autosomal recessive disorder	• **bone deformaties**, short-limbed dwarfism • fine, sparse, light-colored hair • gastrointestinal malabsorption	• **mild to severe anaemia** • defective cellular immunity affecting T-cell response	• *RMRP* mutation	High risk of non-Hodgkin lymphoma and squamous or basal cell carcinoma	1 case in 23,000 live births	No
North American Indian childhood cirrhosis (NAIC)	• pediatric cirrhosis • liver failure • autosomal dominant	• biliary cirrhosis • portal hypertension	Not determined	• *hUTP4/Cirhin*	Not determined	Very rare, 1 in 250 or 750 live births in Ojibway-Cree First nation children from Northwestern Quebec	Yes
Bowen-Conradi	• **bone marrow**	• developmental defects,	Not determined	• *EMG1*	Not	Very rare,	No

syndrome	**failure syndrome** • autosomal dominant • death in early childhood	growth retardation • psychomotor defects • microcephaly, prominent nose • central nervous system defects			determined	1/355 in Hutterite population only	



Figure

1

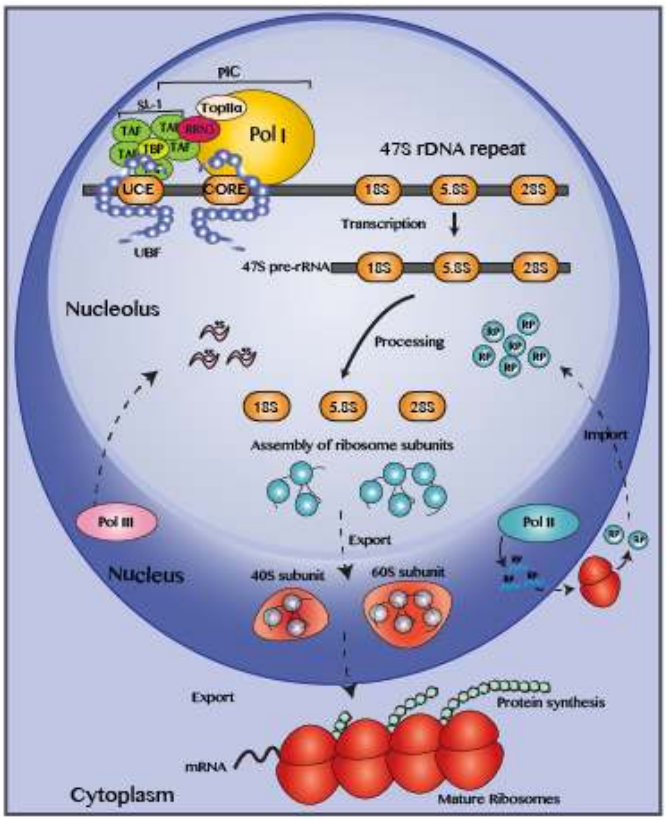


Figure 2

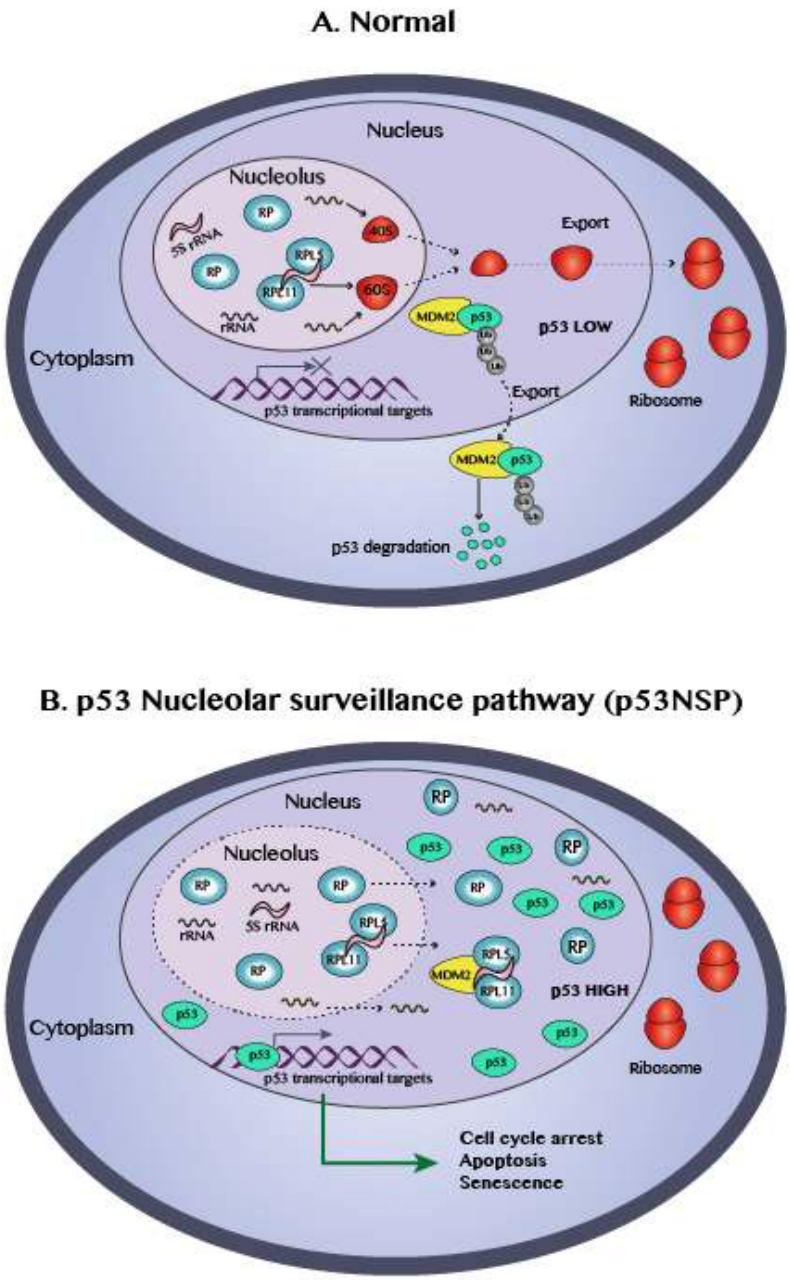


Figure 3