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The nucleolus: a raft adrift in the nuclear sea or the keystone in nuclear structure?

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Abstract

The nucleolus is a prominent nuclear structure that is the site of ribosomal RNA (rRNA) transcription, and hence ribosome biogenesis. Cellular demand for ribosomes, and hence rRNA, is tightly linked to cell growth and the rRNA makes up the majority of all the RNA within a cell. To fulfil the cellular demand for rRNA, the ribosomal RNA genes (rDNA) genes are amplified to high copy number and transcribed at very high rates. As such, understanding the rDNA has profound consequences for our comprehension of genome and transcriptional organization in cells. In this review we address the question of whether the nucleolus is a raft adrift the sea of nuclear DNA, or actively contributes to genome organization. We present evidence supporting the idea that the nucleolus, and the rDNA contained therein, play more roles in the biology of the cell than simply ribosome biogenesis. We propose that the nucleolus and the rDNA are central factors in the spatial organization of the genome, and that rapid alterations in nucleolar structure in response to changing conditions manifest themselves in altered genomic structures that have functional consequences. Finally, we discuss some predictions that result from the nucleolus having a central role in nuclear organization.

Introduction

Nucleoli are the largest non-chromosomal structures present within the eukaryotic nucleus. In yeast, the single nucleolus occupies approximately ¼ of the total nuclear volume in a position that is distal to the spindle pole body and in close contact with the nuclear envelope (1–3). In metazoans there can be multiple nucleoli, formed around distinct chromosomal loci, that differ from yeast in details of morphology but retain the dense staining caused by the prodigious production of ribosomes (e.g. reviewed in (4, 5)). Nucleoli are organised around the core ribosomal RNA (rRNA) gene regions, referred to as nucleolus organizer regions (NORs) (6). NORs can, in some instances, form secondary constrictions on metaphase chromosomes during mitosis.

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In eukaryotes, the NORs usually consist of rRNA genes that are organized into tandem repeat arrays, collectively known as the rDNA (Figure 1). rDNA gene copy number can vary from a few copies up to tens-of-thousands of copies, depending on the species (see (7) for a comprehensive table). For example, the well-characterized single rDNA array in *S. cerevisiae* consists of around 180 copies (8), while in humans there are five rDNA arrays (9) that together comprise 300–400 copies per diploid genome (10). There are very few known exceptions to the tandem repeat rule: the intra-cellular human pathogen *Pneumocystis carinii* (11) and *Tetrahymena* (12) both appear to have just a single rDNA locus, although the latter amplifies this copy in the macronucleus (12). Nevertheless, the vast majority of eukaryotes characterized to date have the canonical rDNA organization, in which the polycistronic rRNA coding region, consisting of 18S, 5.8S and 28S rRNA species (precise nomenclature varies somewhat between species), is interspersed with an intergenic spacer region (IGS) (13). The rDNA genes are the most highly-transcribed in the genome, with rRNA accounting for approximately 80% of total RNA in a cell (14, 15). Despite this, the rDNA is a mosaic of transcribed, typically highly, copies and completely silent copies (16). The organization of active and silent repeats within the linear rDNA array has yet to be determined. Similarly the role of the silent copies has not been completely resolved, although they are required for efficient DNA repair in budding yeast (17).

The nucleolus is a domain of the nucleus, rather than a body delineated by a membrane or the like. Nevertheless, it has a specific structure that, in mammalian nuclei, consists of an inner fibrillar centre, a dense fibrillar component outside of this, and a granular component surrounding this (18, 19), although see (20)). While this is the case in mammalian nuclei, lower eukaryotes, in particular several yeast species, only have two distinctly visible components: a fibrillar component and granules. Furthermore, the fibrillar component in many yeast species is more a collection of strands, rather than a dense body (5). In either case, it has been shown that at least the non-transcribed parts of the rDNA are concentrated in the FC (21).

The nucleolus is very protein dense (e.g. reviewed in (4)) and in humans contains at least 700 different proteins (22), while being relatively DNA sparse. The nucleolus emerges from the complex mixture of proteins that associate with the rDNA, such as upstream binding factor (UBF) (23). Creation of the spatial domain of the nucleolus may result from high concentrations of binding sites in a small volume effectively causing retention of these proteins (24) by preventing movement out of the zone, as shown for ribosome movement (25). However, rapid shuttling of proteins between the nucleolus and nucleus has been observed (18, 19), suggesting that the nucleolus is a dynamic structure.

The nucleolus is not just a site of ribosome biogenesis: it functions in a myriad of other nuclear processes, including cell cycle control (reviewed in (4)). Several proteins are known to localize to the nucleolus in a cell cycle-specific manner, including several that are associated with human disease (26). Furthermore, nucleolar localization of viral proteins involved in viral replication, including HIV, appears to be necessary for replication (19). Additionally, nucleolar structure changes in response to both environmental conditions and the cell cycle (18, 26). Such structural alterations, as well as alterations in the numbers of rDNA repeats, would relieve or exacerbate the retention of proteins sequestered in the

nucleolus as a result of changes in the spatial clustering of binding sites. Strikingly, several noncoding RNA transcripts from the rDNA IGS appear to bind and sequester proteins in the nucleolus, and are regulated by stress (27). Given its dynamic nature, and the central role it plays in responding to cellular and environmental challenges, we hypothesize that the nucleolus has a direct role in coordinating nuclear structural organization.

The nucleolus as an organizer of genome structure

The nucleolus can contribute to nuclear organisation through the sequestration and release of proteins that then, directly or indirectly, affect the organisation of the nucleus. However, for the remainder of this review we are going to consider the issues surrounding the possibility that the nucleolus plays a direct role in the regulation of genome structure and how this might be achieved. In this context, we refer to genome structure as the spatial organisation of the genome within the nucleus, thus this form of organization focuses on the DNA, although obviously all the attendant proteins and other factors are also part of this.

There is growing evidence that the genome takes on a specific structural arrangement within the nucleus. In human cells, different chromosomes are found to occupy chromosome “territories”, which have different positions in different cell types (28, 29). Genes are also observed to inhabit specific locations in the nucleus (3). Gene loops, that bring linearly distant enhancers in close spatial proximity to promoters, are also thought to be important for regulation of gene expression (*e.g.* (30–33)). Recently developed techniques derived from proximity-based ligation (34–36), such as GCC (37) and HiC (38), have been developed to experimentally determine global genome structure. While extremely powerful, these techniques suffer from limitations when it comes to aligning sequences from repetitive elements. Essentially because repetitive elements cannot be positioned to a unique position, they provide potentially confusing information in proximity-based ligation assays and are typically ignored (38–40). However, the rDNA is a special case and useful information can be obtained by collapsing the rDNA sequences to a single locus (37, 41, 42).

Computational-based approaches, utilising proximity-ligation data and biophysical characteristics, have been taken to model global genome structure (*e.g.* (38, 40, 43, 44)). Interestingly, few restraints are required to impart a crude order on *in silico* polymer-based reconstructions of the budding yeast nucleus (43, 44). However, one restraint that is required is the positioning of the nucleolus opposite to the spindle pole body (43), suggesting the nucleolus is a significant landmark for spatial organization of the genome.

Nucleolar localisation of rDNA has been shown to influence the organisation of other genomic loci in the malaria parasite, *Plasmodium falciparum* (45). Despite this, a structured nucleolus is not essential for nuclear function in yeast, as the rDNA genes can be deleted from their chromosomal locus and replaced with plasmid-encoded copies (46). These extra-chromosomally encoded rDNA genes form multiple, tiny dispersed nucleoli (47), and the growth of these strains is compromised. However, it remains unknown whether the growth defects stem from disruption of nuclear organization, or from attenuated rRNA transcription/processing (46). Nucleolar structure is also disrupted when yeast are forced to transcribe the chromosomal rDNA repeats with RNAP II, rather than RNAP I (48). The entire yeast rDNA

array can be shifted to another location within the genome, but in this case only minor phenotypic changes are observed, despite the nucleolus changing its position in the nucleus (49). This is consistent with a limited amount of published data that shows that specific rDNA:non rDNA interactions are sequence specific and independent of the chromosomal position of the non-rDNA locus (42). Thus, more work is required to deduce the effects of changes in nucleolar position on genome structure and function.

If the nucleolus directly regulates nuclear structure then it stands to reason that interactions between the rDNA repeats and other non-nucleolar loci are central to this. This is borne out experimentally in budding yeast where a majority of DNA-DNA interactions involve the rDNA (37). While it can be argued that this interpretation is simplistic and does not take into account the copy number of the rDNA, any interactions between rDNA and non-rDNA loci are candidates for interactions by which the nucleolus shapes genome organization. These interactions should involve rDNA loci that are directly accessible from the nucleoplasm and are not protected by being internalized within the nucleolar structure.

The division of rDNA units into highly transcribed copies and completely silenced copies may reflect a functional distinction between units buried in the nucleolar interior and those located at the nuclear-nucleolar interface, respectively (T. Kobayashi, pers. communication). While it is almost certain that a main driver for nucleolar organization is the centralization of massive biosynthesis of ribosomes, we speculate that the tandem repeat organization of eukaryotic rDNA genes also enables the conservation of contacts at the nuclear:nucleolar boundary while still maintaining dedicated transcription units within the nucleolus. Such a system would allow the flexible assignment of rDNA repeats to the different functional categories: transcription, repair, replication and structural associations, the latter having hitherto largely gone unrecognized. Therefore, the maintenance in eukaryotes of rDNA repeats with identical sequences (notably the non-coding regions (50)) at a much greater copy number than is needed for transcription alone, may ultimately stem from the ability of this system to seamlessly replace one repeat with another, ensuring that critical functions are maintained.

Transcription and nucleolus directed organization

The rDNA is not transcriptionally homogenous; instead, all three classes of RNA polymerase are present in the nucleolus, in at least some organisms. Aside from RNAP I transcription, RNAP II transcription appears to be widespread in eukaryote rDNA (27, 51–55). Furthermore, RNAP III-transcribed 5S rDNA genes are located within the rDNA repeat in several species, including yeast (Figure 1; (56)). Moreover, around thirty small interspersed nuclear element (SINE) retrotransposons that derive from RNAP III-transcribed genes are found scattered throughout the human rDNA intergenic spacer (57). This opens up the question as to the effect of this transcriptional heterogeneity on the spatial organisation of the nucleolus/nucleus.

Transcription-induced clustering represents a simple mechanism for spatial genome organization (58–60). Thus, polymerase class-dependent association of active or primed promoters in the rDNA may contribute to the coordination of nuclear-nucleolar structure. In

support of this idea, structures consistent with RNAP I transcription factories involving rDNA repeats have been observed in metazoan cells (60). Furthermore, RNAP III forms foci within the nucleoplasm, and not the nucleoli, of human cells, although it is possible that this is the result of SINE transcription (61). Transcription by all three eukaryotic RNA polymerases on overlapping regions of the rDNA repeat complicates this picture.

The simplest explanation for the overlapping polymerase activities within rDNA repeats is that the different RNA polymerase activities are temporally and spatially separated. This is supported by evidence that suggests a reciprocal relationship between RNAP I and II transcription in the rDNA (62, 63). Thus the presence of rDNA repeats on the nucleus/nucleolus interface may free them up to be transcribed by RNAP II and/or III. However in a yeast strain where rDNA repeat number is reduced to the extent that most copies are likely to be transcribed by RNAP I (64), RNAP II transcription is also high (51). This suggests that transcription by these two polymerases is not mutually exclusive.

In the case of RNAPs I and III, it is clear that the transcription units can co-exist. Not only are the 5S rRNA genes and 35S rRNA genes (transcribed by RNAP III and I, respectively) interspersed in the linear repeats but there is substantial evidence in the literature that 5S rRNA genes are associated with nucleoli even when located at distant sites in the linear genome (see below). Thus the dynamics of rDNA repeat transcription is an important area for future research.

The nucleolus and RNA polymerase III decoded genes

The spatial organisation of the 5S rDNA genes is one of the clearest examples of the nucleolus affecting nuclear organisation. While in *S. cerevisiae* the 5S rDNA are located with the large rDNA repeats, in most eukaryotes they are not, and instead are present either as one or more clusters of repeats (e.g. *Drosophila melanogaster*, chicken, *Arabidopsis thaliana* and human), in other repeat clusters (e.g. crustaceans, and dinoflagellates), or entirely linearly dispersed (e.g. *Neurospora crassa* and *Schizosaccharomyces pombe*) (56, 65–70). However, these differences in the linear organization of the 5S genes between species belie commonalities in their spatial localization. For example, in mice ectopic 5S rDNA gene sequences have been shown to promote nucleolar localisation (71). Similarly, in humans, one of the transcribed, linear clusters of 5S genes on chromosome I was shown to localize to a perinucleolar compartment (72). Moreover, the linearly dispersed 5S genes in many other eukaryotes have been shown to co-localize with nucleoli in three dimensions (73), suggesting that there are benefits to co-localizing the 5S genes with the other ribosomal genes. This is strong evidence for the nucleolus playing a direct role in the spatial organisation of the nucleus.

The co-localization of RNAP III decoded loci with nucleoli is not restricted to the 5S rDNA: tRNA genes also show interesting patterns of spatial organization. Eukaryotic tRNA genes are generally dispersed throughout the linear genomes, although in rare cases there are isolated linear clusters of tRNA genes. *Xenopus laevis* oocytes have developmentally regulated tRNA genes that are found in clusters (74), and multiple clusters of tRNA genes in *S. pombe* are located within the centromeric heterochromatin (73, 75, 76). They are also

frequent sites of genomic rearrangements (77, 78). In *S. cerevisiae* both microscopy and crosslinking proximity analysis show that tRNA genes cluster together and co-localize with the nucleolus (37, 42, 79–81). In addition, a smaller cluster of tRNA genes has also been identified at the centromere of *S. cerevisiae* (40, 43), consistent with the observation that the tRNA genes in *S. pombe* are primarily clustered at the centromere at a position offset from the nucleolus (82). As previously noted for 5S rDNA sequences, yeast tRNA coding regions confer interaction specificity with the nucleolus (42) indicating that position alone is insufficient to explain this phenomenon.

Little is known about the three-dimensional organization of tRNA genes in most eukaryotes, however, and whether they co-localize with nucleoli. This is important to determine, as metazoan nuclei can be 100 times larger than yeast but have only 2–3 times as many tRNA genes (83). Thus, there is a significantly greater structural problem to overcome, and the relative effect of tRNA gene clustering on overall genome organization will be much less. In this context, if RNAP III transcription units are key components for spatial organization, a significantly more frequent DNA element would be needed in complex eukaryotes. In this context it is interesting to consider that SINES, retrotransposons derived from RNAP III transcripts (usually tRNA and 7SL RNA), are found in great quantities in large eukaryotic genomes (84–86). There is evidence that SINES can form clusters in mammalian nuclei (87, 88) and substantial evidence that at least some SINES bind RNAP III complex components *in vivo* (89). It will be interesting to test whether some subset of these SINE clusters co-localize with nucleoli, especially in light of the finding that *A/tu* SINES are processed in the nucleolus (90).

By definition rDNA:non rDNA interactions must involve interplay between different loci, but it need not be direct and may involve RNA, proteins or other factors (*e.g.* epigenetic modifications) that facilitate either directed or self-assembled interactions. Irrespective of how the associations are stabilized, they must be flexible enough to allow reassignment of the rDNA repeat to another function without interfering with the primary function of the nucleolus: ribosome production. A simple model to explain the origin of these interactions is that the act of transcription or transcriptional regulation is responsible for interaction formation and/or maintenance. This is consistent with polymerase class-dependent association of different regions of the rDNA, and more generally with the idea of transcription factories. However, in the yeast strain where all the rDNA repeats are transcriptionally active due to enforced reductions in copy number, little or no phenotype is observed (17). It is possible that interactions with the rDNA may function to position non-nucleolar loci during nuclear division (91), when the rDNA are transcriptionally or replicatively inactive and accessible to other factors. In this case, transcription would not be the sole driver of interactions that involve the rDNA repeats.

The nucleolus and heterochromatin

The nucleolus appears to influence the chromatin structure of the DNA that surrounds it. In metazoans the nucleolus is commonly observed to be surrounded by shell of late-replicating heterochromatin. Similarly, tephritids (fruit flies) and other dipterans (true flies) also exhibit preferential associations of the rDNA with heterochromatin-rich chromosomes (92). In

Drosophila, there appears to be a direct relationship between the nucleolus and non-rDNA heterochromatin (93, 94). Furthermore, nucleolar association seems to be an important factor to maintain the heterochromatic state of the inactive human X chromosome (95), with the Barr body originally being known as the “nucleolar satellite” (96). Analysis of nucleolus-associated chromatin domains (NADs) in two human cell lines (*i.e.* HeLa and HT1080) identified satellite repeats as being the major components of the NADs (97). Repetitive elements have also been implicated as forming part of the NAD in yeast (98).

Overlap between some metazoan NADs and reported lamina-associated domains suggests that specific genomic regions could alternate between associating with the nucleolus and the nuclear periphery, either in different cells or at different times (97, 99). The regulation of this recruitment would necessarily affect the organization of the remainder of the genome too. However, large-scale relocations are not a necessity if relative long-range positioning can be maintained through alterations to the compaction levels of intervening regions, rather than simply by physical relocation of the DNA. In effect some contacts can be broken while others are maintained. However, direct recruitment of non-rDNA loci to the nucleolar boundary remains to be demonstrated. Therefore regulation of these interactions in response to specific signals or pathways is still a hypothesis that requires testing.

Do bacteria have nucleoli and do they also function to organize the nucleoid?

It has traditionally been thought that bacteria lack the equivalent of a nucleolus as their repetitive ribosomal DNA genes are organised as dispersed repeats. However, it is clear that the bacterial nucleoid is structured (100–108), and recent evidence suggests that the rRNA genes in *E. coli* may be transcribed in specific foci in the cell, opening up the idea that bacteria contain a nucleolus-like structure (109, 110) to facilitate recycling of RNA polymerase and coordination of ribosome assembly(111).

The different copies of bacterial ribosomal RNA genes, including the spacer regions, have high levels of sequence similarity. This finding was unexpected given the apparent dispersal of these genes in the genome. It has been proposed that sequence similarity is maintained through a process of gene conversion (112). Therefore putative bacterial nucleoli may serve not only to optimise rRNA transcription and hence growth (111), but also to juxtapose ribosomal DNA genes to facilitate gene conversion between the disparate copies.

Whether the bacterial equivalent of a nucleolus actually exists is an important area for future study as it will shed light on critical aspects of bacterial growth rate regulation(111).

Conclusions

Accepting that the nucleolus is not simply a raft adrift the nuclear landscape, what advantage is there in the nucleolus controlling nuclear structure? We contend that the answer lies in the central position that ribosomes have within cellular metabolism (Figure 2). Stresses of all kinds affect ribosome activity (*e.g.* reviewed in (113)), the production of ribosomes, and consequently the nucleolus itself. Responses to stress (*e.g.* reviewed in (114)) may

sometimes involve gross alterations to nucleolar structure (e.g. (115)). These alterations have been related to the release and stabilization of proteins from the nucleolus (e.g. (115) and reviewed in (114)) therefore it is likely that alterations to the NADs associated with the nucleolar boundary also occur during stress response, but direct evidence for this is lacking. Our hypothesis predicts that such alterations occur and cause stress related alterations to the associated genes, and these events are part of how the stress response is relayed to appropriate transcriptional networks outside the nucleolus (Figure 2). Thus, nucleolar structure acts as an intermediary between the genomic structural network that co-ordinates transcription, and the cytoplasmic translational network (Figure 2). The fact that regions of the nucleolus are acted on by the three different polymerases supports the rDNA's sensory role. This model is conceptually similar to the rDNA theory of aging proposed by Kobayashi (2008) (116). In this theory, the repetitive nature of the rDNA makes it uniquely prone to instability, and this instability acts as an early warning system for general genomic instability, triggering the aging pathway. Therefore we propose that nucleolar structure is the keystone that synchronizes expression and cellular responses by linking the distinct genomic and cytosolic protein networks within cells.

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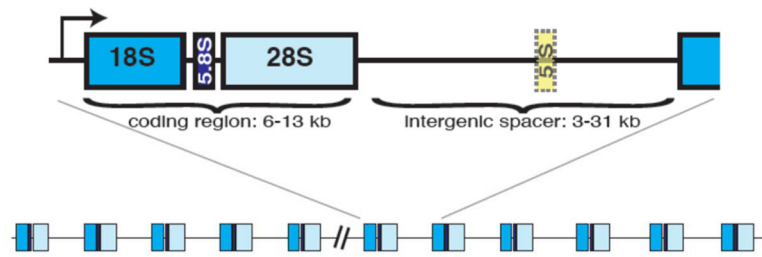
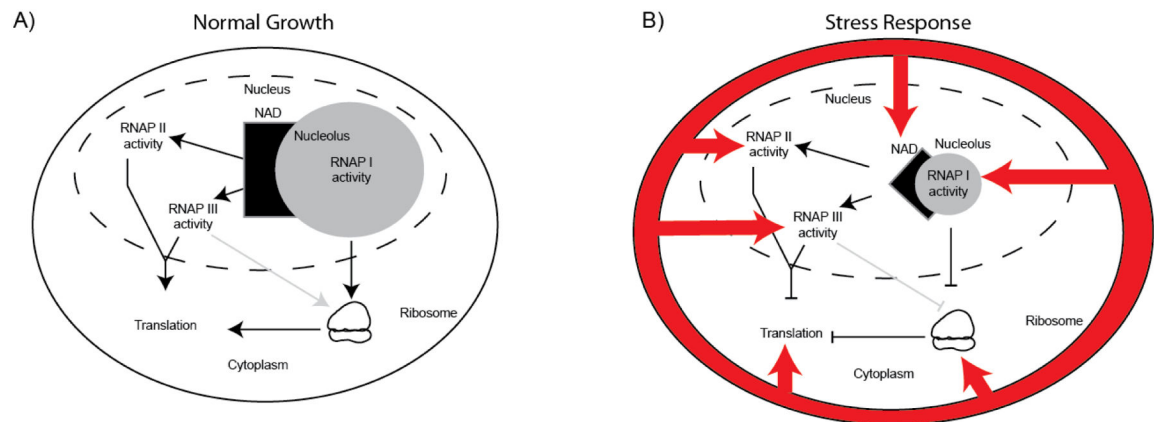


Figure 1.

Structure of the eukaryotic rDNA repeat. The structure of a typical eukaryotic rDNA repeat unit is shown in the upper part of the figure (not to scale), with the regions encoding the three major rRNA species (18S, 5.8S and 28S) illustrated as blue boxes. The inclusion of the 5S rRNA gene (hatched box) within the rDNA repeat unit is variable and depends on the organism being investigated. The direction of RNA pol-I transcription is indicated, as is the known variation in size of the coding region and the intergenic spacer amongst eukaryotes. Individual rDNA repeats are usually arranged into arrays of tandem as illustrated.

**Figure 2.**

Nucleolar structure and NADs act as an intermediary between the genomic structural network that coordinates transcription and the cytoplasmic translational network. A) In a permissive environment, the structure of the nucleolus is dictated largely by the RNAP I transcription levels. In turn, this sets the organization of the rDNA repeats and interactions with the genomic loci in particular the NADs, which affect RNAPII and RNAPIII transcription patterns (*i.e.* genes that are transcribed and also whether this transcription is efficient [occurring in factories] or less efficient [dispersed]) and levels (depicted by black arrows). Cytoplasmic translation also feeds back to the nucleus and all facets of RNAP activity (for simplicity these linkages have been omitted from this cartoon). The net effect of this is that nucleolar structure acts as a link to help coordinate nuclear processes while the nucleolar product (the ribosome) is the central facet in the cytoplasmic network. B) In a non-permissive (*i.e.* stress) environment (depicted by the red zone), environmental signals (red arrows) target nuclear (*i.e.* RNAP I RNAP II, RNAP III transcription) and cytoplasmic processes. By targeting RNAP I transcription, alterations are affected in nucleolar structure (depicted by smaller nucleolus) including changes in the NADs (depicted by alteration to shape). The net effect of this is to reinforce the signalling to the RNAP II and RNAP III transcription and subsequently effect a change in cytoplasmic translation.