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Review

Targeting the nucleolus as a therapeutic strategy in human disease

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The nucleolus is the site of ribosome biogenesis, one of the most resource-intensive processes in eukaryotic cells. Accordingly, nucleolar morphology and activity are highly responsive to growth signaling and nucleolar insults which are collectively included in the actively evolving concept of nucleolar stress. Importantly, nucleolar alterations are a prominent feature of multiple human pathologies, including cancer and neurodegeneration, as well as being associated with aging. The past decades have seen numerous attempts to isolate compounds targeting different facets of nucleolar activity. We provide an overview of therapeutic opportunities for targeting nucleoli in different pathologies and currently available therapies.

Highlights

Changes in nucleolar activity and/or morphology are often associated with human disease, opening new opportunities to develop novel biomarkers and therapies.

Although cancer cells often present enlarged nucleoli and increased ribosome biogenesis, mutations impairing translation can also lead to tumor development.

An extended lifespan has been associated with smaller nucleoli, and partial reduction in nucleolar activity increases longevity in multiple animal models.

Nucleolar stress-generating agents targeting different steps of ribosome biogenesis are been developed for their use in cancer therapy, although the specificity of currently available drugs is limited.

Nucleolar functions

The nucleolus is the most visible structure within the nucleus, and was first described over two centuries ago [1]. It is a **membraneless organelle** (see Glossary) whose canonical function is the coordination of ribosome biogenesis, a highly regulated process in which rRNA is synthesized, modified, and assembled with ribosomal proteins (r-proteins) to form mature ribosomes (see Figure I in Box 1) [2]. Ribosome biogenesis represents the most metabolically demanding process of the cell [3], which makes it highly sensitive to a variety of cellular stimuli and stressors. As such, the nucleolus is a central hub that integrates both intrinsic and external growth and stress cues, and coordinates cellular responses [4].

Despite their highly organized architecture (Box 1), nucleoli retain liquid-like properties. They are able to split and coalesce, like oil drops on the surface of water, as well as showing open exchange dynamics with the nucleoplasm, a characteristic of liquid-like behavior [5]. The current model of nucleolar formation describes it as a biological condensate that is composed of three immiscible phases compartmentalized by **liquid–liquid phase separation** (extensively reviewed in [5]) (Box 1). These three layers are formed in concert with each step of ribosome biogenesis, and are sustained by a steady-state flux of ribosomal components. Accordingly, proteomics has revealed an enrichment of intrinsically disordered domains, that are often involved in phase transitions, among nucleolar proteins (20%) compared to cytosolic factors (14%) in humans [6].

Its fluid dynamics makes the nucleolus an ideal node for rapidly adapting to changes in cellular homeostasis because these organelles can change in size, number, and protein composition on demand [5]. As such, nucleolar morphology is intrinsically linked to, and is a readout of, nucleolar activity, which includes rRNA transcription and processing, as well as the assembly and output of ribosomes (Box 2). In addition to ribosome biogenesis, nucleoli are also involved in several non-canonical processes such as protein quality control, RNA editing, telomerase assembly, cell cycle progression, viral infection, and DNA replication and repair [7,8]. Consistent with its multiple

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functions, nucleolar alterations have been linked to numerous pathologies such as cancer and neurodegeneration, as well as to overall aging [8–10].

In this article we describe the links between nucleolar activity and human pathologies, and provide a summary of currently available and emergent therapies to target nucleoli and ribosome biogenesis.

Nucleoli and disease

Nucleolar morphology is highly responsive to a wide array of cellular perturbations, including direct insults to the nucleolus, such as the depletion of nucleolar factors or r-proteins [5], as well as more general stressors such as cytotoxic agents, nutrient starvation, heat shock, UV radiation, hypoxia, and viral infection (reviewed in [11]). These morphological alterations are stressor-specific, and can include changes in nucleolar number or size, translocation of nucleolar components to the nucleoplasm and cytosol, and the formation of other structures such as **nucleolar caps** or **beaded necklaces** [5,12]. 'Nucleolar stress' is a loose term used to refer to alterations in nucleolar morphology and function [13], and makes no formal distinction between whether nucleolar alterations are the cause of stress or are a secondary response to stressors.

Initial work suggested that nucleolar stress is primarily signaled by a P53-dependent checkpoint. In this model, nucleolar perturbations lead to the extranucleolar accumulation of r-proteins and/or nucleolar factors, such as NPM1, which in turn stabilize P53, leading to cell cycle arrest and apoptosis [11]. However, *TP53*-deficient cell lines are also susceptible to nucleolar stress, indicating that it must lead to cell death by P53-independent mechanisms which are still poorly understood [14].

As mentioned, in addition to changes triggered by external agents, alterations in nucleolar morphology and function have been associated with various diseases and aging [9,11], as briefly outlined below (Figure 1).

Cancer

Increases in nucleolar size or number are regarded as a clinically relevant marker of poor prognosis in several tumor types [15]. These morphological alterations were classically attributed to an

Box 1. Overview of ribosome biogenesis

The nucleolus contains hundreds of proteins that concentrate around tandem repeats of rDNA at so-called nucleolar organizing regions (NORs) [106]. The number of NOR-bearing chromosomes varies among species, from one in haploid yeast cells to ten in human somatic cells (acrocentric chromosomes 13, 14, 15, 21, and 22) [1]. In the nucleolus of mammalian cells, ribosome biogenesis is initiated with the transcription of rDNA by RNA Pol I into a precursor polycistronic rRNA (47S, pre-rRNA), which is subsequently processed into 28S, 18S, and 5.8S rRNAs. The rRNAs are modified and assembled with ribosomal proteins (r-proteins) and the 5S rRNA, produced by RNA polymerase III, to form 40S and 60S pre-ribosomal subunits [107]. Once assembled, ribosomal complexes are released into the nucleoplasm and transported to the cytoplasm [108] (Figure IA).

Three main events take place within the nucleolus: RNA Pol I-driven transcription, processing and modification of rRNA, and the assembly of rRNA-containing ribonucleoprotein (RNP) complexes, which is reflected in its **tripartite structure** (Figure IC). Hence, nucleoli are organized in three concentric layers or subcompartments: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). The GC is delimited by a ring of dense perinucleolar chromatin (PC). Interrogation of nucleolar structure by confocal microscopy coupled to proteomics has proposed a novel distinct rim structure separating the GC and PC, which is postulated to act as a surfactant [5,6].

FCs are enriched in components involved in RNA Pol I-dependent transcription, such as upstream binding factor 1 (UBF1) and the SL-1 complex which contains the transcription intermediary factor 1α (TIF-1A). It is generally accepted that pre-rRNA transcription occurs either in the FC or at the border between the FC and DFC. The DFC harbors pre-rRNA processing factors such as small nucleolar RNPs (snoRNPs), fibrillarin (FBL1), and nucleolin (NCL1) and, together with the FC, is enclosed by the GC, where pre-ribosome subunit assembly takes place assisted by proteins such as the histone chaperone nucleophosmin (NPM1) (Figure IB) [2,5].

Glossary

Beaded necklaces: severe nucleolar disruption in which the dense fibrillar component (DFC) nucleolar markers resemble 'unfolded beaded necklaces', immersed in an expanded granular component (GC). One of the known inducers of this morphology is caused by the depletion of some ribosomal proteins (r-proteins).
G-quadruplexes: four-stranded helical structures formed by the presence of guanines in close proximity in DNA and RNA.
High-throughput: a method of experimentation that allows large-scale genetic, chemical, or pharmacological testing.
Liquid–liquid phase separation: a physical process in which an initially homogeneous liquid, upon reaching a sufficient concentration of one of the constituents, separates into two distinct phases.
Membraneless organelles: non-membrane-enclosed biomolecular condensates that are usually composed of proteins and nucleic acid, and which form a cellular compartment through liquid–liquid phase interactions.
Nucleolar caps: bipartite structures containing the fibrillar center (FC) and DFC surrounding the components of the GC, and that invert the canonical structure of the nucleolar layers. The formation of nucleolar caps occurs upon inhibition of RNA Pol I.
Nucleolar integrity: preservation of the structural and functional activities of the nucleolus, including its dynamic properties which allow transient changes in nucleolar morphology in response to different stimuli.
Repeat expansion disorders: a group of hereditary diseases, usually affecting the nervous system, that are characterized by the presence of unstable stretches of repetitive DNA (microsatellite repeats) within the genome. These repeats may be present in non-coding regions, such as introns, or in coding regions that already contain some of these repeats. In the latter, extension of these stretches of DNA beyond a threshold number of repeats results into the pathological phenotype. Frequently the length of the repeats correlates with the severity of the disease. Huntington's disease (HD) and *C9ORF72*-linked amyotrophic lateral sclerosis (ALS) are well-known examples.

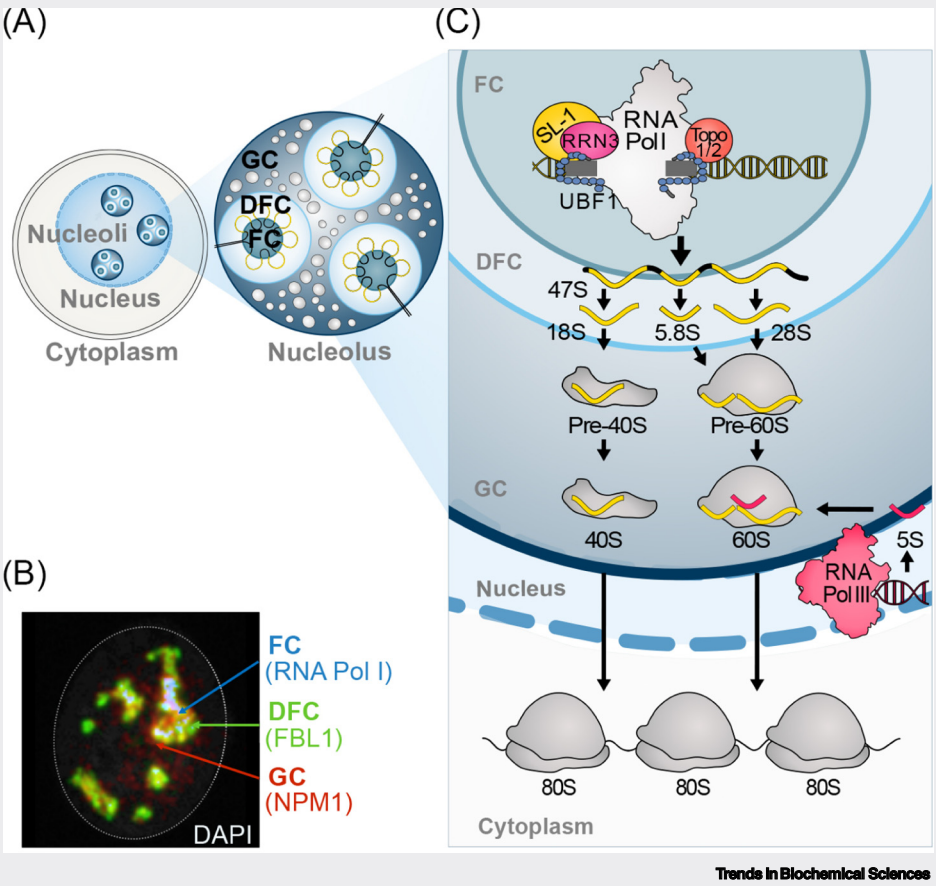

Figure I. Nucleolar structure and function. (A) Representation of the tripartite structure of the nucleolus (left panel). The innermost compartment is the fibrillar center (FC), followed by the DFC and the GC. Transcription of rDNA to pre-rRNA (47S) by RNA Pol I occurs in the FC or at the FC–DFC border, whereas processing of the pre-RNA into the 18S, 5.8S, and 28S rRNAs takes place in the DFC, which is followed by pre-ribosome subunit assembly in the GC. The 5S rRNA is transcribed by RNA Pol III outside the nucleolus, and is incorporated into the large ribosomal subunit in the GC. Both the small (40S) and large (60S) ribosomal subunits are exported into the cytoplasm for further maturation, after which they can assemble into active ribosomes (80S). (B) Microscopy image showing the different nucleolar layers. The FC is marked by one of the largest catalytic RNA Pol I subunits POLR1A (blue), the DFC by FBL1 (green), and the GC by NPM1 (red). The white line delimits the nucleus. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; Topo 1/2, DNA topoisomerases 1 and 2.

Ribosomopathy: a heterogeneous group of pathologies caused by downregulation or mutations in factors necessary for ribosome biogenesis. The phenotypic manifestations of ribosomopathies are diverse, but they tend to converge on defects in hematopoietic cells and in skeletal tissues.

Tripartite structure: the internal organization of the nucleolus based in the disposition of its components in different immiscible layers (FC, DFC, GC) in which nucleation occurs spontaneously within the different layers and follows an ordered patterned established by the biophysical properties of its components. This organization is highly conserved in eukaryotes over evolution.

increased translational demand of rapidly proliferating tumor cells [4,16]. Indeed, several potent oncoproteins, including RAS, MYC, and mTOR, are known activators of translation and ribosome biogenesis [17]. Likewise, tumor suppressors, such as P53, PTEN, or RB1, participate in surveillance systems which constrain ribosome biosynthesis and cell growth [18]. Along these lines, human cancers frequently show altered expression of r-proteins [19]. For instance, overexpression of individual r-proteins correlates with poor prognosis in multiple cancer types: RPLP0, RPLP1, and RPLP2 in ovarian cancer [20], RPS11 and RPS20 in glioblastoma [21], and activated ribosome biogenesis and translation signatures in metastatic breast cancer [22]. In fact, overexpression of a single r-protein, RPL15 or RPL35, was sufficient to increase metastatic burden in mouse models of breast cancer [22].

Of note, this view has been challenged by findings in aggressive tumors with inconspicuous nucleoli or low rates of protein synthesis, raising caution about how to interpret morphological data [15,23,24]. Along these lines, **ribosomopathy** patients, who are characterized by impaired ribosome biogenesis and downregulated protein synthesis [25], have a greater risk of developing malignancies [26]. Interestingly, ribosomopathies are often characterized by the presence of unbound r-proteins [11], suggesting that the disease phenotypes might be driven by stoichiometric imbalance in the components of the ribosome rather than by the absence of a specific component [27]. Nevertheless, how compromised ribosomal function leads to cancer is not understood.

Neurodegenerative disorders

Although mature neurons are post-mitotic cells with limited growth potential, their metabolic demand is extremely high owing to their large size and the need to support a large number of synaptic contacts. Thus, neurons require a higher production of rRNA than other cell types [28]. Neurons often display a single prominent nucleolus [29]. Interestingly, human sensory ganglion neurons show a positive linear correlation between nucleolar and cell body volumes, indicating that nucleolar mass is dictated by the requirements for ribosome biogenesis instructed from the cell [30].

Owing to their specific high demand of ribosome biogenesis, nucleolar alterations have been extensively linked to neurodegenerative diseases, although, unlike cancer, no general trend is observed [29,31]. There are examples of neurodegenerative conditions where nucleoli are smaller than those found in healthy cells, such as Alzheimer's disease (AD) and Parkinson's disease (PD) [29,32]. Although reduced ribosome biogenesis is a common factor in these pathologies, how this contributes to neurodegeneration remains unclear. Conversely, enlarged nucleoli have been reported in familiar cases of amyotrophic lateral sclerosis (ALS) driven by *C9ORF72* mutations, although this phenotype did not correlate with an increased ribosome output [33,34]. In fact, we and others have shown that toxic peptides associated with *C9ORF72* mutations in

Box 2. Phenotypic characterization of modulators of nucleolar integrity

As discussed in the text, novel therapies targeting different aspects of ribosome biogenesis have been identified my monitoring nucleolar activity through different readouts. For instance, inhibitors of rDNA transcription can be identified by detecting the levels of 47S rRNA relative to RNA Pol II and Pol III transcripts [109]. Although classically followed through RT-qPCR, scaled-up high-content screening applications require the use of rDNA transcription reporters, either in cell lines or cell-free assays (Figure I, yellow panel left). As previously described, RNA Pol I inhibitors are often DNA intercalators, and any novel RNA Pol I inhibitors should therefore be evaluated for DNA damaging activity, which is common in this class of compounds [67,68]. To further characterize the mechanism of action of novel molecules, some useful secondary assessments include determining whether the compounds interfere with preinitiation complex (PIC) formation or destabilize POLR1A (Figure I, yellow panel, right). High-throughput microscopy (HTM) is a suitable technique to assess the impact of molecules or genes on the stability and localization of PIC components such as UBF1 and POLR1A. Furthermore, techniques to label RNA, such as the use of 5-ethynyluridine (EU) or fluorescent nucleic acid stains, can easily be adapted to measure rRNA synthesis by HTM [110,111].

At low throughput, rRNA processing can be monitored by RT-qPCR or northern blotting, techniques which can detect the levels of 47S RNA and of the different intermediates in pre-rRNA processing (Figure I, orange panel). Although there are no high-throughput techniques available to monitor rRNA processing, r-protein distribution assessed by HTM or mass spectrometry (MS) has been used as an indirect readout of problems in ribosome assembly. Accumulation of r-proteins in the nucleus or nucleoli reflects an impairment in r-protein cytoplasmic export and/or issues related to rRNA synthesis and assembly (Figure I, green panel left). Accordingly, reporter systems have been very useful for studying r-protein localization. Examples include yeast strains expressing RPL7/RPS9–GFP [80], mammalian cells expressing GFP–RPL37 that were used in the discovery of CID-765471 [69], and tetracycline-inducible GFP–RPL29 and YFP–RPS2 HeLa cells that were used in the characterization of metarrestin and in several RNAi screens [70,91,112] (Figure I, green panel, right). Finally, CRISPR/Cas9 knock-in HaloTag fusion proteins have been shown to be efficient for monitoring nucleolar r-protein distribution and flux (Figure I, green panel, right) [59] as well as for evaluating changes in nucleolar morphology through the use of HaloTagged nucleolar proteins [73,94] (Figure I, blue panel).

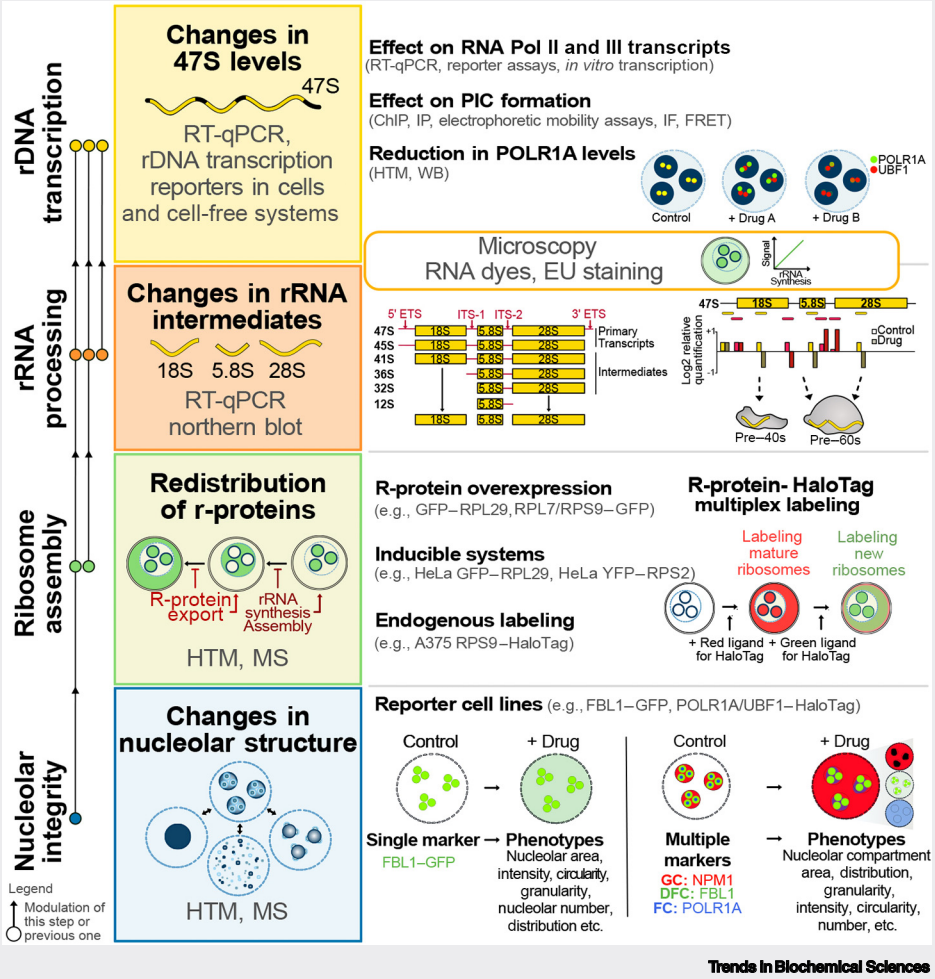

Figure I. Summary of experimental methodologies to characterize modulators of nucleolar activity and morphology at low and high throughput. Abbreviations: ChIP, chromatin immunoprecipitation; ETS, external transcribed spacer; EU, 5-ethynyluridine; FRET, fluorescence resonance energy transfer microscopy; HTM, high-throughput microscopy; IF, immunofluorescence, IP, immunoprecipitation; ITS, internal transcribed spacer; MS, mass spectrometry; WB, western blot.

ALS severely compromise translation and ribosome biogenesis, suggesting that enlarged nucleoli in this context do not reflect an increase in nucleolar function but rather the presence of nucleolar stress [35,36].

Nucleolar stress was formally shown to drive neurodegeneration in a mouse model of Huntington's disease (HD), where TIF-1A was genetically ablated in medium spiny neurons, and in a model of PD, upon TIF-1A depletion in dopaminergic neurons [37]. These models, together with the previously mentioned observations in ALS, suggest that nucleolar morphology is not an absolute readout for ribosome biogenesis, nucleolar stress can be a driver of neurodegeneration, and the specific pathology is determined by the neuronal type that is affected.

Despite the diversity of morphological alterations of nucleoli in neurodegenerative diseases, common features among these disorders include the epigenetic silencing of rDNA promoters and an

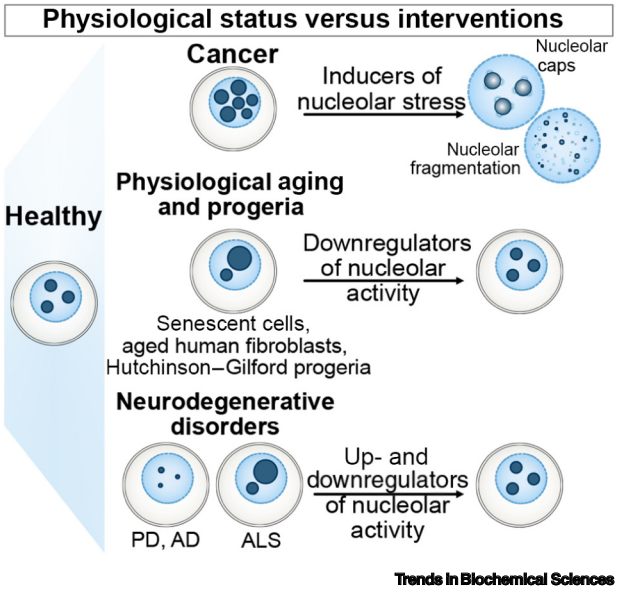

Figure 1. Disease-associated changes in nucleolar morphology. Cancer cells are often characterized by bigger and more numerous nucleoli than somatic cells, likely because of an increased demand for ribosome biogenesis. This makes tumor cells sensitive to compounds that cause nucleolar stress, which can be detected by perturbations in nucleolar morphology such as nucleolar segregation or the formation of nucleolar caps. Nucleolar alterations have also been linked to aging. Cells from physiological and premature aging models, such as senescent cells or cells from Hutchinson–Gilford progeria patients, show enlarged nucleoli. In addition, strategies lowering nucleolar activity have been successful in extending lifespan and improving health status in several model organisms. In the context of neurodegeneration, there are examples of both smaller and enlarged nucleoli in patients compared to healthy individuals, and both phenotypes might indicate underlying perturbations in nucleolar function. The efficacy of strategies focused on increasing or decreasing ribosome biogenesis in the context of cancer, neurodegeneration, and aging is currently being investigated. Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; PD, Parkinson's disease.

aberrant distribution of nucleolar proteins, often due to their sequestration or displacement by protein aggregates containing RNA; this latter phenotype is particularly associated with **repeat expansion disorders** such as ALS and HD [38,39]. Interestingly, the neurotoxicity of some cancer therapeutics, such as platinum derivatives, has also been related to the effects of these compounds in nucleoli [40]. Currently, why nucleolar alterations have a particular impact on the nervous system is also poorly understood. Nevertheless, preclinical approaches directed to either stimulate or reduce nucleolar activity are being tested for the treatment of neurodegenerative pathologies, the latter being more successful so far [29,41].

Aging

Changes in nucleolar size or activity are emerging as a novel hallmark of aging (reviewed in [8]). Nucleolar activity progressively decreases with age, and this has been proposed to be the result of changes in nutrient-sensing pathways, nucleolar protein composition, or epigenetic modifications of the nucleolar organizing regions (NORs) [8]. Overall, modifications that limit nucleolar activity correlate with an extended lifespan, whereas activation of ribosome biogenesis is associated with its shortening. Supporting this view, increased nucleolar area and upregulation of ribosome biogenesis have been described in premature aging pathologies such as Hutchinson–Gilford progeria [42]. Furthermore, enlarged nucleoli have also been found in aged human fibroblasts [42] and senescent cells [43]; these often present a single and large nucleolus. By contrast, reduced nucleolar activity and/or size have been correlated with extended lifespan in yeast, worms, flies, and mice (reviewed in [8]). For instance, an elegant study in *Caenorhabditis elegans* revealed that long-lived mutants had smaller nucleoli, and the increased longevity mediated by several interventions was abolished by simultaneously boosting nucleolar activity through *ncl-1* depletion [44]. Interestingly, signs of nucleolar hardening have been described in aged *C. elegans*, suggesting that changes in the physical properties of nucleoli might also be related to aging [7].

It should be noted that some of the initial connections between nucleoli and aging derive from studies showing that rDNA instability leads to the accumulation of extrachromosomal rDNA circles in aged yeast cells [45], and that mutations in helicases involved in the maintenance of genomic integrity at repeated loci such as Werner (*WRN*) lead to the expansion of rDNA repeats [43] and accelerated aging in humans [46]. These yeast studies led to the proposal that safeguarding the genomic integrity of rDNA, by stimulating sirtuin deacetylases through the use of resveratrol, a compound that is particularly abundant in red grapes, could have life-extending properties [47]. However, this idea has been largely disputed and remains one of the most controversial aspects of aging research (extensively reviewed in [48,49]). Regardless of resveratrol, the concept of manipulating nucleolar function to delay age-related pathologies is an exciting field which has only recently started to show its potential.

Targeting the nucleolus and ribosome biogenesis

In this review, we provide an overview of strategies and chemicals that target the different steps of ribosome biogenesis (Box 1), as well as their potential limitations and uses for the treatment of human disease (Figure 2).

Modulators of the preinitiation complex

Several factors assemble with RNA polymerase I (Pol I) at rDNA promoters to form the preinitiation complex (PIC) (Figure 2). Briefly, RRN3 mediates the conformational transition of RNA Pol I to its transcriptionally active form, which interacts with the SL1 complex, recruiting the PIC to the rDNA promoter. In addition, UBF1 binding to rDNA regulatory regions is necessary to increase chromatin accessibility and enable transcription [50]. Targeting the formation of the PIC is one of the earliest therapeutic approaches directed to nucleoli.

A selective impact on PIC assembly is indicated by alterations in the levels of the 47S rRNA precursor, the primary product of RNA Pol I, without affecting the transcripts produced by RNA Pol II or III. Using this strategy, a chemical screen identified CX-5461 as an RNA Pol I inhibitor, and this molecule has recently received the fast-track designation by the FDA for the treatment of homologous recombination-deficient breast cancer. CX-5461 reduced rRNA transcription in cell-free assays, decreased cellular 47S levels, and induced nucleolar stress and cell death. Mechanistically, the compound was first proposed to destabilize the interaction between RNA Pol I and SL1 [51], as were the structurally related 9-hydroxyellipticine (9HE) and other ellipticines which also affect SL1 occupancy at rDNA promoters [52], although this mechanism of action is currently in dispute [53]. Similarly, the commonly used platinum-derived chemotherapeutics, cisplatin and oxaliplatin, displace UBF1 from rDNA promoters and also disrupt PIC assembly [51,54]. Importantly, most of these drugs are DNA intercalators that often trigger genotoxic effects, casting doubt on how these agents specifically affect rDNA transcription or trigger cell death [55]. Regardless of their limited specificity, many of these compounds have proved their efficacy in the clinic and have set the stage for the discovery of additional specific modulators of ribosome biogenesis.

Publication of the crystal structure of the *Saccharomyces cerevisiae* RNA Pol I complex enabled rational design of RNA Pol I inhibitors through structural and homology-based studies [56]. For instance, sodium cerivastatin was identified through a virtual screen aiming to identify FDA-approved compounds that can potentially interfere with the RRN3–RNA Pol I interaction, and which were subsequently validated in yeast using a human rDNA–luciferase reporter [57]. In another study, a small peptide was designed to disrupt the RNA Pol I and RRN3 interface [58]. All these strategies reduced rRNA transcription and viability in cellular models.

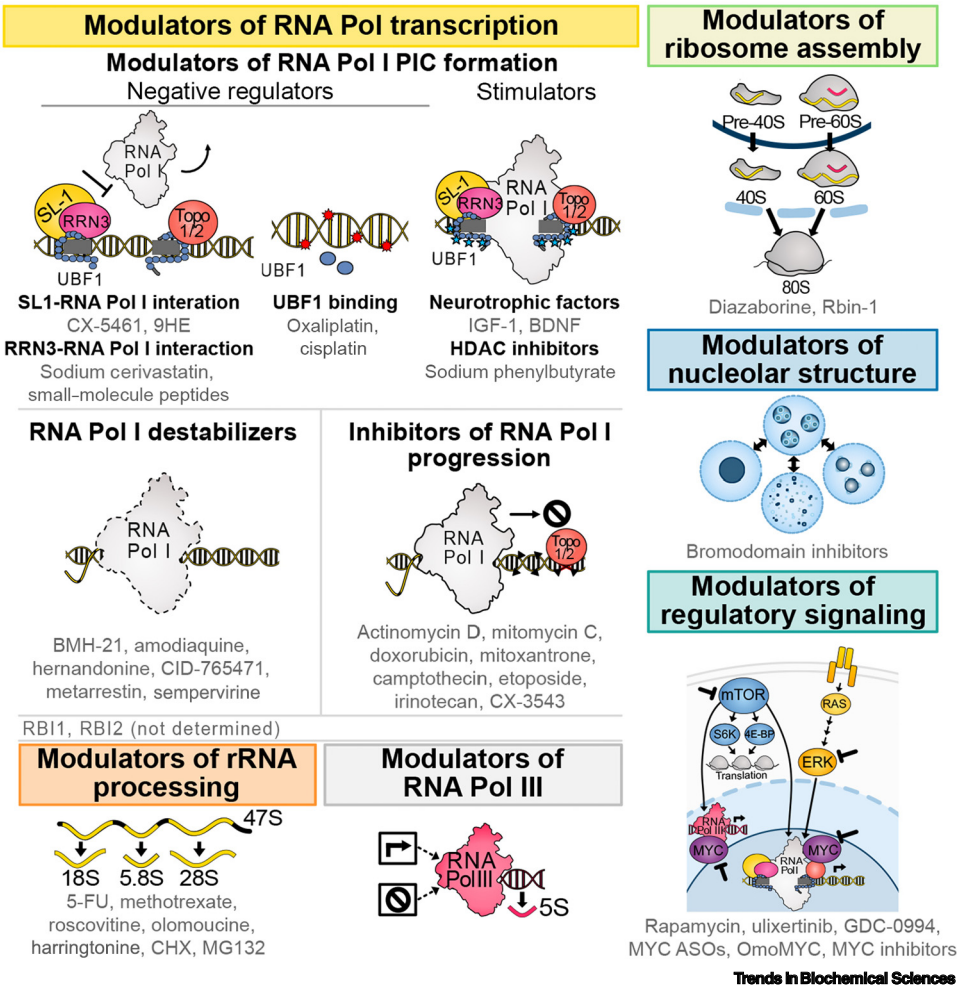

Figure 2. Targeting nucleoli. The scheme provides an overview of the compounds described to have an effect on different steps of ribosome biogenesis. Abbreviations: ASOs, antisense oligonucleotides; CHS, cycloheximide; 5-FU, 5 fluorouracil; HDAC, histone deacetylase; 9HE, 9-hydroxyellipticine; OmoMyc, dominant negative MYC-inhibitor mini-protein; PIC, preinitiation complex; Pol I/III, RNA polymerases I and III, Topo 1/2, DNA topoisomerases 1 and 2.

In addition to targeted approaches, RBI1 and RBI2 were isolated in a chemical screen by monitoring the distribution of an endogenously tagged r-protein RPS9 because this protein is only stable when assembled in ribosomes [59]. A secondary screen evaluated the effects of these compounds on 47S levels, and revealed that they are inhibitors of rDNA transcription. Although how RBI1 and RBI2 perturb the PIC demands further work, both compounds were shown to reduce viability of cancer cells and are promising preclinical agents [59].

In contrast to drugs targeting nucleolar activity for their use in oncology, compounds stimulating PIC formation have been explored in the context of neurodegenerative diseases, which as previously mentioned, are frequently associated with nucleolar dysfunction (reviewed in [29]). For instance, growth stimulation by neurotrophic factors requires TIF1A activity [29], whereas histone deacetylase inhibitors (HDACi) promote PIC formation by enhancing UBF1 acetylation and opening rDNA chromatin [60]. Although neurotrophic factors failed in clinical trials for ALS [61], the HDACi sodium phenylbutyrate is currently progressing in clinical trials for the same

disease [39,62]. Interestingly, we also found sodium phenylbutyrate in a chemical screen oriented to identify compounds that limit the toxicity of ALS-associated arginine-rich neurotoxic peptides [63] which accumulate at nucleoli and perturb nucleolar function [35]. Noteworthy, compounds interfering with the interaction between nucleolar proteins and RNA repeats, such as P3 and BIND, have also showed benefits *in vitro* and *in vivo* in RNA-repeat expansions associated disorders such as HD and ALS [64,65]. Nevertheless, and as in the case of cancer, these therapies are still in their early days of preclinical development, and more work needs to be done to decipher which nucleolar targeting strategy is best suited for a particular disease.

RNA Pol I destabilizers

BMH-21 and its derivatives were the first drugs discovered to trigger the degradation of POLR1A (also RPA194), one of the catalytic subunits of RNA Pol I (Figure 2). They were first identified in an image-based **high-throughput** phenotypic screen looking for activators of P53 [66,67]. These drugs reduce RNA Pol I association with rDNA, cause nucleoplasmic translocation of nucleolar proteins, generate nucleolar caps, and trigger proteasomal degradation of POLR1A [66]. However, although decreased RNA Pol I stability correlates with the antitumoral activity of BMH-21, the stabilization of POLR1A by proteasomal inhibition does not improve rRNA transcription in cells exposed to the drug [66]. Furthermore, these compounds are also DNA intercalators with a high affinity for GC-rich DNA structures, once again raising doubts as to whether their effects on RNA Pol I are direct or are mediated by their impact on chromatin. Nonetheless, the main appeal of these compounds is that, in contrast to the other available RNA Pol I inhibitors, they do not cause extensive DNA damage [66]. In addition to BMH-21, other destabilizers of POLR1A have been reported, including amodiaquine [68], hernandonine [13], acridine derivatives CID-765471, aminacrine, and ethacridine [69], metarrestin [70], and sempervirine [71]. Of note, and regardless of the potential off-target effects of these drugs, auxin-inducible degron (AID) alleles [72], that enable the conditional degradation of endogenous POLR1A [73] and POLR1B (previously RPA135) [74], recapitulated the effects observed with the chemicals on nucleolar morphology, the formation of nucleolar caps, and inhibition of rRNA transcription.

Given the essentiality of RNA Pol I, an obvious concern for these therapies is their potential toxicity [51]. However, it should be noted that, although most available genotoxic chemotherapies are also widely toxic, they show benefits when applied to the right cohort of cancer patients. In this regard, genetic studies aiming to clarify whether targeting RNA Pol I could be beneficial in the context of specific mutations are crucial. Furthermore, partial inhibition of RNA Pol I function can have beneficial effects, in contrast to the toxicity derived from acute RNA Pol I inhibition. For instance, RNA Pol I haplosufficiency is compatible with viability in *Drosophila* and, when restricted to specific cell populations, was shown to improve longevity [75]. These results support the emerging connection between nucleolar activity and aging [8], and indicate that partial limitation of nucleolar activity has the potential to attenuate age-related pathologies.

Inhibitors of RNA Pol I progression

Selective inhibition of rRNA transcription elongation is limited by the fact that this process does not involve many RNA Pol I-specific factors. Accordingly, most compounds modulating this process often intercalate into rDNA or stabilize rRNA/rDNA secondary structures, such as **G-quadruplexes**, leading to DNA damage [51]. These effects are particularly toxic to cancer cells because of their high rRNA transcription rates [76]; indeed, these compounds are commonly used chemotherapeutics (reviewed in [51]). A prominent example of this class of drugs is the previously mentioned CX-3543 which, despite its effects on the PIC, was originally discovered in an *in vitro* screen for chemicals that can dissociate NCL1 from G-quadruplex complexes [77] (Figure 2).

Targeting rRNA processing

Processing of the 47S precursor into 28S, 5.8S, and 18S rRNAs is a multistep process that produces different rRNA intermediates [78]. Accumulation of these intermediates, relative to the total levels of the 47S rRNA, is used as a readout of defective rRNA processing. Among compounds affecting rRNA processing are several antimetabolites used as chemotherapeutics such as 5-fluorouracil (5-FU) and methotrexate, the cyclin/CDK2 and CDK9 inhibitors roscovitine and olomoucine, the translation inhibitors harringtonine and cycloheximide, and the proteasome inhibitor MG-132 [51,79] (Figure 2). Additional compounds interfering with rRNA processing were recently identified upon counter-screening hits from a chemical screen in yeast cells expressing r-proteins fused to GFP [80].

RNA Pol III inhibitors

RNA Pol III transcribes 5S rRNA, an essential component of ribosomes, among other small RNAs [81]. However, 5S is the dominant RNA Pol III transcript, making it a feasible target for the inhibition of ribosome biogenesis (Figure 2). Consistent with r-proteins and Pol II activity, upregulation of RNA Pol III transcription is frequent in cancer, and RNA Pol III mutations are causative of several neurodevelopmental disorders [82]. Moreover, conditional ablation of RNA Pol III catalytic subunits extended lifespan in *S. cerevisiae*, *C. elegans*, and *Drosophila* models, further demonstrating that partial inhibition of ribosome biogenesis can have beneficial effects [83]. Although no specific RNA Pol III inhibitors are yet available [84], the structure of the human RNA Pol III has recently been published [85,86], which should enable the rational design of new drugs through *in silico* methodologies. Two potential targets are the C-terminal extension of the RNA Pol III POLR3E (also RPC5) subunit, which is necessary for RNA Pol III stability [85], and MAF-1. MAF-1 is an mTORC1-controlled inhibitor of RNA Pol III and an interesting therapeutic target because it has been shown to regulate lifespan in yeast, worms, flies, and mice [87]. However, the exact role of MAF-1 in aging remains unclear, as does the contribution of 5S transcription relative to the rest of the RNA Pol III targets.

Inhibitors of ribosome assembly

Alterations in ribosome assembly lead to aberrant distribution of r-proteins, and this has been used to measure perturbations of rRNA processing (discussed in Box 2). A chemical screen using yeast strains expressing either GFP-tagged RPL7 or RPS9 discovered diazaborine, the first described inhibitor of ribosome maturation in yeast [88] (Figure 2). Diazaborine inhibits the formation of the 60S ribosomal subunit by blocking ATP hydrolysis by the AAA-ATPase DRG1, which is essential for recycling of the ribosomal assembly factor RPL24 from pre-60S ribosomes [89]. Similarly, Rbin-1 inhibits the AAA-ATPase that is crucial for maturation of the 60S subunit in fission yeast [90]. Analogous r-protein–reporter systems have been generated in mammalian cells, either by overexpressing tagged r-proteins or by endogenous labeling of r-proteins by CRISPR/Cas9, and these have been used in chemical and genetic screens [69,70,91]. However, hits from these screens have not yet been tested for their effects on ribosome production or maturation.

Modulators of nucleolar structure

Nucleolar morphology is arguably the main readout for **nucleolar integrity** and activity, and is altered by most of the aforementioned drugs. However, most chemical screens aiming to identify nucleolar modulators did not use nucleolar size or morphology as an endpoint, in contrast to genetic screens, several of which have been performed based on these properties. For instance, the evaluation of changes in nucleolar number in human MCF10A breast epithelial cells [92], or nucleolar size in *S. cerevisiae* and *Drosophila* models [93], helped to identify factors involved in ribosome biogenesis by RNAi screens. Of note, image-based characterization of nucleolar

phenotypes demands the development of standardized parameters for quantitative analyses. One of these is the iNo score, a numerical index defined by the five most notable features of nucleolar morphology [94]. This score was used to evaluate nucleolar alterations in a FBL1–GFP reporter cell line after depleting each r-protein. These data are publicly available in a database which also includes information about the effects of the depletion of each r-protein on nucleolar structure, rRNA processing, and P53 levels (http://www.ribosomalproteins.com). Nucleolar morphology has also been successfully used in chemical screens. For instance, our group performed a chemical screen based on nucleolar number, and found that BET bromodomain inhibitors (BETi) prevented nucleolar stress caused by different insults, including actinomycin-D or arginine-rich peptides [63], and provided the first example of a drug that can rescue nucleolar stress (Figure 2). Given the beneficial effects observed in genetic studies partially targeting ribosome biogenesis in aging [8], the discovery of drugs that can reduce nucleolar stress or activity may provide new tools to be explored as anti-aging interventions.

Targeting signaling pathways that regulate nucleolar activity

As one of the key elements of cell growth, ribosome biogenesis is controlled by several growth signaling routes, the most prominent of which are mTOR, MYC, and MAPK. This further supports the nucleolar link to aging because inhibition of each of these pathways can extend lifespan in animal models [8]. Of note, and in contrast to the previously described compounds which cause stoichiometric imbalances in ribosomal components, interventions targeting regulatory pathways downregulate the entire ribosome biogenesis program rather than a specific factor (Figure 2).

The mTOR complex is the central hub of metabolism and growth that integrates and coordinates cellular anabolic activity in response to stress, growth factors, IGF-1/insulin signaling, and nutrient availability (reviewed in [95]). The key effectors of mTOR are S6K1, which controls ribosome biogenesis both directly and indirectly through translation, and 4E-BP1, which also regulates nucleolar activity through translation [95]. Activity of this pathway can be attenuated pharmacologically with compounds targeting mTOR, such as rapamycin, or by an array of inhibitors of targets upstream of mTOR, such as PI3K or AKT (reviewed in [96]). Many of these compounds have been extensively tested in oncology, and mTOR inhibition is one of the best characterized anti-aging interventions [95,96]. There are also high hopes that mTOR inhibition may be applicable to neurodegenerative disorders [97], and a Phase 2 clinical trial to evaluate the efficacy of rapamycin in ALS patients is currently underway [98]. Of note, although regulation of ribosome biogenesis is a prominent target of mTOR signaling, the pleiotropic effects of this route call into question the relative contributions of each phenotype, such as the activation of autophagy or translation inhibition, to the *in vivo* effects observed upon mTOR inhibition, and further work will be necessary to elucidate this.

MYC is one of the few transcription factors that can regulate all three RNA polymerases, and drives the expression of rRNAs, r-proteins, and nucleolar factors (reviewed in [99]). Targeting MYC is mainly explored in the context of cancer, although this strategy may also prove to be applicable in neurodegeneration or aging. Accordingly, MYC haploinsufficiency increases longevity in mice [100]. Although MYC has long been considered to be undruggable, several strategies targeting MYC have recently been published, including chemical MYC inhibitors, a dominant negative peptide (OmoMYC), and antisense oligonucleotides (ASOs) targeting MYC (extensively reviewed in [101]). Interestingly, selective susceptibility of MYC-driven tumors to CX-5461 has been described [102], suggesting that high levels of MYC could serve as a biomarker for treatment with nucleolar stressors in oncology [103].

Finally, mitogen-activated protein kinase (MAPK) signaling is a potent driver of proliferation, and MAPKs are among the most commonly mutated or hyperactivated factors in cancer [104].

Ribosome biogenesis specifically receives input from this route via its downstream mediator, ERK, which can phosphorylate RNA Pol I transcription factors TIF-1A and UBF1 [8]. Several ERK inhibitors, such as ulixertinib and GDC-0994, are now available and are in clinical trials (reviewed in [104]), and the rest of the MAPK signaling pathway can be targeted pharmacologically at multiple levels (extensively reviewed in [105]). As with mTOR or MYC, MAPK signaling controls many aspects of cell growth, and further work is necessary to clarify whether an impact on nucleolar activity contributes to the therapeutic effects of these agents.

Concluding remarks

Because a large proportion of cellular energy is invested in ribosome biogenesis [3], nucleoli are a visible marker of overall cellular health. In this context, and given that ribosome biogenesis is often elevated in some diseases, such as cancer, and compromised in others, such as in neurodegenerative disorders, nucleoli are an interesting but largely unexploited therapeutic target (see Outstanding questions). Importantly, although still in its early days, the specificity of available drugs is limited and more specific therapeutic agents are necessary to selectively target nucleoli. Regarding cancer, we believe that efforts should be dedicated to identifying specific mutations that confer sensitivity to therapies that generate nucleolar stress, thereby enabling better personalized therapies and helping to mitigate potential widespread toxicities of this strategy. Finally, the emergent connection between nucleolar activity and aging is of particular interest because fine-tuning of ribosome biogenesis might enable the development of interventions aiming to collectively counteract age-related pathologies.

Outstanding questions

Why are neurons and hematopoietic cells particularly sensitive to perturbations in nucleolar function?

What are the mechanisms that drive P53-independent cell death upon nucleolar stress?

Are the alterations in nucleolar morphology observed in cancer and neurodegeneration the cause or the consequence of the disease?

How do non-canonical nucleolar functions beyond ribosome biogenesis contribute to the impact of nucleolar dysfunction on human disease?

Which specific mutations provide sensitivity or resistance to nucleolar stress and hence provide clues to potential chemotherapies?

Can the material properties of the liquid phases of nucleoli be pharmacologically modified to modulate nucleolar activity?

To what extent are the effects on cell growth and lifespan – triggered by inhibition of mTOR, MYC, and MAPK signaling – mediated by their modulation of ribosome biogenesis?

Does the presence of high levels of nucleolar stress sensitize cancer cells to a specific therapy?

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Declaration of interests

No interests are declared.

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