

The nucleolus: structure/function relationship in RNA metabolism

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The nucleolus is the ribosome factory of the cells. This is the nuclear domain where ribosomal RNAs are synthesized, processed, and assembled with ribosomal proteins. Here we describe the classical tripartite organization of the nucleolus in mammals, reflecting ribosomal gene transcription and pre-ribosomal RNA (pre-rRNA) processing efficiency: fibrillar center, dense fibrillar component, and granular component. We review the nucleolar organization across evolution from the bipartite organization in yeast to the tripartite organization in humans. We discuss the basic principles of nucleolar assembly and nucleolar structure/function relationship in RNA metabolism. The control of nucleolar assembly is presented as well as the role of pre-existing machineries and pre-rRNAs inherited from the previous cell cycle. In addition, nucleoli carry many essential extra ribosomal functions and are closely linked to cellular homeostasis and human health. The last part of this review presents recent advances in nucleolar dysfunctions in human pathology such as cancer and virus infections that modify the nucleolar organization. © 2010 John Wiley & Sons, Ltd. *WIREs RNA* 2010 1 415–431

INTRODUCTION

The nucleoli are specific nuclear domains present in all eukaryotic cells. The nucleolus, a membraneless organelle, is the ribosome factory of the cell.¹ In cycling cells, nucleoli assemble at the exit from mitosis, they are functionally active throughout interphase, and they disassemble at the beginning of mitosis. The nucleolus is the site where different steps of ribosome biogenesis are grouped together, i.e., transcription of ribosomal genes (rDNAs), maturation/processing of ribosomal RNAs (rRNAs), and assembly of rRNAs with ribosomal proteins.² It was proposed that the nucleolus is ‘an organelle formed by the act of building a ribosome’.³ Indeed, the organization and size of the

nucleoli are directly related to ribosome production.⁴ Consequently, the size of the nucleolus is a diagnostic marker of highly proliferative cancer cells.⁵ The variability of the nucleolar organization has been intensively examined in different biological contexts such as proliferation, differentiation, development, and disease. The comparison of the nucleolar organization across evolution revealed both the conservation in the basic ‘building blocks’ and a higher complexity in modern eukaryotes.⁶ The nucleolus constitutes a model to understand the principles of the organization of nuclear domains, the dynamics of protein trafficking, as well as the interplay between nuclear bodies dedicated to related functions (Cajal body, promyelocytic leukemia body, and nuclear speckles).

Throughout the past 50 years, nucleolar complexity was deciphered using multiple approaches. This was possible thanks to technological breakthrough: specific *in situ* labeling, three-dimensional resolution, and improved isolation procedures of nucleoli for biochemical characterization and proteomic analysis. Thus, it was discovered that other ribonucleoproteins (RNPs) in addition to ribosomal subunits are assembled or processed in the nucleolus. The best example is the nucleolar assembly proposed for the signal recognition particle.⁷ In plant cells but not in animal cells, nucleoli have been implicated as

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sites of silencing RNA biogenesis.⁸ In addition, the comparative proteomics of animal and plant nucleoli demonstrated the nucleolar function adaptation (in humans^{9,10} and in plants¹¹).

Today, the nucleolus is considered a multifunctional domain. Extra ribosomal functions assigned to the nucleolus include the involvement in cell cycle and cell proliferation control, stress sensing and tumor surveillance pathways, apoptosis, telomere formation, transfer RNA modifications, viral life-cycle, etc. Unsurprisingly, nucleolar dysfunction has severe consequences for human health.^{5,12,13} These extra ribosomal functions of the nucleolus have been reviewed elsewhere and will not be discussed here (reviewed in Refs 14–16).

This review will essentially cover three topics: (1) nucleolar organization depending on ribogenesis activity and across evolution, (2) the principles of nucleolar assembly in cycling cells, and (3) the alterations in nucleolar organization in diseases.

NUCLEOLAR ORGANIZATION REFLECTS rDNA TRANSCRIPTION AND PRE-rRNA PROCESSING EFFICIENCY

Nucleoli assemble around the nucleolar organizer regions (NORs), as first proposed by McClintock in *Zea mays*.¹⁷ The NORs are chromosomal regions where multiple rDNA copies cluster in arrays. The number of NOR-bearing chromosomes varies depending on the species, ranging from 1 in haploid yeast cells to 10 in human somatic cells (acrocentric chromosomes 13, 14, 15, 21, and 22). On mitotic chromosomes, the active NORs are detected by a specific silver staining procedure, designated Ag-NOR staining,¹⁸ reflecting their continuous association throughout mitosis with a subset of argyrophilic proteins belonging to the rDNA transcription machinery.¹⁹

At the end of mitosis, when rDNA transcription by the RNA polymerase I (pol I) resumes (see below), active NORs are directly involved in nucleolar reassembly. Within each active NOR, only a subset of rDNA units are transcribed. In contrast, inactive NORs are not bound by argyrophilic proteins, they are not associated with the pol I machinery, and they are not involved in nucleolar formation. The nucleolus is either organized around a single NOR or alternatively several active NORs coalesce in a single nucleolus once rRNA synthesis has initiated.²⁰ For instance, in *Xenopus laevis* or *Potorous tridactylus* cells, there are two nucleoli per cells each corresponding to a single NOR, whereas in human HeLa cells, there are two to three nucleoli per cell corresponding to six active NORs.

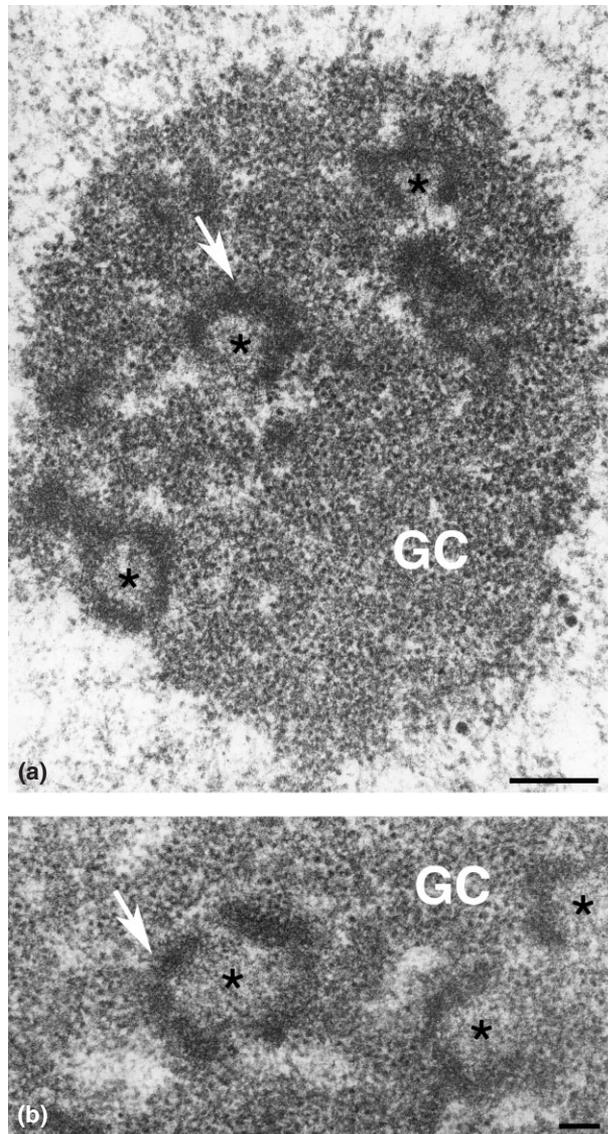
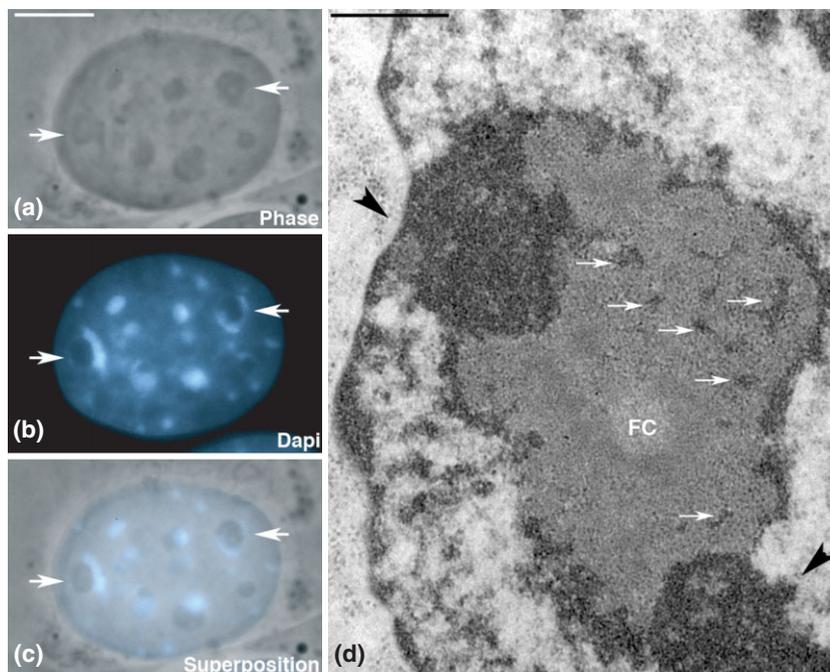


FIGURE 1 | Nucleolar organization of a human HeLa cell prepared by conventional methods for electron microscopy. The cells were fixed by glutaraldehyde and osmic acid. The sections were contrasted by uranyl acetate and lead citrate. (a) Section of one nucleolus and (b) details of the three nucleolar components. In (a) and (b), the three nucleolar components are visible: the fibrillar centers (asterisks), the dense fibrillar component (white arrow), and granular component (GC). Scale bar: (a) = 0.5 μm and (b) = 0.1 μm .

Nucleolar Organization in Higher Eukaryotes: A Tripartite Organization

The nucleoli observed by electron microscopy (EM) appear to be mainly composed of fibrils and granules on sections of fixed (formaldehyde and osmium) samples contrasted by uranyl and lead (Figure 1(a) and (b)). A great variability of the nucleolar morphology was described based on the types or functions in animal and plant cells.^{2,21}

FIGURE 2 | Perinucleolar heterochromatin in mouse NIH3T3 nuclei observed in light microscopy and EM are shown in left and right panels, respectively. (a–c) The heterochromatin is observed after DNA Dapi staining especially at the nucleolar periphery (arrows); the nucleoli (contrasted structure in phase) appeared as black holes with Dapi. (d) A protocol to preferentially reveal the nucleic acids in EM was used. The DNAs and RNAs were contrasted with uranyl after methylation and acetylation of the amino and carboxyl groups. Around the nucleolus, two large clumps of chromatin (arrow heads) are visible as well as the perinucleolar chromatin. White arrows indicate intranucleolar chromatin localized in the GC. One FC is visible in the middle of the nucleolus. Scale bar = 0.5 μm . EM, electron microscopy; FC, fibrillar center.



However, this variability resides in the arrangement of three fundamental components defined by their texture and contrast by EM and designated fibrillar centers (FCs), the dense fibrillar component (DFC), and the granular component (GC) (reviewed in Ref 22). The FCs are clear fibrillar areas of different sizes ranging from 0.1 to 1 μm containing fibrils (Figures 1(b) and 2(d)). They are partly surrounded by the highly contrasted DFC of compact texture. The FCs and DFC are embedded in the GC that mainly consists of granules 15–20 nm in diameter in a loosely organized distribution (Figure 1(a) and (b)). Using complementary approaches, a spatiotemporal map of ribosome biogenesis in these three nucleolar components was obtained including the localization of rDNAs, rRNAs, small nucleolar RNAs (snoRNAs), as well as several proteins belonging to transcription and processing machineries and ribosomal proteins. It was established that the sites of active pol I transcription are localized at the interface between the FCs and the DFC, where early processing of the pre-rRNAs occurs in the DFC and late processing in the GC. The nontranscribed part of the rDNAs as well as the pol I complexes and the transcription machinery such as the upstream binding factor (UBF) and topoisomerase I are localized in the FCs.²³ What is the role of FCs? The FCs appear to be pivotal elements to understand how pol I transcription organizes the nucleoli. It was first proposed that the FCs are the interphasic counterparts of the mitotic NORs because the nucleoli are reformed around the FCs at the end of mitosis (see *Nucleolar Assembly* section). The variability of the

FCs (number and volume) was then correlated with the transcriptional activity of the rDNAs in defined biological conditions. In the nucleolus of peripheral mature human lymphocytes with low activity, a single large (diameter = 0.2–0.4 μm) FC is visible.²⁴ Upon stimulation, the lymphocytes enter the cell cycle, ribosome biogenesis is stimulated, and the nucleolus becomes enlarged while the numerous small FCs are formed. It was proposed that when ribosome production is activated, the FCs unfold because a fraction of the rDNA copies present in the single FC are transcribed and the DFC is generated.²⁵ This conclusion is also supported by the observation of nucleoli corresponding to the activity of a single NOR-bearing chromosome as in *Potorous tridactylis* cells; the clusters of transcribed rDNAs are intercalated with repressed genes in the same rDNA tandem [repeat].²⁶ Consequently, we suggest that it would be interesting to re-evaluate the variability in size of FCs as a percentage of the total nucleolar volume and not only their number and size in EM sections. The prediction is that in small resting nucleoli (ring-shaped nucleoli of lymphocytes or remnant nucleoli of chick erythrocytes),⁴ the FC volume is high compared to the total nucleolar volume. We anticipate that this criterion could be a useful index of ribosomal gene activity.

Presently, it is not possible to exclude that FCs have additional uncharacterized functions. In nucleoli of stimulated rat neurons, the volume of only one FC increases to 10-fold^{27,28}; the causes for this cyclic variability are still unknown. In the nucleoli of rat

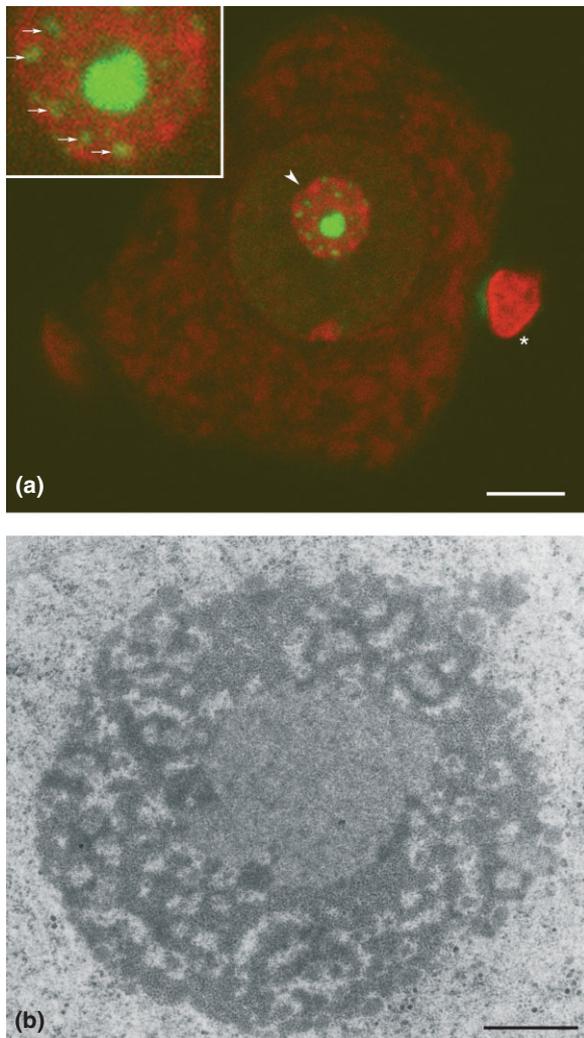


FIGURE 3 | GFC in neuron nucleolus. (a) The confocal image illustrates a trigeminal ganglia neuron immunostained for UBF (green) and counterstained with propidium iodide for nucleic acids. Two cells of different size are visible: the large cell corresponds to the neuron and the asterisk indicates the nucleus of a satellite glial cell. The nucleolus (arrow head) of the large neuron contains a prominent GFC visible in green and several FCs of normal size. In the enlargement (left corner) of the nucleolus, the FCs of normal size are indicated by arrows and the GFC is visible in the center. Scale bar = 5 μm . (b) Nucleolus in a trigeminal ganglia neuron observed in EM. A typical GFC is visible in the center of the nucleolus. Scale bar = 1 μm . Unpublished data from the group of M. Lafarga (I. Casafont and M. T. Berciano). EM, electron microscopy; FC, fibrillar center; GFC, giant FC; UBF, upstream binding factor.

sensory ganglia neurons, one giant FC (GFC) was observed (Figure 3(a) and (b)). The accumulation of UBF was demonstrated in this GFC (Figure 3(a)) as well as the absence of nascent RNAs. GFCs also contain components of the SUMO-1 conjugation pathway (SUMO-1 and Ubc9), but their role in GFC has not been determined.²⁹

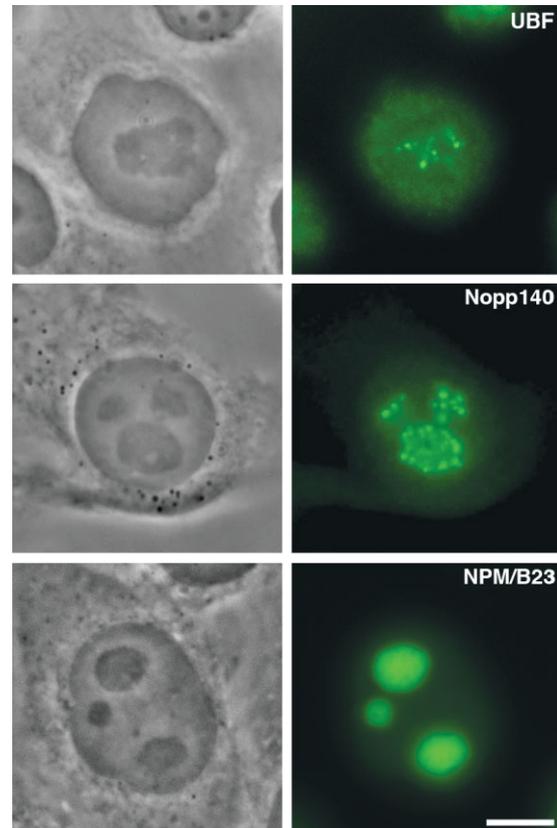
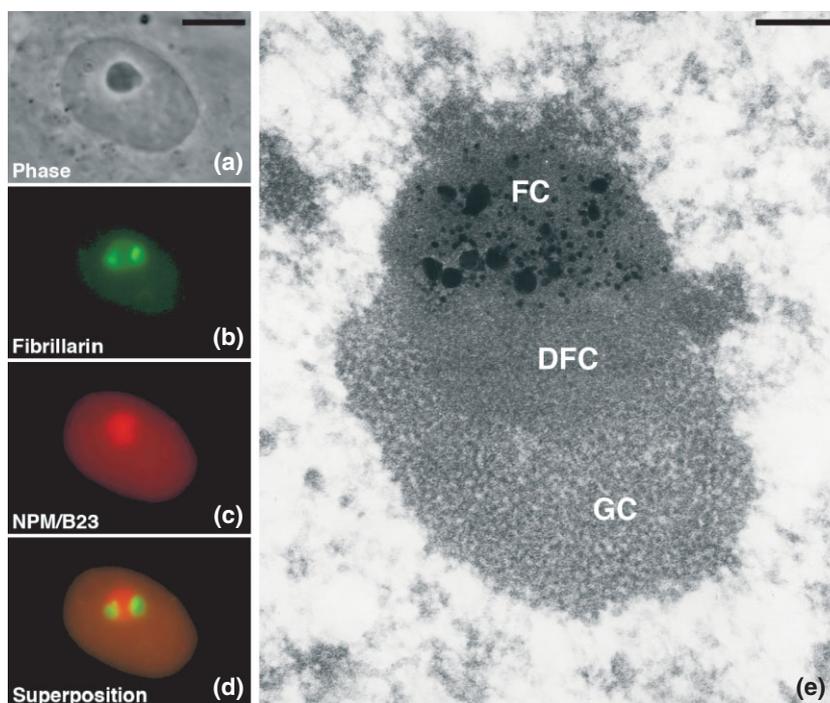


FIGURE 4 | Localization of nucleolar markers in the three nucleolar components in human HeLa cells. In the right panel, the green labelings show the distribution of the proteins and in the left panel in the same cells the nucleoli are visible in dark by phase contrast. Antibodies against UBF decorate several foci in the nucleolar interior corresponding to FCs. Nopp140-GFP (Nopp140) fusions exhibit a dotted labeling characteristic of the DFC of nucleoli. NPM/B23-GFP fusions (NPM/B23) decorate the GC. Scale bar: 5 μm . DFC, dense fibrillar component; FCs, fibrillar centers; GC, granular component; UBF, upstream binding factor.

The nucleoli are visible in the nucleus using phase contrast light microscopy (Figures 2, 4, and 5). The three basic nucleolar components can be mapped by immunolabeling or using fluorescent proteins corresponding to a precise step in the ribosome assembly pathway. For example, antibodies against fibrillarin or fibrillarin-reporter constructs identify the DFC and antibodies against nucleophosmin/initially B23 nucleolar protein (NPM/B23) and NPM/B23-GFP identify the GC.²⁵ This renders the three-dimensional analysis of the nucleolar organization in fixed or living cells possible by confocal microscopy. Typically in cells in which ribosome synthesis is active, the three nucleolar components are intermingled reflecting the vectorial formation of ribosomes: the FCs and DFC are distributed in the foci in the internal part of the nucleoli surrounded at the nucleolar periphery by the GC (Figure 4).

FIGURE 5 | Organization of the nucleolus after inhibition of pol I transcription. In light microscopy, the nucleolar segregation in a human HeLa cell treated with a low concentration of actinomycin D is observed in the left panel. (a) The nucleolus is visible in the nucleus by phase contrast. (b–d) The DFC visualized by fibrillarin–GFP fusion and the GC visualized by NPM/B23–DsRed fusion disengage and form two juxtaposed structures. In the right panel, (e) the segregation of the three nucleolar components observed in electron microscopy. This HeLa cell was treated with low concentration of actinomycin D and the Ag-NOR staining (black dots) revealed the Ag-NOR proteins in FC. DFC, dense fibrillar component; FC, fibrillar center; GC, granular component. Scale bar = 1 μ m.



Nucleolar Organization in Lower Eukaryotes: A Bipartite Organization

Despite the above classical description of a tripartite nucleolar organization in mammalian cells, many eukaryotes, including the genetically tractable yeast *Saccharomyces cerevisiae*, have only two morphologically distinct nucleolar components (discussed in Ref 6). Several features distinguish yeast from human nucleoli. A major difference lies in the internal organization of the organelle (number of components) and occurrence of intranucleolar bodies (*Subnucleolar Structure in Budding Yeast* section). In addition, yeast nucleoli lack condensed perinucleolar chromatin and show extensive nuclear membrane attachment (see below). Finally, yeast is characterized by a closed mitosis implying that its nucleolus does not disassemble during mitosis.

In budding yeast, there is a single nucleolus that occupies one third of the nuclear volume. In haploid yeast cells, depending on the growth conditions about 100–200 rDNAs cluster in a single NOR that localizes to the left arm of chromosome XII. There is about a 10-fold range difference in size between yeast and human nucleoli ($\approx 0.5 \mu\text{m}$ and from ≈ 0.5 to $9 \mu\text{m}$, respectively) and a human nucleolus is about the size of a yeast nucleus. In yeast nucleoli only two nucleolar components are detected: fibrillar strands (F) and granules (G) (Figure 6(a)). In contrast to the situation in humans where the fibrillar constituent generates distinctive FC/DFC modules

(Figures 1(a), (b), and 6(c)), in yeast, F is the only fibrillar component. A good demonstration of a bipartite nucleolar organization in yeast is provided upon nucleolar segregation conditions (Figure 6(b) and section *Nucleolar Organization Related to the Activity of Ribosome Biogenesis*).

Nucleolar Structure Across Evolution: Seeking the Transition Between Bipartite and Tripartite Nucleoli

The emergence of tri-compartmentalized nucleoli that coincides with the transition between anamniotic and amniotic vertebrates correlates with a striking expansion in the size of intergenic rDNA spacers that separates the pol I transcription units in rDNA arrays and this has been suggested to underlie the specialization of a single fibrillar component in two distinct compartments (discussed in Ref 6). Extended spacers might have allowed the specific exclusion of one form of chromatin by ‘looping it out’ from a defined nucleolar location into a novel compartment. At this transition lies the reptile group comprised of turtles, lizards, sphenodonts, snakes, birds, and crocodiles (Figure 7). Recent analyses indicate that turtle nucleoli are bipartite, whereas lizard, snake, bird, and crocodile nucleoli show three subnucleolar compartments (MT and DLJL, unpublished and illustrated for turtles and lizards in Figure 7). The emergence in higher eukaryotes of a third nucleolar compartment, the fibrillar center, a repository of pol I

FIGURE 6 | Nucleolar organization across evolution. Lower and higher eukaryotes are characterized by a bipartite (F and G) versus a tripartite (FC, DFC, and GC) nucleolar organization, respectively, as illustrated under physiological (a and c) and segregation (b and d) conditions. (a) A wild-type yeast nucleolus with fibrillar strands (F) and granules (G). (b) A yeast nucleolus from a cell deleted for *srp40*, the two nucleolar components are segregated and adopt a 'Ying-Yang' configuration. (c) A wild-type human nucleolus with several FC/DFC (asterisks and arrows) modules embedded into a single GC. (d) A human nucleolus following actinomycin D treatment (0.5 $\mu\text{g}/\text{mL}$, 2 h) with all three components segregated. All samples were treated by acetylation and inspected by EM. (a, b) Yeast *Saccharomyces cerevisiae*; (c) HEP-2 larynx carcinoma; and (d) Jurkat T lymphocyte. Scale bars = 0.2 μm .

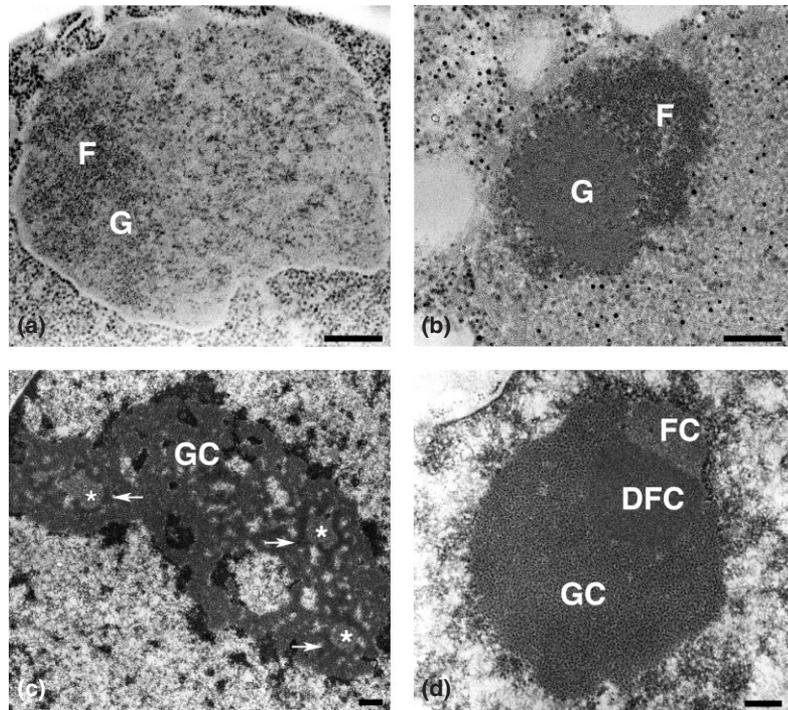
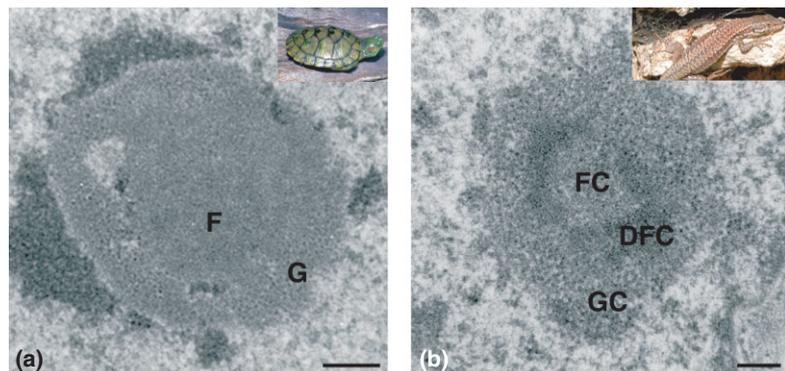


FIGURE 7 | Nucleolar organization at the transition between bipartite and tripartite organization. (a) A nucleolus from *Trachemys scripta* (red-eared slider) and (b) a nucleolus from *Podarcis muralis* (common wall lizard). All samples treated by acetylation and inspected by EM. Scale bars = 0.4 μm . EM, electron microscopy.



complexes ready to engage pol I transcription, might impart regulatory functions to nucleolar processes.

Subnucleolar Structures in Budding Yeast

In addition to nucleolar fibrillar strands and granules, several specialized subnucleolar domains have been described in budding yeast. These include the nucleolar body (NB) and the 'No-body', involved in snoRNA biogenesis and ribosome surveillance, respectively, as well as a nucleolar domain enriched in poly(A) RNAs. Subnucleolar compartmentalization might facilitate specific reactions such as RNA modification, RNA processing, and RNA degradation.

Nucleolar Body

The NB is a spherical body, Ag-NOR positive, of about 300 nm in diameter that emanates from fibrillar

strands (Ref 30 and M. T. and D. L. J. L., unpublished data). There is one NB per nucleolus. The NB has primarily been involved in snoRNA maturation. SnoRNAs are transcribed in the nucleoplasm as precursors carrying noncoding extensions, requiring specific maturation, and targeting the nucleolus where they function in RNA processing, in RNA modification, and, possibly, in RNA folding. Several box C + D snoRNAs were shown to initially concentrate in the NB prior to distributing to the overall nucleolar volume.³⁰ The transient accumulation of snoRNAs, such as U3, in the NB is thought to allow 5'-cap trimethylation by the trimethyl guanosine synthase Tgs1 and 3'-end processing. Ectopically expressed human survival of the motor neuron protein (SMN), a prototypic Cajal body/gem antigen, accumulates in the NB and led to

the suggestion that NB and Cajal body are orthologous structures. A visual screen for altered patterns of U3 nucleolar distribution led to the identification of mutations that affect NB localization³¹: three mutations were identified that led to the formation of a nucleolar ring corresponding to NB exclusion and two to snoRNA accumulation in the NB. Strikingly, mutations that led to NB accumulation affected both box C + D and box H + ACA snoRNAs indicating a certain level of commonality in snoRNA intranuclear trafficking pathways.

No-body

Each of the many steps in ribosome synthesis is subjected to an error rate and the possibility of producing misassembled ribosomes with potentially impaired translational capacity, and deleterious consequences for cell viability are immense (reviewed in Ref 32). To circumvent such problems, cells have evolved multiple quality control pathways that recognize and target defective RNP particles for rapid clearance. One of the best characterized nuclear surveillance pathway is the TRAMP-Exosome pathway where defective pre-RNPs are targeted for degradation following the addition of short poly(A) tails at the 3'-end of their RNAs by TRAMP. In this surveillance, polyadenylation acts as a recruitment and stimulatory signal for the RNA exosome that turns over the RNA (reviewed in Refs 33,34). The RNA exosome is a multiprotein complex endowed with both 3'-5' exoRNase and endoRNase activity that operates in RNA synthesis (formation of mature RNA 3'-end), RNA degradation (physiological RNA turnover), and RNA surveillance (clearance of defective RNPs).

The 'No-body' is a nucleolar focus, distinct from the NB, enriched in pre-rRNAs and RNA surveillance components that was detected in strains defective in the HEAT-repeat containing protein Sda1, a ribosome synthesis factor involved in pre-60S synthesis and export of both small and large subunits.³⁵ The following components have been localized to the 'No-body': small and large ribosomal subunits, TRAMP components, core exosome as well as nuclear specific exosome subunits.³⁵ Intact TRAMP and exosome complexes are required for 'No-body' formation consistent with a role of this organelle in the surveillance of nuclear-restricted pre-ribosomes.

Other Nucleolar RNA Surveillance Centers

Other putative nucleolar 'surveillance centers', distinct from the NB and 'No-body', are composed of foci enriched for polyadenylated snRNAs and snoRNAs³⁶ and a focus detected upon Rnt1 (yeast RNase III)

mild overexpression that juxtaposed with primary rRNA transcripts.³⁷ In human, nucleolar-associated foci enriched for the export factor Crm1 and the translational repressor CPEB1 have been described and referred to as CNoBs.³⁸ Whether CNoBs are related to NBs, No-bodies, or other nucleolar surveillance centers remain to be determined.

Nucleolar Organization Related to the Activity of Ribosome Biogenesis

Nucleoli are characterized by a great variability in size, number, and position within the nuclear volume and this variability depends on cellular metabolic activity. In cycling cells, the volume of the nucleoli increases between the G1 and G2 phases and the number of FC doubles in G2.³⁹ In quiescent cells at the terminal stage of differentiation when ribosome biogenesis is stopped, small ring-shaped nucleoli or nucleolar remnants (diameter = 0.3 μ m) are typically observed in lymphocytes or erythrocytes.⁴ These nucleoli are formed by one clear area containing chromatin and dense fibrils at the periphery. In erythrocyte nucleoli, active pol I transcription was not detected,⁴⁰ but a modified form of UBF was found as well as fibrillar, nucleolin, NPM/B23, U3 and U8 snoRNAs, and partially processed pre-rRNAs.⁴¹ Cell cycle stimulation of erythrocytes fused with cycling cells induced the reactivation of ribosome biogenesis in nucleolar remnants.⁴² Typical nucleolar organization with FCs, DFC, and GC is restored, whereas the reversibility of the repression was not observed in *Xenopus* erythrocytes upon incubation in extracts that failed to restore the cell cycle.⁴¹

A variety of drug treatments leading to transcriptionally arrested cells typically induce a phenotype of segregation in which the fibrillar and granular components of nucleoli disengage and form three juxtaposed structures (Figures 5(a), (e), and 6(d)). Following nucleolar segregation, nucleolar components are reorganized in such a manner that the two fibrillar components appear as individual caps juxtaposed to a central body corresponding to the GC.^{43,44} Likewise, nucleolar segregation can be achieved in yeast, for instance with mutations affecting ribosome *trans*-acting factors such as Srp40, the yeast homolog of mammalian Nopp140; here, consistent with a bipartite organization, only two nucleolar components become segregated (Figure 6(b)). Remarkably, there are physiological occurrences of nucleolar segregation that also correspond to the inhibition of rRNA synthesis at defined periods of differentiation and cell maturation.⁴ This process is thought to be directly linked to the

inhibition of pol I transcription and indeed it is observed during transcriptional arrest by inhibitors such as the intercalating agent actinomycin D that exhibits high binding affinity for GpC sites in rDNAs and preferentially affects pol I transcription at low doses. Interestingly, studies have shown that nucleolar segregation in cells treated with high doses of actinomycin D inhibiting both pol I and pol II transcription not only implicates reorganization of nucleolar components but also of an energy-dependent relocalization of molecules from Cajal bodies such as the p80 coilin, Cajal body-specific RNAs (scarNAs) and nucleoplasmic proteins.^{10,45}

The adenosine analog 5,6-dichloro-1-ribofuranosylbenzimidazole (DRB), a casein kinase (CK2) inhibitor, has a repressive effect on pol II transcription, decreases pol I transcription, and impairs pre-rRNA processing.⁴⁶ DRB reversibly induces unraveling of nucleoli into necklace structures.⁴⁷ On one hand, EM studies revealed that the nucleolar necklace is composed of small FCs partially surrounded by and connected to each other by the DFC, indicating that each bead of the necklace most probably corresponds to one functional transcription domain.⁴⁸ On the other hand, the nucleolar proteins involved in pre-rRNA processing are mislocalized in large bodies derived from the GC. When DRB is removed, reassembly of the nucleoli occurs. This process is CK2-driven and ATP/GTP-dependent.⁴⁹

Similar effects are obtained when cells are treated with the highly selective cyclin-dependent kinase (CDK) inhibitors, roscovitine, olomoucine, purvalanol, or alsterpaullone.⁵⁰ These CDK inhibitors modify both pol I transcription and pre-rRNA processing and induce a dramatic but reversible disorganization of active nucleoli, whatever the interphase stage of the cells. Because the transcription factor UBF is regulated by CDKs, e.g., CDK2–cyclin E,⁵¹ pol I transcription decreases after CDK inhibitor treatment but remains active. Remarkably, in addition to the decrease in pol I transcription, these treatments impair pre-rRNA processing.⁵⁰ Thus, the typical organization of nucleoli in the three major components, i.e., FCs, DFC, and GC, is undoubtedly linked to both pol I transcription and pre-rRNA processing.

The link between pol I transcription, pre-rRNA processing, and nucleolar structure is highlighted by studies based on the depletion of nucleolar proteins such as the transcription initiation factor TIF-IA, the mammalian homolog of yeast *Rrn3p*. In *TIF-IA*^{-/-} cells, the amount of pol I associated with rDNAs is severely reduced as well as pre-rRNA synthesis. After TIF-IA depletion, the size of

the nucleoli decreases, nucleolar structures disappear, and nucleolar proteins (observed for the p19^{Arf} tumor suppressor, the transcription factor UBF, and NPM/B23) are released from the nucleoli and localized in the nucleoplasm.⁵² Similarly, depletion of p19^{Arf}, a nucleolar protein reported to inhibit production of rRNAs by delaying the processing of 47S/45S and 32S pre-rRNAs,⁵³ results in morphological nucleolar changes.⁵⁴ Ag-NOR staining of *Arf*^{-/-} cells showed an increased number of Ag-NORs per nucleus and an irregular shape compared to control cells. At the EM level in *Arf*^{-/-} cells, the authors observed multiple, elongated, irregular nucleoli exhibiting larger FCs in comparison with the round nucleoli of wild-type cells. The depletion of the NPM/B23 multifunctional nucleolar protein was also reported to cause distortion of the nucleolar structure and fragmentation of nucleoli.⁵⁵ Recently, the GTP-binding nucleolar protein nucleostemin (NS) was shown to play a role in pre-rRNA processing as its depletion delays the processing of 32S pre-rRNAs to 28S rRNAs and induces the relocalization of proteins involved in pre-rRNA processing, i.e., DDX21 and EBP2, from nucleoli to nucleoplasm.⁵⁶ Interestingly, NS depletion leads to the dissociation of the components of snoRNPs and the telomerase complex, and to the disruption of the DFC and FCs in the nucleolus.⁵⁷

The Perinucleolar Domain

The Perinucleolar Chromatin

In most animal and plant cells but not in budding yeast, a heterochromatin layer is observed at the nucleolar periphery by EM^{4,58} (Figure 2(d)). This heterochromatin is visible with DNA Dapi staining (a positive ring surrounding a black hole) demonstrating the high DNA content at the periphery compared to within the nucleolus (Figure 2(a) and (c)). Incidentally, the first protocols established to isolate nucleoli from rat hepatocytes for biochemical purposes included a DNase treatment to remove this chromatin layer.⁵⁹ Around the nucleolus in human cells, it was demonstrated that chromatin motion is constrained in a manner similar to that of perinuclear chromatin.⁶⁰ It would be important to characterize the genes or sequences located in the chromatin layer around the nucleolus to understand the complexity of the interactions of the nucleolar domain in the nucleus. Recently, nucleolus-associated chromatin domains (NADs) were isolated, sequenced, and characterized in human cells.⁶¹ Different gene families and certain satellite repeats were identified as being the major blocks of NADs; altogether they correspond to not less than 4% of the total genome sequences.⁶¹ In

addition at the periphery of the nucleolus, a specific domain was designated the perinucleolar compartment (PNC).⁶² The PNC is associated with a specific DNA locus and is highly enriched in RNA-binding proteins and pol III transcripts.⁶³

The Nucleolus and the Nuclear Envelope

There is an intimate and evolutionarily conserved relationship between nucleoli and the nuclear envelope. This connection has long been known, but its exact significance remains elusive. Why does the yeast nucleolus contact so extensively the nuclear membrane? In fast-growing yeast cells, not less than 2000 ribosomes are exported every minute, and one possibility is that under these circumstances a fraction of maturing pre-ribosomes directly transit from the nucleolus to the cytoplasm through this interface. Nuclear membrane attachment might also serve a regulatory function under unfavorable growth conditions by promoting transfer of the nucleolar material to the vacuole for bulk degradation and recycling by piecemeal microautophagy of the nucleus.⁶⁴ In higher eukaryotes, nucleoli are also frequently located close to the nuclear envelope and this location might serve a similar function.⁶⁵ There are also cases in higher eukaryotes where centrally located nucleoli are directly connected to the cytoplasm through invaginations, the so-called 'nucleolar canal', of the nuclear membrane (discussed in Ref 66). Strikingly, 'nucleolar canal' formation strictly depends on the presence of rDNA transcription in micronuclei containing one active NOR,⁶⁷ consistent with a function in ribosome export. Furthermore, dynamic tubular nuclear channels comprised of invaginations of the nuclear envelope, in essence cytoplasm incursions, have been detected in many human cell types.⁶⁸ These channels which are fenestrated by nuclear pores either intersect the nuclei completely resulting in 'doughnut-like' structures occasionally associated with nucleoli or terminating close to or at nucleoli; in both cases such topology is consistent with a role in ribosome export. The number of channels and their complexity (branching) vary widely, but remain characteristic of a given cell type. It is not known whether channel occurrence and complexity reflect cell proliferation rates or whether it increases in disease situations.

NUCLEOLAR ASSEMBLY

Nucleolar assembly during the cell cycle in higher eukaryotes and nucleologenesis during embryonic development have been abundantly described during the past two centuries and in the last 20 years the

molecular mechanisms regulating these processes were progressively unraveled (reviewed in Refs 69,70). It was decided to focus this chapter on some principles that govern the establishment of the nucleolar function after mitosis in cycling cells or during embryogenesis.

Inherited Machineries

The assembly of nucleoli in higher eukaryotes is directly dependent on pre-existing machineries and complexes inherited through mitosis from the previous interphase. The processing machineries derived from nucleolar disassembly transit through mitosis and become the building blocks for the new nucleoli. At the onset of mitosis in early prophase, the pre-rRNA processing machineries are released from the nucleoli concomitantly with condensation of chromatin into mitotic chromosomes and before the arrest of pol I transcription.^{71,72} The nucleolar processing proteins preferentially localize around the chromosomes and remain attached to the surface of isolated chromosomes forming a peripheral chromosome layer.⁷³ The colocalization of the different factors involved in pre-rRNA processing (GC and DFC proteins and snoRNAs) suggests that processing complexes are at least partly maintained during mitosis.⁷⁴

Pol I transcription is repressed at the beginning of mitosis and reactivated in telophase. During mitosis, the pol I transcription machinery remains associated to rDNAs within NORs that were transcriptionally active during the previous interphase.¹⁹ As demonstrated in HeLa cells, the six active NORs are inherited and will participate in nucleolar assembly in the following G1 phase. Recent quantitative kinetic analyses have revealed that some pol I subunits, including RPA39, RPA16, and RPA194, might transiently dissociate from the NORs during metaphase and reappear in anaphase.^{75,76} A key issue is the characterization of 'active' versus 'inactive' NORs, i.e., NORs not associated with the pol I transcription machinery and not involved in nucleolar formation at the exit from mitosis.¹⁹ It was established that when pol I transcription is arrested during mitosis, UBF remains associated with noncondensed rDNA in active NORs.⁷⁷ By integrating large arrays of heterologous UBF-binding sequences at ectopic sites on nonacrocentric human chromosomes, McStay and collaborators⁷⁸ described the formation of pseudo-NORs. They established that UBF binding and the subsequent protein-protein interactions are responsible for the formation of structures that exhibit the characteristics of active NORs, i.e., the Ag-NOR stainable secondary constriction and the association with the pol I transcription machinery. As pseudo-NORs

are transcriptionally silent, the pol I transcription activity in the previous interphase is not a prerequisite for the formation of active NORs. Conversely, the pol I transcription inactivity is insufficient by itself to explain the existence of 'inactive' NORs. How UBF discriminates between transcriptionally active and silent rDNAs remains to be elucidated to understand the existence of both active and inactive NORs.

Inherited 'Unprocessed' rRNAs

At the time of nucleolar assembly, in addition to transcription and processing machineries inherited from the previous cell cycle, we demonstrated that inherited 'unprocessed' rRNAs are involved in two biological situations. In *X. laevis* embryos, transcriptions are successively activated, i.e., pol II and pol III transcriptions during mid-blastula transition (MBT) and later pol I transcription. During MBT unprocessed 40S pre-rRNAs containing 5'-external transcribed spacer sequences were detected in embryonic nuclei, localized with UBF and fibrillarin before activation of pol I transcription.⁷⁹ These pre-rRNAs of maternal origin, stored in the cytoplasm, enter the nucleus and participate in the structural organization of the nucleolus prior to acquiring its transcription competence.^{79,80} In particular, they are localized in foci called prenucleolar bodies (PNBs) and are associated with the NORs. On the contrary, these pre-rRNAs were not imported into erythrocyte nuclei incubated with egg extracts containing these pre-rRNAs.⁴¹ It is still unknown how these maternal 40S pre-rRNAs are stabilized in the cytoplasm of embryonic cells and what is the signal allowing their nuclear import prior to nucleolar assembly.

In cycling cells, the arrest of pre-rRNA processing occurring at the onset of mitosis takes place before the arrest of pre-rRNA synthesis.^{70,77} Consequently, partially processed 45S pre-rRNAs are generated at the G2/M transition.⁸¹ This confirms previous observations that 45S and 32S rRNAs are present in metaphase-arrested cells.⁸² These 45S pre-rRNAs localize around the chromosome during mitosis.⁸¹ In telophase they are associated with processing proteins in PNBs (see below) and are recruited to UBF-associated NORs independently of pol I transcription.⁸¹

PNB Formation During Nucleolar Assembly

The pre-rRNA processing complexes persist throughout mitosis mostly at the chromosome periphery. During telophase and early G1, when nuclear functions are reactivated, these pre-rRNA processing complexes are regrouped in PNBs.^{83,84} PNB formation

is a general process described in all higher eukaryotic cells inspected at this period of the cell cycle (Figure 8). In addition, in some cells containing abundant pre-rRNA processing machineries, the formation of nucleolar-derived foci corresponding to nucleolar processing complexes are observed in the cytoplasm during mitosis and these complexes are imported into nuclei in early G1.^{85,86} Processing proteins (fibrillarin, NPM/B23, nucleolin, Nop52, etc.) from DFC and GC are localized in the PNBs as well as the box C + D snoRNA U3⁸⁴ and 45S pre-rRNAs.⁸¹ Thus the PNBs are transitory structures that gather the building blocks of the nucleolus machineries. What could be the function of this intermediate step in the delivery of processing machineries during nucleolar assembly? It was proposed that PNBs move to the sites of pol I transcription to deliver the pre-rRNA processing complexes. PNB dynamics in living cells do not reveal such directed movement of PNBs toward the NORs.^{20,87} Analyses by time-lapse fluorescence resonance energy transfer demonstrates that proteins of the same pre-rRNA processing machinery interact with each other within PNBs, but not when they are localized at the chromosome periphery.⁸⁸ The timing of these interactions suggests that PNBs could be preassembly platforms for pre-rRNA processing complexes.⁸⁸ This notion is compatible with the recent description of autonomous preassembled protein modules comprised of several individual ribosome synthesis factors and the recent description of their stochastic recruitment to nascent transcripts (discussed in Ref 32,89). Using photoactivation, the flux of proteins between NORs and PNBs was measured in living cells at different periods of the nucleolar assembly. It appears that the recruitment of the processing complexes first of DFC and then of GC during nucleolar assembly is due to PNBs.⁹⁰

Cell Cycle Control of Nucleolar Assembly

The mechanism that governs the DFC disassembly of nucleoli in prophase is linked to the repression of pol I transcription, induced at least in part by CDK1-cyclin B-directed phosphorylation of components of the pol I transcription machinery.^{91,92} In prophase, the repression of pre-rRNA processing most probably occurs before the repression of pol I transcription. These observations raise the possibility that pol I transcription and pre-rRNA processing are repressed in prophase either by distinct mechanisms or by similar reactions operating with different kinetics. In favor of the second possibility is the observation that the RNA-binding affinity of B23/NPM is decreased following CDK1 phosphorylation and that this is thought to trigger its release from the nucleolus.⁹³

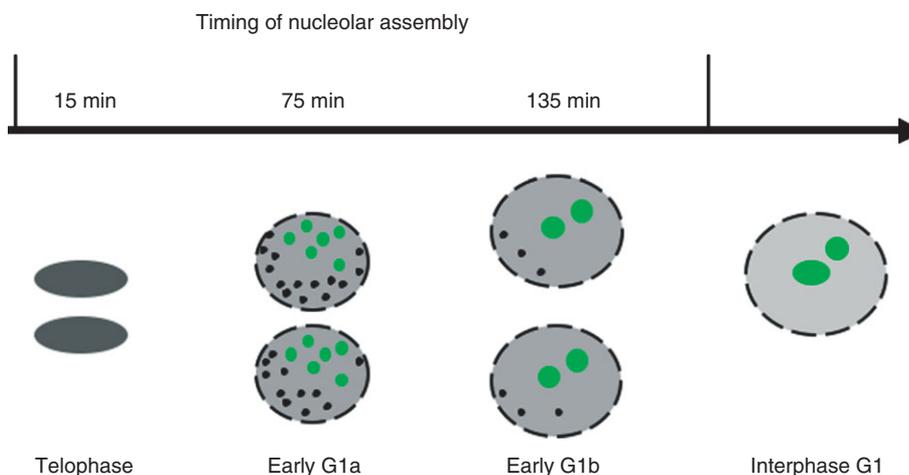


FIGURE 8 | In cycling cells, nucleolar assembly takes about 2 h. In HeLa cells, transcription by pol I starts in telophase in the six active NORs, whereas the mitotic chromatin is still condensed (illustrated as two oval dark structure). In early G1a, the mitotic chromatin decondenses (illustrated in grey), the nuclear envelope (broken line) is assembled, numerous PNBs (dark foci) are formed, and the active NORs recruit the processing proteins in DFC (green). In early G1b, the processing proteins are almost completely transferred from PNBs to GC, and NORs regrouped in two to three nucleoli. Interphasic cells are generated, when the cytoplasmic bridge (not shown) between the two daughter cells is broken. GC, granular component; NOR, nucleolar organizer regions; PNBs, prenucleolar bodies.

At exit from mitosis, the formation of nucleoli (Figure 8) is also a regulated process: inactivation of CDK1-cyclin B occurring at the end of mitosis induces the first events of nucleologenesis. This corresponds to release from mitotic silencing of pol I transcription, PNB formation around mitotic 45S pre-rRNAs, and traffic of early pre-rRNA processing components to transcription sites. In addition to inactivation of CDK1-cyclin B, another CDK activity is indispensable in early G1 to promote the last events of nucleologenesis and to form a functional nucleolus. Indeed, cells exiting from mitosis in the presence of a CDK inhibitor exhibit neither relocalization of the late pre-rRNA processing components from PNBs to pol I transcription sites, resumption of proper rRNA processing, nor formation of functional nucleoli.⁵⁰ The balance between the CDK1 kinase and PP1 phosphatase activities certainly regulates cell cycle dissociation and re-association of the nucleolar component. Moreover, PP1 is also regulated by CDK1 during mitosis.⁹⁴

Nucleoli assemble at the exit from mitosis concomitantly with the resumption of pol I transcription at the level of active NORs.¹⁹ However, the formation of functional nucleoli is not governed solely by the resumption of pol I transcription. Indeed, (1) the reactivation of pol I transcription in mitotic cells does not lead to the formation of nucleoli,⁹⁵ (2) initiation of nucleolar assembly occurs independently of pol I transcription,⁸¹ and (3) at the exit from mitosis nucleologenesis is impaired in the presence of either a CDK inhibitor or leptomycin B even if rDNAs are transcribed.^{50,90}

NUCLEOLUS AND DISEASE

As a testimony to its great plasticity, the occurrence, shape, and size of nucleoli are frequently altered in disease situations involving increased cell proliferation rates or viral infections. These morphological differences are often correlated with both quantitative and qualitative differences in ribosome synthesis. In addition, defective ribosome surveillance recently emerged as a possible causal effect for several human diseases with the suggestion that the accumulation of chemically modified ribosomes, e.g., oxidized particles, might contribute to the progression of neurodegenerative diseases such as Alzheimer and Parkinson diseases (reviewed in Ref 32). Ribosome oxidation might alter ribosome function and might result from intracellular exposure to reactive oxygen species or environmental exposure to UV or other debilitating treatments. Finally, several nonribosomal functions of the nucleolus, for instance in cell cycle regulation or telomerase trafficking, are directly required for cellular homeostasis (reviewed in Ref 15,16). Here we have focused on cancer and viral infections owing to space limitation. Other ribosomopathies are described in recent reviews.^{96–98}

Cancer

In aggressive human breast cancer cell lines, the average number of FC/DFC modules increases from four to six and overall rRNA production by $\approx 20\%$.^{99,100} It is not only higher amounts of ribosomes that are produced but alternative pre-rRNA

processing pathways that are activated resulting in the accumulation of pre-rRNA precursors that are not normally detected, as well as the production of specialized ribosomes characterized by differential RNA modification patterns and altered translational capacities.⁹⁹ The detection in cancer cell lines of rRNA positions that are specifically hypermodified by 2'-O methylation (i.e., positions which are modified in more ribosomes), including several positions that map to functionally relevant ribosomal sites, indicates that under physiological conditions a certain level of hitherto unsuspected hypomodification prevails (i.e., positions that are not normally modified in all ribosomes). Although it is currently not understood how distinctive rRNA modification patterns are generated, it is possible it reflects the use of alternative pre-rRNA processing pathways and that these impinge the specific sequential recruitment of snoRNP complexes and RNA modifying complexes. From this point of view, rRNA modification patterns might be considered as ribosomal assembly stigmata. *In vivo*, the translational capacity of these 'cancer ribosomes' is affected as both translational fidelity and IRES-dependent translation are reduced. Regulation of IRES-dependent translation might be directly relevant to cell transformation as several tumor suppressors and proto-oncogenes depend on internal initiation for their expression (reviewed in Silvera et al.¹⁰¹). Recently, 36 chemotherapeutic agents were discriminated for their effects on nucleolar morphology, pre-rRNA synthesis, and pre-rRNA processing; strikingly drugs that affect most strongly RNA synthesis and early steps of ribosome synthesis (e.g., actinomycin D, cisplatin, and roscovitine) are those that most markedly alter nucleolar morphology while those that affect later stages of ribosome assembly (e.g., 5-fluorouracil or MG-132) leave the nucleolus relatively intact.¹⁰²

The PNC (*The Perinucleolar Domain* section) is predominantly present in cancer cells derived from solid tumors.¹⁰³ The association of PNC with metastasis capacity was demonstrated in several cancers.¹⁰⁴ The role of PNC in malignancy is not fully characterized. However, its formation depending on pol III transcription correlates with the fact that elevated pol III synthesis can drive oncogenic transformation.¹⁰⁵

Viral Infections

Like many viral infections, Herpes simplex virus type 1 (HSV-1) leads to striking nucleolar morphology alterations^{99,106} (reviewed in Greco¹⁰⁷). Ribosomes synthesized in HSV-1 infected cells differ from those

produced in uninfected cells by their protein composition (Ref⁹⁹ and references therein). The amount of individual ribosomal proteins and their level of post-translational modification is altered and additional nonribosomal proteins are associated with ribosomes; these include cellular proteins of nonribosomal origin, such as the poly(A) binding protein 1 (Pab1) and most strikingly, proteins of viral origin. In the initial phases of HSV-1 infections ribosome synthesis is sustained and later decreases, an effect that until recently was thought to reflect pol I down regulation. It appears that there is in fact no alteration in rRNA synthesis and no gross increase in cytoplasmic rRNA degradation but that upon viral infection novel, and likely unproductive, pre-rRNA processing pathways are activated leading to the accumulation of three novel virally induced rRNA intermediates.⁹⁹ Furthermore, the global level of rRNA methylation is severely affected, a possible consequence of fibrillarin redistribution outside the nucleolus.¹⁰⁸ Under physiological conditions, the tumor suppressor p53 is highly unstable owing to its ubiquitination by Hdm2 and degradation by the proteasome. Following a variety of nucleolar stresses, such as those that stop the production of ribosomes, unincorporated ribosomal proteins are free to interact with and titrate Hdm2 resulting in p53 stabilization, cell cycle progression defect, or apoptosis (Ref 109 and references therein). It is possible that in virally induced ribosome assembly pathways, unfaithful, and likely short-lived, pre-ribosomes partially counteract the effects of nucleolar stress by somehow limiting the amount of free ribosomal proteins available to interact with Hdm2; consistently, p53 is unstable upon HSV-1 infection.⁹⁹ From an evolutionary standpoint, this can be considered a viral strategy to delay the host cell death. It is not yet known if other viruses use such a strategy.

CONCLUSIONS

Does the nucleolus solely result from the act of building a ribosome? A major contributor to nucleolar formation is undoubtedly the rDNA itself which, present in multiple and highly clustered copies, is heavily transcribed resulting in the recruitment of elevated local concentrations of dozens of specialized proteins and RNPs that transiently interact with each other. There are several reactions that precisely regulate the dynamic assembly–disassembly of the nucleolus in cycling cells and these are intimately linked to the control of rRNA synthesis by phosphorylation and to the timely recruitment of pre-existing, inherited, nucleolar components. This combined with the rapid establishment of a dynamic

flux of RNAs and proteins to the sites of RNA synthesis and RNP assembly is likely to result in the morphologically detectable structures that we have reviewed here. Prokaryotes and Archaea have much fewer rDNA copies, usually dispersed in the genome as individual units rather than clustered in arrays, they have much fewer *trans*-acting factors and no detectable nucleolar structure. The transient nature of the interactions that take place between nucleolar constituents underlies the dynamics and great plasticity of the nucleolus as often illustrated in disease situations. Whether in addition some nucleolar constituents act more directly within an underlying structural framework is not yet known and this is a fascinating question for future research. Another outstanding question is to address what intrinsic physicochemical properties underlie the remarkably clear-cut boundaries between each subnucleolar compartment and to establish whether they correspond to specific steps in the ribosome synthesis pathway. The DFC–GC transition might, for instance, correspond to the separation between the 40S and 60S subunits. Several nucleolar subdomains have been described in yeast and human, their quality as ‘RNA surveillance centers’ requires further work.

The nucleolus and its organization have been selected and highly conserved during evolution, and further, nucleolar complexity has increased with the emergence of amniotic vertebrates and the acquisition of a third nucleolar compartment. Among the evolutionary benefits that one can think of are: (1) an increased overall efficacy in RNA synthesis and ribosome assembly owing to increased local concentrations of specific *trans*-acting factors, (2) trafficking and assembly of nonribosomal classes of RNPs, and (3) the opportunity to sequester specific *trans*-acting factors within the confines of a dynamic nuclear domain whose assembly–disassembly precisely oscillates in relation to the cell cycle (e.g., extra ribosomal functions in cell cycle control).

Finally, the concept that ribosomes come in different ‘flavors’ and that there is no such thing as a single ‘ribosome make’ is emerging from recent analyses in pathological situations. Future research will uncover that this probably pertains to physiological conditions as well as to fine-tuning ribosome abilities (e.g., target-specific transcripts, elicit stress responses, etc.). Whether the production of specialized ribosomes involves specific subnucleolar structures is an entirely open question.

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