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New insights into nucleolar structure and function

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Abstract

The nucleolus is a non-membrane-bound nuclear organelle found in all eukaryotes. It is the quintessential 'RNA-seeded' nuclear body, forming around specific chromosomal features called nucleolar organizing regions that contain arrays of ribosomal DNA. Assembly is triggered by activation of RNA polymerase I-mediated transcription and regulated in mammalian cells in a cell cycle-dependent manner. Although the nucleolus is best known for its role in coordinating ribosome biogenesis, biochemical and proteomic analyses have revealed a much wider functional complexity than previously appreciated, including roles in cell cycle regulation, DNA damage sensing and repair, pre-mRNA processing, telomere metabolism, processing of non-coding RNAs, and coordination of the cellular response to various stresses. Despite these advances, much remains to be learned about the full range of biological processes that occur within, or involve, this organelle and how its assembly/disassembly and functional reorganization in response to various stimuli are regulated. Here, we review the impact of recent studies that provide major insights into these fundamental questions, and we highlight the therapeutic potential of targeting nucleolar pathways.

Nucleolus and ribosome biogenesis

The interphase nucleolus is a functionally compartmentalized structure with a classic 'tripartite architecture' defined by electron and light microscopy and comprising fibrillar centers (FCs) surrounded by dense fibrillar components (DFCs) embedded in a granular component (GC). The FC contains pools of unengaged RNA polymerase I (Pol I) transcription factors such as the upstream binding factor (UBF), whereas the DFC contains early pre-RNA processing factors. Transcription is believed to occur at the border of these two regions, and the GC is the site of later pre-rRNA processing steps and ribosome subunit assembly (for review, see [1]). Nucleolar proteins with non-ribosomal roles have been localized both to these compartments and to novel compartments [2,3], and their structure/function relationships within the architectural context of the nucleolus remain to be defined.

In higher eukaryotes, there is an ordered disassembly/re-assembly of the nucleolus at each cell division, with the increase in cyclin-dependent kinase 1 (CDK1)/cyclin B activity at the onset of mitosis triggering a repression of rRNA transcription and sequential nucleolar breakdown. Certain factors remain associated with nucleolar organizing regions (NORs), whereas others move to the chromosome periphery or are released. When CDK1/cyclin B activity decreases at mitotic exit, rRNA transcription resumes within the NOR, downstream processing factors are recruited, and the nucleolus is re-assembled (for a comprehensive review, see [4]). Although this suggests a simple structure/function model in which the onset of rRNA transcription signals recruitment of downstream processing factors and formation of the nucleolus (and inhibition triggers the reverse), studies have shown that transcription can be disconnected both structurally and functionally from downstream processing [5,6]. This suggests a more complex regulation than simply turning rRNA transcription on and off. Furthermore, diploid cells, which have NORs on each of the five different acrocentric chromosomes and thus the

potential for up to 10 nucleoli, have only one to three. Although it is known that some NORs remain silent while others fuse in early G_1 [7], the underlying control mechanisms remain unclear.

Nucleolus as a self-organizing system

The transcription factor UBF is a key component of the Pol I pre-initiation complex that remains associated with NORs during mitosis when rRNA transcription halts and nucleoli are disassembled (see [8] for review). Using chromosome engineering, McStay and colleagues [9,10] constructed artificial arrays containing multiple copies of UBF-binding DNA sequence arrays on non-NOR-bearing human chromosomes. These so-called 'pseudo-NORs' recruited endogenous UBF, along with the entire Pol I transcriptional machinery, and adopted key morphological features of active NORs. However, they could not be transcribed (no promoter sequences) and thus failed to recruit pre-rRNA processing factors and form nucleoli. This demonstrated an additional transcription-independent role for UBF in nucleolar formation. The same group has now extended these studies to the construction of functional synthetic nucleoli in human cells through the integration of ectopic arrays of a 'neo-NOR' cassette into various chromosomal contexts [11]. These neo-NORs, which comprise an engineered human rDNA promoter, mouse pre-rRNA coding sequences, and mouse transcription terminator, were shown to be transcriptionally active (albeit at a lower level than endogenous NORs) and produce mature rRNAs and polysome-associated ribosomes. Neo-NORs and their transcripts could be distinguished from endogenous NORs/transcripts, revealing that approximately 40% of neo-NORs associate with endogenous NORs in large nucleoli in a compartmentalized manner. Interestingly, this suggests that there may be 'NOR territories' comparable to the chromosome territories that contribute to compartmentalized nuclear architecture (see [12] for review). Taken together, these studies have revealed the central role that UBF plays both in maintaining NOR competency and in establishing the mitotic hallmarks of competent NORs and have confirmed that the nucleolus is a self-organizing structure triggered by signals encoded in the genome.

Surprisingly, organization of rDNA by cohesin has also been shown to be critical for nucleolar structure and function, and cohesin complexes are believed to play roles in chromatin organization [13] and promotion of rRNA production [14]. Roberts syndrome (RBS) is a cohesinopathy in which mutations in the acetyltransferase ESCO2 abolish its activity, resulting in reduced acetylation of the cohesin subunit Smc3. Cells show fragmented nucleoli and have profound defects in rDNA transcription and ribosome production [15]. Cohesin binds rDNA, and in yeast the comparable RBS mutation (eco-W216G) induces disorganized nucleoli and reduced looping at rDNA, suggesting a functional role [16]. Pol I occupancy is not affected, but rDNA cleavage is slower. Furthermore, ribosomal defects could be induced by mutating any subunit in the cohesin ring, while depletion/destruction of cohesin in a single cell cycle led to loss of nucleolar integrity. Thus, nucleolar dysfunction in RBS almost certainly contributes to the global changes in gene expression and cell physiology that are observed.

Physical properties of membrane-less organelles

The self-organization model for assembly of macromolecules into higher-order cellular structures was originally proposed on the basis of *in vivo* microscopy observations at the beginning of this century [17]. As a general rule, non-membranous cellular bodies like the nucleolus serve to concentrate proteins (and, in most cases, RNAs) involved in similar processes in a constrained space, presumably to enhance reaction efficiency and facilitate regulation. Recent analysis of the physical properties of RNA-protein complexes has provided a possible molecular mechanism behind this phenomenon, and membrane-free RNA-rich organelles have been shown to form via sol-gel phase transitions. These transitions are mediated by multiple weak binding interactions between intrinsically disordered low-complexity sequences (LCSs), which are enriched in many RNA- and DNA-binding proteins. McKnight and colleagues [18,19] demonstrated that interaction of LCSs in a range of cytoplasmic RNA-binding proteins can drive phase transitions to hydrogel droplets that are capable of sequestering target mRNAs. Although electron microscopy analysis showed them to be composed of uniformly polymerized amyloid-like fibers, they differ from pathological amyloid fibrils in that they are reversible and dynamic, can be heterotypic, and are detergent-soluble. Extending these observations to nuclear RNA-binding proteins, they demonstrated LCS-mediated polymerization of the transcription factor TAF15 and recruitment of the C-terminal domain of RNA polymerase II [19]. Cech and colleagues [20] used the multifunctional RNA-binding protein FUS to demonstrate the formation of RNA-protein granules at more physiological concentrations through cooperative binding to RNA, with the protein's RNA-binding domain mediating the initial nucleation step and its LCSs mediating the protein-protein binding-induced phase transition. Similar to TAF15 hydrogels [19], these higher-order assemblies of FUS could also bind the C-terminal domain of RNA polymerase II, suggesting a scaffolding/recruitment role with the potential to directly affect transcription. Importantly, phase transitions have also

been shown to be subject to control by cellular signaling pathways [21,22], which lends further support to the idea that they are common events that serve to spatially organize and biochemically regulate key processes.

Although phase transitions have not yet been directly correlated with nucleolar formation, they are likely to represent a unifying principle of compartmentalization without membranes. In support of this, Hyman and colleagues [23] showed that nucleoli in *Xenopus laevis* oocytes exhibit liquid droplet-like behavior, freely diffusing and fusing with each other in a manner consistent with that of liquid-like configurations of RNA and proteins. They further demonstrated that nucleolar viscosity is ATP-dependent, suggesting that the internal fluidity of this structure relies on active processes. Liquid droplet-like behavior is consistent with interference microscopy measurements showing that the viscosity of the nucleolus, originally described as dense and compact, is only about twice that of the surrounding nucleoplasm [24]. This apparent lower molecule density has been shown to accommodate virus assembly while normal nucleolar function continues [25], demonstrating that views about the "compact" nucleolus are changing (see [26] for review). The nuclear lamina protein Lamin B1 has been implicated in maintenance of nucleolar plasticity, and atomic force microscopy studies have demonstrated that steady-state stiffness of isolated nucleoli relies on ongoing ribosome biogenesis while Lamin B1 is required for flexibility [27,28].

Nucleolar detention

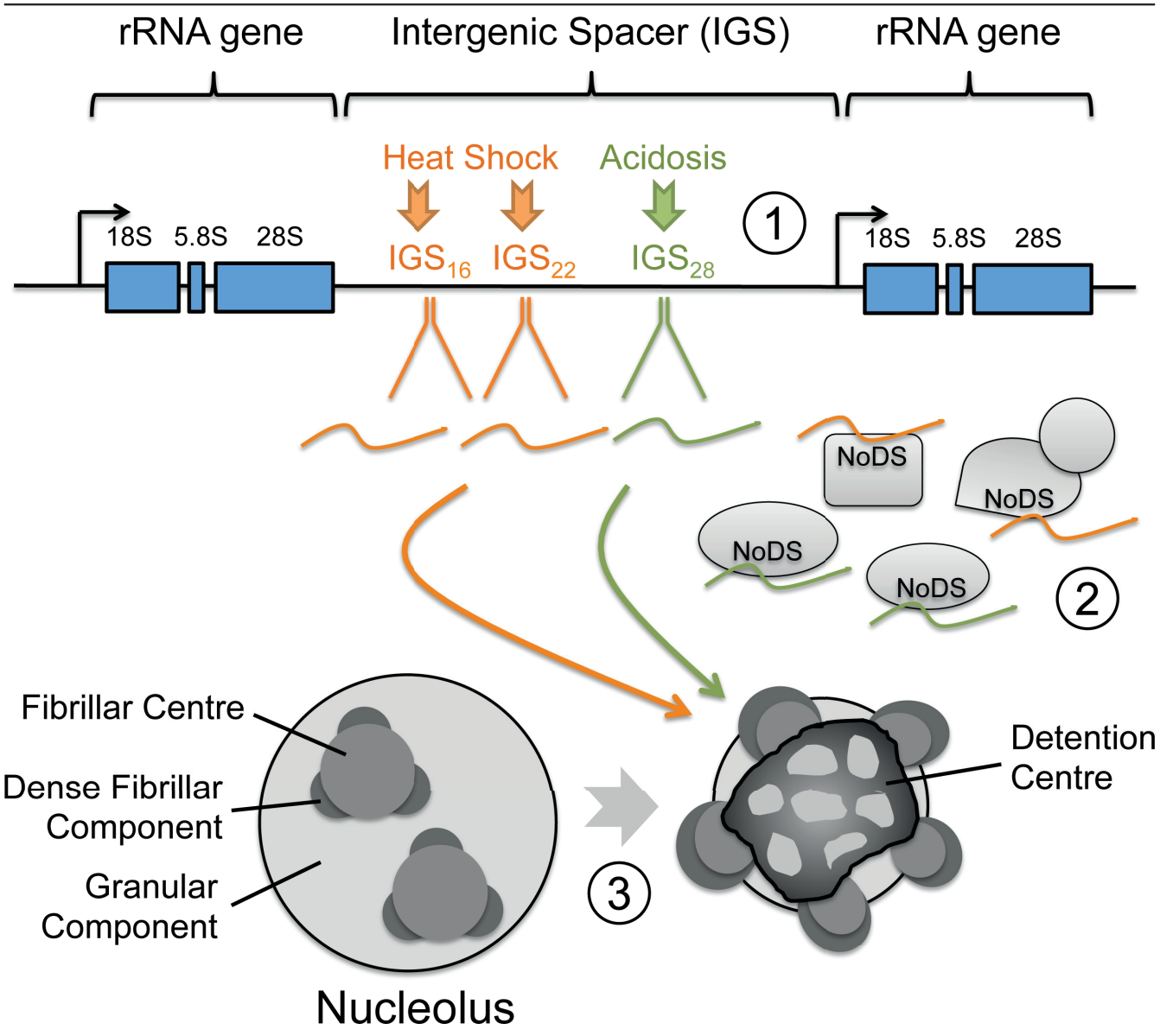
Although organization of macromolecules into cellular compartments can increase efficiency and specificity of molecular processes, such a compartmentalization may offer additional flexibility in the management of cellular reactions. Lee and colleagues [29] recently identified a post-translational regulatory mechanism based on the capture and immobilization of a diverse range of cytoplasmic and nuclear proteins within the nucleolus in response to cellular stresses such as reduced pH and heat shock (Figure 1). This nucleolar sequestration is driven by induction of Pol I-mediated transcription of stress-specific long non-coding RNAs from discrete regions of the rDNA intergenic spacer [29]. The processed transcripts interact directly with proteins that contain a discrete code termed a nucleolar detention sequence, and this initial nucleation event is further stabilized via high-affinity hydrophobic interactions. By immobilizing the proteins within the nucleolus and depriving them of their intrinsic dynamic nature and access to their cellular effectors, this temporary imprisonment allows rapid (and reversible) inhibition of numerous cellular processes in response to stress [30]. Formation of the spatially distinct nucleolar detention center was shown to involve restructuring of nucleolar architecture and silencing of ribosome biogenesis, highlighting the plasticity and dynamic nature of this nuclear organelle [30]. These results also provide further evidence of the generality of RNA-based seeding events (see [31] for review).

Nucleolar microRNA

Just as analysis of the protein and DNA constituents of the nucleolus has shed light on its structure/function and identified previously unknown roles in regulation of cellular homeostasis [32–36], analysis of the RNA constituents has also thrown up a few surprises. In particular, the identification of nuclear and nucleolar-targeted microRNA (miRNA) species and the demonstration that RNA interference (RNAi) can function in the nucleus [37] have forced a rethink of what was previously believed to be a solely cytoplasmic role for these factors (see [38] for review). miRNAs are genome-encoded small RNAs (about 22 nucleotides) generated via both canonical (involving Drosha, Dicer, and the RISC complex member AGO2) and non-canonical pathways (see [39] for review). By pairing to the mRNAs of protein-coding genes, miRNAs can direct their post-transcriptional repression via RNAi.

Though primarily a cytoplasmic process, RNAi factors have been found in complex in the nucleus, and RNAi has been shown to function in this compartment. Its regulation deviates from that of cytoplasmic RNAi, however [37]. Nuclear miRNAs have also been shown to regulate transcript stability, modulate alternative splicing, and induce epigenetic alterations to silence or activate specific transcripts [38]. Politz and Pederson [40] first detected a specific miRNA, miR-206, in both the cytoplasm and the nucleolus in 2006, and they and others have identified more nucleolar miRNAs since then [41–43]. Furthermore, differences observed in the localization of these miRNAs between cells suggest that nucleolar targeting might be a transient or regulated process or both [41]. Consistent with this idea, Lam and colleagues [43] identified 11 mature nucleolar-enriched miRNAs and demonstrated that their nucleolar/cytoplasmic partitioning involves XPO1-mediated shuttling. They further showed that cell stress induced by the introduction of foreign nucleic acids or infection with the influenza A virus could trigger translocation of these miRNAs from the nucleolus to the cytoplasm. This suggests that, similar to its role in regulated protein sequestration, the nucleolus might also function as a detention site for miRNAs, in this case keeping them inactive until they are released by specific stress signals. Interestingly, the Pederson group [44] has now demonstrated nucleolar retention of specific mRNAs, including a spliced IGF2 mRNA that contains target sites for all five of the miRNAs that they have localized to the

Figure 1. Structural/functional reorganization of the nucleolus in response to stress



(1) Stress stimulus-specific induction of long non-coding RNA transcripts from distinct regions of the intergenic spacer (IGS). (2) IGS transcripts bind and sequester/immobilize a diverse range of cellular proteins within the nucleolus. Interaction is mediated via a discrete nucleolar detention sequence (NoDS). (3) Formation of the nucleolar detention center leads to restructuring of nucleolar architecture and silencing of ribosome biogenesis.

nucleolus. Although speculative at this point, such observations may further the proposed miRNA sequestration role of this structure to that of a staging platform for the pre-assembly of certain mRNA-miRNA regulatory complexes.

Ribosome biogenesis-targeted chemotherapy

Following the first descriptions of the nucleolus in the 1830s by German physiologists Rudolph Wagner and Gabriel Valentin [44–46], the Italian pathologist Giuseppe Pianese in 1896 noted its excess volume within the nuclei of various malignant tumor cell samples [47]. Though not a diagnostic indicator, macronucleoli (and an increase in their number per cell) were later shown to reflect the high energy demands of hyper-proliferative cells and continue to be useful prognostic indicators for aggressive tumors (see [48] for review).

Decades of research have given a much greater appreciation of the complexity of nucleolar regulation, and this

organelle is now known to function within the cell as a central hub or 'control center' that coordinates cell growth and proliferation with metabolic and stress signals. In a recent review, Tsai and Pederson [49] summarize our current understanding of the nucleolar surveillance systems that are in place to monitor ribosome biogenesis and nucleolar integrity and to coordinate ribosome production with metabolic demand and cell cycle progression. Given that impaired transcription or processing of ribosomes can halt the cell cycle and trigger the p53 tumor suppressor pathway, the nucleolus is now considered not only a prognostic indicator but also a viable therapeutic target.

The push to develop drugs that specifically target RNA Pol I activity has produced promising candidates, such as the small-molecule inhibitors CX-3543 and CX-5461. Hannan and colleagues [50] demonstrated the selective destruction of B-lymphoma cells *in vivo* by CX-5461, with maintenance of a viable wild-type B-cell population. They used a murine model of spontaneous lymphoma in which the oncogene MYC is overexpressed in B-lineage lymphocytes, and demonstrated that accelerated rRNA transcription and ribosome biogenesis in these cells were essential for their survival. When mice were treated with CX-5461 to reduce this activity, rapid activation of p53 was triggered, leading to programmed cell death by apoptosis. Specifically, the ribosomal protein (RPL11 and RPL5)-MDM2-p53 nucleolar surveillance pathway was activated (see [51] for review). Importantly, CX-5461 did not trigger this response in normal spleen cells, nor did it affect spleen size or B-cell numbers. This work suggests that the dependence of certain tumor cells on hyper-activated rDNA transcription for survival can be exploited to selectively target these cells for destruction.

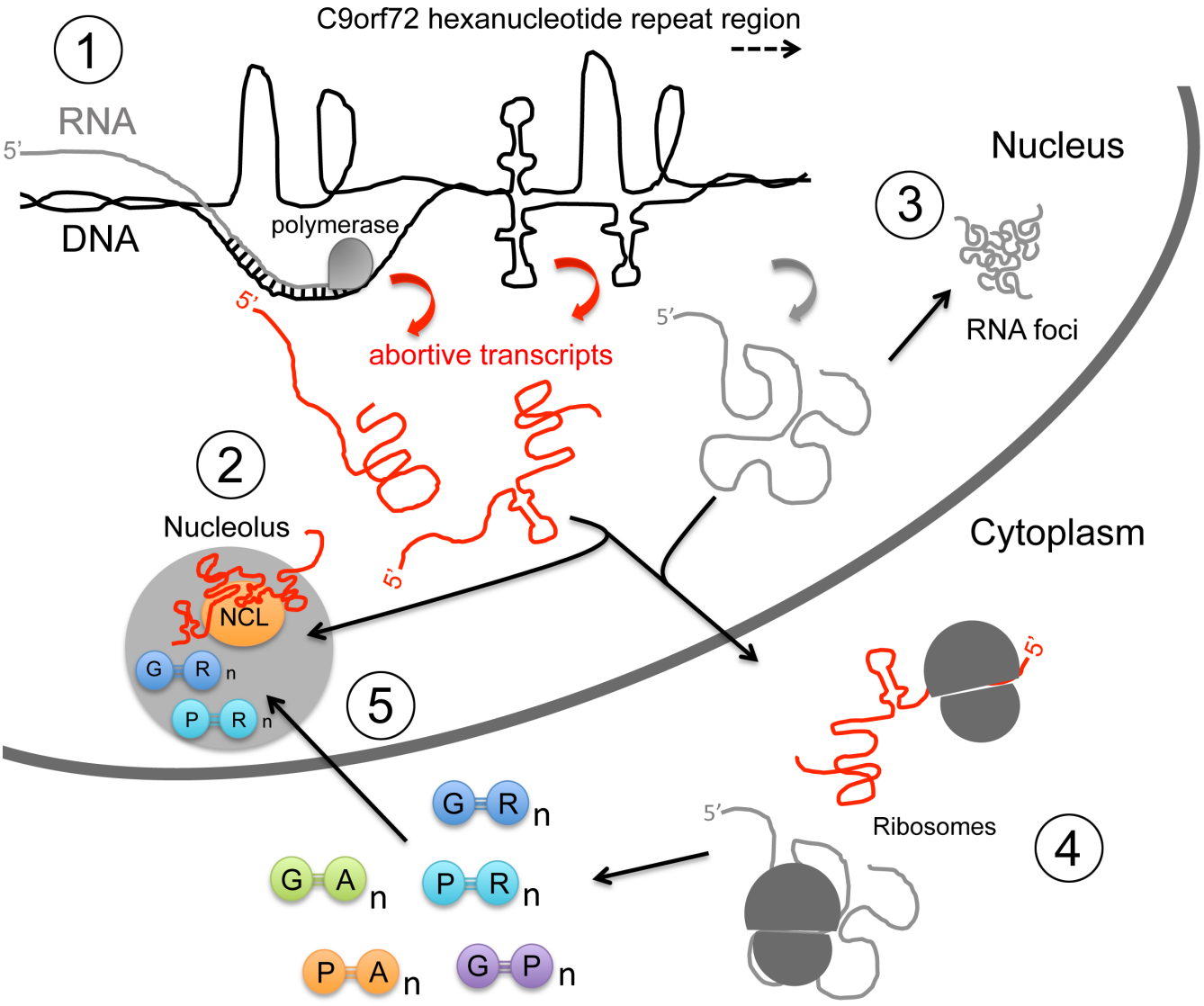
Using a chemical library screen to identify small molecules that activate the p53 tumor suppressor pathway, Laiho and colleagues [52] discovered and validated six compounds, two of which were shown to stabilize p53 by activating DNA damage repair pathways. They first showed that, of the remaining four, BMH-21 activates p53 by binding to GC-rich sequences (present at a high frequency in rDNA genes) and inhibiting RNA Pol I activity [53]. Interestingly, they found that BMH-21 treatment also induces proteasome-dependent destruction of RPA194, the large catalytic subunit of the Pol I complex. Assessment of the three remaining compounds—BMH-9, BMH-22, and BMH-23—showed that they similarly inhibited RNA Pol I activity and destabilized RPA194 in a proteasome-dependent manner [54]. Though targeting the same pathway, these drugs are mechanistically distinct from CX-5461, which appears to inhibit formation of the Pol I pre-initiation complex [55], and CX-3543, which selectively inhibits Pol I elongation [56]. And although p53-dependent nucleolar stress response pathways have been the most extensively studied to date, nucleolar proteins have also been linked to p53-independent pathways that culminate in cell cycle arrest and apoptosis (see [57] for review). Given that more than 50% of known cancers lack functional p53, there is particular interest in the possibility of activating such pathways.

Nucleolus and neurodegenerative diseases

Cancer is not the only disease state associated with nucleolar dysfunction. Nucleolar proteins have also been implicated in cardiac pathophysiology (see [58] for review), and nucleolar stress is the one common feature shared by neurodegenerative disorders that include Parkinson's disease, Alzheimer's disease, Huntington's disease, and spinocerebellar ataxias (see [59] for review). The last two belong to a group of poly-glutamine (polyQ) diseases that are caused by CAG repeat expansions within particular genes. Interestingly, these repeat regions have been shown to confer toxicity not only on the translated peptides but also on the mutant transcripts (see [60,61] for review). One outcome of this dysregulation is altered rDNA transcription, and Chan and colleagues [62] recently showed that expanded CAG RNAs can induce apoptosis by activating nucleolar stress pathways. This appears to be mediated, at least in part, via their interaction with the essential protein nucleolin (NCL), which results in titration of NCL away from the Pol I machinery and perturbed rRNA transcription [62].

A similar connection has been made for other neurodegenerative diseases, suggesting that this may be a common pathophysiological mechanism (see [63] for review). Specifically, the number of repeats in the hexapeptide repeat region (GGGGCC) in the DNA sequence of a gene designated c9orf72, normally present in 2-23 copies, can be expanded in patients with amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD) to 700-1,600 copies. ALS involves loss of motor neurons, whereas FTD is associated with degeneration of the frontal and temporal lobes of the brain; however, they overlap both genetically and pathologically, and the expansion accounts for 25-40% of all cases. In an effort to understand the underlying molecular mechanisms of its pathophysiology, Wang and colleagues [64] showed that the hexapeptide repeat expansion (HRE) forms DNA and RNA G-quadruplexes and promotes RNA-DNA hybrids (R-loops) that cause repeat length-dependent accumulation of abortive transcripts (Figure 2). They went on to identify a structure-dependent interaction between these transcripts and

Figure 2. The hexapeptide repeat expansion (HRE) in c9orf72 contributes to cellular toxicity at multiple levels



(1) Repeat region DNA and RNA form structures that can cause repeat length-dependent accumulation of abortive transcripts. (2) Abortive transcripts migrate to the nucleolus, bind, and mislocalize nucleolin (NCL), leading to impaired rRNA transcription and induction of nucleolar stress pathways. (3) Hexapeptide repeat expansion (HRE) transcripts accumulate in nuclear foci that can sequester RNA-binding proteins and disrupt RNA processing. (4) Repeat-associated non-ATG-dependent translation of HRE transcripts produces aggregative polydipeptides. (5) HRE-encoded polydipeptides can migrate to the nucleolus, resulting in inhibition of ribosome biogenesis and cell death.

NCL, which results in mislocalization of the protein and likely contributes to the nucleolar stress observed in patient cells.

The c9orf72 HRE transcripts can also be translated in an ATG-independent manner into GA_n, GP_n, or GR_n polymers (and the antisense into PA_n, PG_n, and PR_n polymers), which are disordered and hydrophobic and aggregate into foci in affected cells [65]. The McKnight lab [65] showed that exogenously applied GR_n and PR_n repeat polypeptides can enter cells and migrate to the nucleus, where they bind nucleoli and inhibit ribosome biogenesis, leading to cell death (Figure 2). Furthermore, Isaacs and colleagues [66] used *in vitro* and *in vivo* models to demonstrate that HREs promote neurodegeneration through translated dipeptide repeat (both poly-GR and

poly-PR) proteins. Taken together, these studies confirm that both the HRE transcripts and polypeptides are toxic to cells and suggest that their interaction with nucleolar proteins contribute to this toxicity and likely explains the nucleolar stress observed in patients.

Summary

As shown here, recent technological advances in the molecular dissection of the composition, assembly, and maintenance of the nucleolus, coupled with a growing appreciation of the pathological implications of its dysfunction, are providing new insights into its role as a multifunctional signaling hub that plays a key role in preservation of cellular homeostasis.

Importantly, its untapped potential as a therapeutic target is finally being appreciated, with ribosome biogenesis-targeted cancer therapeutics already in phase I clinical trials. The link between nucleolar stress and neurodegenerative disorders, while potentially identifying novel risk factors, may also help to develop therapeutic strategies that shift the life-and-death balance of the neurons to slow down their progressive loss.

Abbreviations

ALS, amyotrophic lateral sclerosis; CDK1, cyclin-dependent kinase 1; DFC, dense fibrillar component; FC, fibrillar center; FTD, frontotemporal dementia; GC, granular component; HRE, hexapeptide repeat expansion; LCS, low-complexity sequence; miRNA, microRNA; NCL, nucleolin; NOR, nucleolar organizing region; Pol I, RNA polymerase I; RBS, Roberts syndrome; RNAi, RNA interference; RPL, ribosomal protein; UBF, upstream binding factor.

Disclosures

The authors declare that they have no disclosures.

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