

EXTRA VIEW

Regulation of nucleolus assembly by non-coding RNA polymerase II transcripts

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ABSTRACT

The nucleolus is a nuclear subcompartment for tightly regulated rRNA production and ribosome subunit biogenesis. It also acts as a cellular stress sensor and can release enriched factors in response to cellular stimuli. Accordingly, the content and structure of the nucleolus change dynamically, which is particularly evident during cell cycle progression: the nucleolus completely disassembles during mitosis and reassembles in interphase. Although the mechanisms that drive nucleolar (re)organization have been the subject of a number of studies, they are only partly understood. Recently, we identified Alu element-containing RNA polymerase II transcripts (*aluRNAs*) as important for nucleolar structure and rRNA synthesis. Integrating these findings with studies on the liquid droplet-like nature of the nucleolus leads us to propose a model on how RNA polymerase II transcripts could regulate the assembly of the nucleolus in response to external stimuli and during cell cycle progression.

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The nucleolus is the cellular factory for producing rRNAs (rRNAs) and assembling ribosome subunits. In response to cellular stress and environmental cues its activity and structure can change, and enriched factors involved in cellular functions such as cell cycle regulation, DNA repair or mRNA processing can be released.^{1–6} The dynamic nature of nucleolar structure and function is particularly evident during cell cycle progression as reviewed previously^{6,7}: At the onset of mitosis nucleoli completely disappear and rRNA production is turned off. Following cell division, the nucleolus reassembles and rRNA production is restarted.

To rationalize the plastic conformation of the nucleolus, the model of a liquid droplet-like structure established via a liquid-liquid phase separation process has been proposed.^{8–10} This description accounts for the self-assembly of the nucleolus as well as that of PML and Cajal bodies, splicing speckles and other nuclear bodies. The partitioning of the nucleus into organelles with specific activities cannot be explained by viewing the nucleus simply as a membrane-confined container for the nucleoplasm – an aqueous solution with high concentrations of

proteins and nucleic acids macromolecules. At the same time, the above nuclear subcompartments are variable in size and shape and their constituting protein and RNA components are in rapid exchange with factors from the nucleoplasm.¹¹ Thus, like in a liquid, molecules in these nuclear subcompartments constantly rearrange. The apparent similarity of this aspect of nuclear organization with two liquids that do not mix and form separate phases, as for example oil drops in water, has led to the view that features of nuclear subcompartments within the nucleoplasm can be described by a liquid-liquid phase separation process as reviewed previously.¹² Another equivalent term used in this context is that of a liquid-demixing phase separation.¹³ Within a cell, this process frequently involves partially unstructured proteins,¹⁴ and can be regulated by their interaction with RNA.^{15,16} When considered in the context of this physico-chemical framework, our recent findings on the role of *Alu* element-containing RNA polymerase (Pol) II transcripts in nucleolus structure and function have a number of implications.¹⁷ They suggest that the interaction of RNA Pol II transcripts with unstructured nucleolar protein shifts the equilibrium between the two liquid phases, the nucleolus

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and the nucleoplasm and acts as a factor that affects the (dis)assembly of the nucleolus. Based on these findings, we discuss here how these RNA-protein interactions could be modulated in the cell to provide an additional regulatory layer for controlling structure and function of the nucleolus.

RNA-driven nuclear subcompartment assembly and maintenance

In the past few years, several studies have illustrated the ability of specific coding and non-coding RNA transcripts to initiate the formation of nuclear bodies. In a number of these, cell lines that had *lacO* operator repeats stably integrated into their genome were used to tether specific RNAs to these genomic loci. The RNA transcripts containing several MS2 stem-loops were bound to the *lacO* arrays via the high affinity MS2 coat protein fused to *lac* repressor. For example, it was reported that tethering the replication-dependent histone gene H2B RNA to *lacO* operator repeats array induced the formation of histone locus bodies-associated Cajal bodies and the accumulation of coilin, a Cajal body-associated protein.¹⁸ The same study found that β -globin transcripts tethered to *lacO* arrays formed *de novo* speckles as identified by the enrichment of the serine-arginin rich splicing factor SC35, a nuclear speckle-associated protein. In addition, the *lacO*-bound non-coding RNA NEAT1 triggered the formation of *de novo* paraspeckles, which were associated to paraspeckle-specific proteins such as PSP1, NONO and PSF.^{18,19} Likewise, *lacO*-tethered non-coding satellite III repeat RNA recruited several proteins associated to nuclear stress bodies, for example heat shock-specific transcription factor HSF1 or the splicing factor SF2/ASF.¹⁸ These results suggest that specific RNA transcripts can serve as a structural scaffold for the assembly of various nuclear subcompartments.

In our recent work, a related RNA-mediated assembly process was proposed for the organization of the nucleolus.^{17,20} Both the inhibition of Pol I and II induced structural changes in the nucleolus. However, a dispersion of nucleolar domains throughout the nucleoplasm was specific for Pol II inhibition. By analyzing the RNA content of the nucleolus, we found that it was enriched in *Alu* element-containing RNA transcripts (*aluRNAs*) originating from intronic regions of Pol II transcribed genes. We also found that depletion of *aluRNAs* led to the same

dispersion phenotype of the nucleolus as Pol II inhibition. Furthermore, overexpression of *aluRNAs* resulted in larger nucleoli suggesting that the nucleolus size and the available amount of Pol II-produced *aluRNAs* were linked. Inhibition of Pol II or depletion of *aluRNAs* not only resulted in nucleoli dispersion, but also induced a strong reduction of Pol I transcriptional activity. This supports the view that nucleolus structure and function are closely connected as discussed previously.²¹ In addition, our study points to an RNA polymerase ‘cross-talk’ where the product of Pol II transcription influences the transcriptional activity of Pol I and the maintenance of the nucleolar subcompartment.

RNA-driven liquid-liquid phase separation during nucleolus formation

To describe the principles underlying the dynamic organization of the nucleolus the model of a liquid-liquid phase separation has been applied, where liquid droplet-like structures become separated from the nucleoplasm.⁸⁻¹⁰ Furthermore, a mechanism has been proposed according to which this type of liquid-liquid phase separation involves unstructured domains in RNA-binding proteins.¹⁶ The latter are referred to as low complexity domains or intrinsically disordered domains.^{16,22} Although liquid droplet-like structures form also in the absence of RNA, it was recently shown that the presence of RNA could induce such a phase separation at much lower protein concentration. The presence of a short RNA sequence from the promoter region of DNMT3b efficiently induced FUS (fused in sarcoma) protein self-assembly, a process that was also observed at much higher concentrations of FUS in absence of RNA.²³ In addition, a study by Zhang et al. showed that specific mRNAs drive Whi3 (a known RNA-binding protein and regulator of the cell cycle) assembly into droplets with distinct biophysical properties that were dependent on the mRNA transcript.²⁴ Thus, RNAs binding to an unstructured protein domain can promote liquid droplet-like assembly by shifting the equilibrium toward protein association.

As shown in our recent work, *aluRNAs* interacts with at least three nucleolar proteins, nucleolin (NCL), nucleophosmin (NPM) and fibrillarin, which are essential for nucleolus structure and function.²⁵⁻²⁷ All three of them contain unstructured domains. The C-terminal region of NCL²⁸ and fibrillarin²⁹ both contain a low complexity domain, a glycine-arginine rich (GAR) domain, which is

reported to interact with RNA transcripts.³⁰ NPM contains basic and acidic sequences, which were reported to be intrinsically disordered and to regulate its interaction with RNA.³¹ For NCL and NPM, their unstructured domains were linked to the ability of the proteins to self-associate,^{29,32} which is an essential part of the process of liquid droplet formation.^{13,16} These known properties of NCL, NPM and fibrillarin led us to propose that *alu*RNAs support nucleolar assembly by interacting with those proteins and promoting a liquid-liquid phase separation. In support of this view, recent *in vitro* work has shown the ability of fibrillarin to phase-separate at lower concentrations if yeast RNA was present.¹⁰ It was also demonstrated that the RNA-binding domains and the GAR domain of NCL are important for its localization to the nucleolus.^{33,34} This could mean that those domains are important for the protein to phase-separate in an RNA-dependent manner.

Remarkably, some specific proteins found in the RNA-enriched nuclear bodies mentioned above also contain disordered domains as for example coilin in Cajal bodies,³⁵ which was also reported to self-associate.³⁶ In paraspeckles, the N- and C-terminal regions of PSP1, NONO, and PSF are predicted to be disordered,³⁷ and in speckles, SC35 can self-associate³⁶ and contains a serine-arginine rich domain,³⁸ which like the GAR domain is a low complexity domain. Interestingly, a recent study by Frege et al.³⁹ reported the presence of intrinsically disordered proteins in many membrane-less nuclear subcompartments. This suggests that a mechanism based on RNA-protein interaction-mediated liquid-liquid phase separation could also promote the assembly of other RNA-containing nuclear bodies.

RNA pol II transcripts during cell cycle-dependent nucleolus (dis)assembly

In our recent work, we focused on the effect of depleting or overexpressing *alu*RNAs on nucleoli during the interphase of the cell cycle. This raises the question whether *alu*RNAs or other Pol II transcripts could also be relevant for the disassembly of nucleoli at the onset of mitosis and their reassembly at the end of cell division. By tracing the location of the NCL and NPM nucleolar marker proteins during the cell-cycle the (dis)assembly of nucleoli has been dissected in a number of studies, e.g. refs.^{26,40} In Figure 1 we have reproduced these

experiments via simultaneous visualization of the NCL and NPM distributions in HeLa cells to illustrate that this process involves droplet-like nucleolar substructures. At telophase, the NCL and NPM images indicated that nucleoli were still disrupted. During cytokinesis some accumulation into areas was observed for NCL and to a lesser extent also for NPM, while fully assembled nucleoli with colocalization of NCL and NPM were only present during interphase.

If Pol II transcripts are essential for nucleolus formation, the inhibition of Pol II during mitosis and early interphase should prevent the proper assembly of nucleoli after mitosis completion. To test this hypothesis, we synchronized cell by thymidine block⁴¹ followed by a nocodazole block. During nocodazole block, cells were treated with specific Pol I (low concentration of actinomycin D) and Pol II (low concentration of α -amanitin) inhibitors for 5 hours as well as with the protein translation inhibitor cycloheximide. During this treatment, cells remained blocked in prometaphase, the chromosomes were condensed but not aligned due to the lack of microtubules. Without inhibitor as well as in presence of actinomycin D, α -amanitin, and cycloheximide, the nucleoli remained disassembled and the NCL and NPM marker proteins redistributed to the chromatin free regions of the cell (Fig. 2A). Next, nocodazole was washed out and cells were further incubated with the respective Pol I or Pol II inhibitors, or translation inhibitor. The cells progressed normally to form the metaphase plate (Fig. 2B). The inhibition of Pol I did not prevent the formation of nucleoli, as reported previously,⁴² which still contained both NCL and NPM (Fig. 2C). For some of the cells, additional NCL dots formed in the nucleoplasm, which were not co-localizing with NPM. These nucleoplasmic foci might originate from aberrant accumulation of NCL that could not be integrated into nucleoli due to Pol I inhibition. In contrast to this relatively minor structural phenotype, cells treated with a Pol II inhibitor completely failed to form nucleoli after release of the nocodazole block and nucleolar particles remained dispersed (Fig. 2C). In addition, a previous study reported that roscovitine, another inhibitor of Pol II transcription,⁴³ impaired post-mitotic nucleolus assembly.⁴⁴ The phenotype of persistent dispersed nucleolar subdomains observed upon Pol II inhibition did not appear to be due to a lack of protein synthesis (Fig. 2C). The inhibition of protein translation for several hours did not

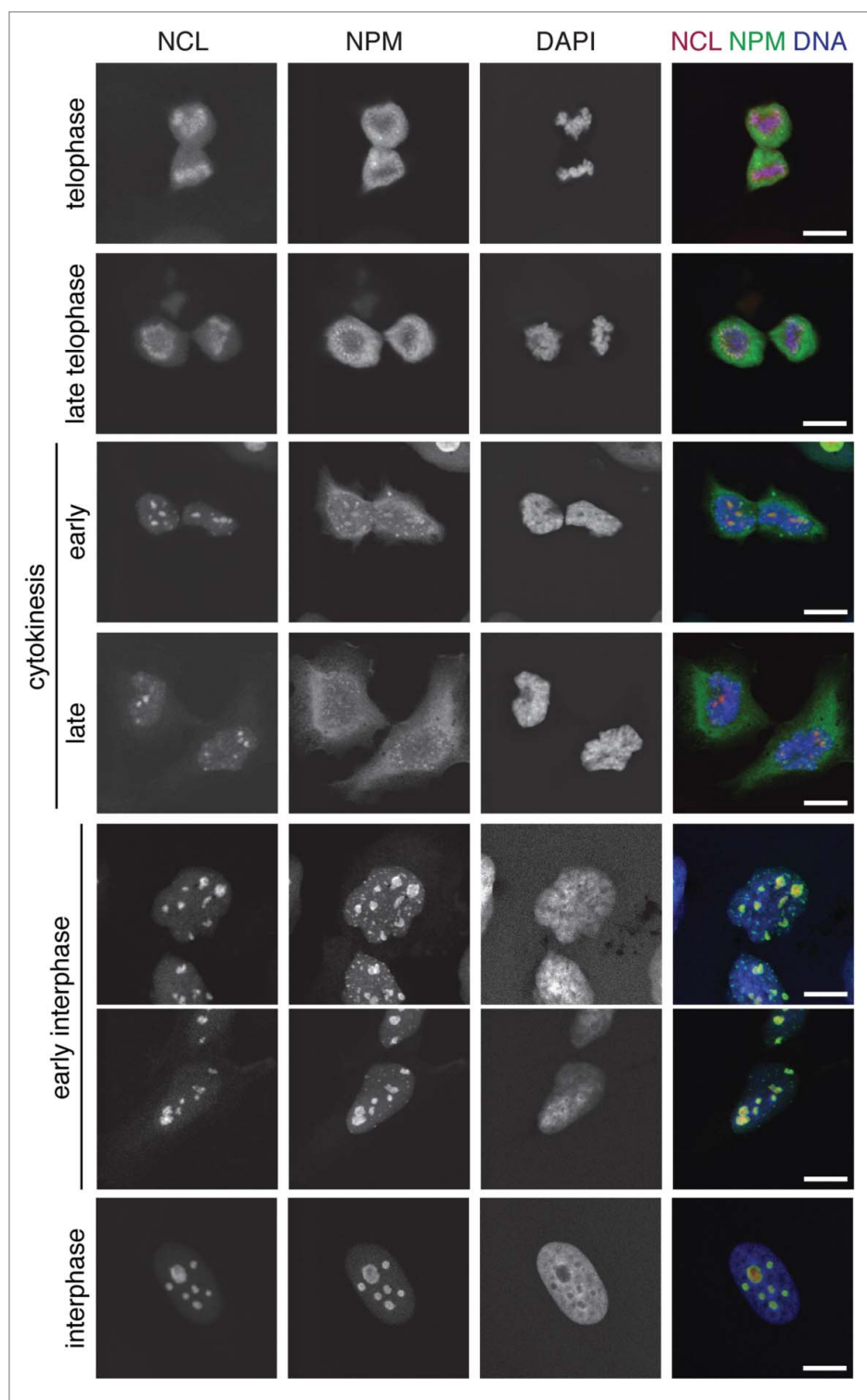


Figure 1. Cell cycle-dependent structural changes of the nucleolus. Confocal laser scanning microscopy (CLSM) images showing the nucleolar marker proteins nucleolin (NCL, red, stably expressed RFP-NCL) and nucleophosmin (NPM, green, immunofluorescence) with DNA (DAPI, blue) counterstaining in U2OS cells at different stages of the cell cycle. As evident from the NCL and NPM distribution, nucleoli are still completely disrupted during telophase and fully assembled during interphase. Scale bars, 10 μm .

give rise to disintegrated nucleolar subdomains. Taken together, these observations suggest that Pol II transcription and the resulting Pol II transcripts such as *alu*RNAs could be important for the post-mitotic

assembly of nucleoli. However, to corroborate this conclusion one would have to dissect the role of specific Pol II transcripts in further experiments to exclude indirect effects of the global Pol II transcription.

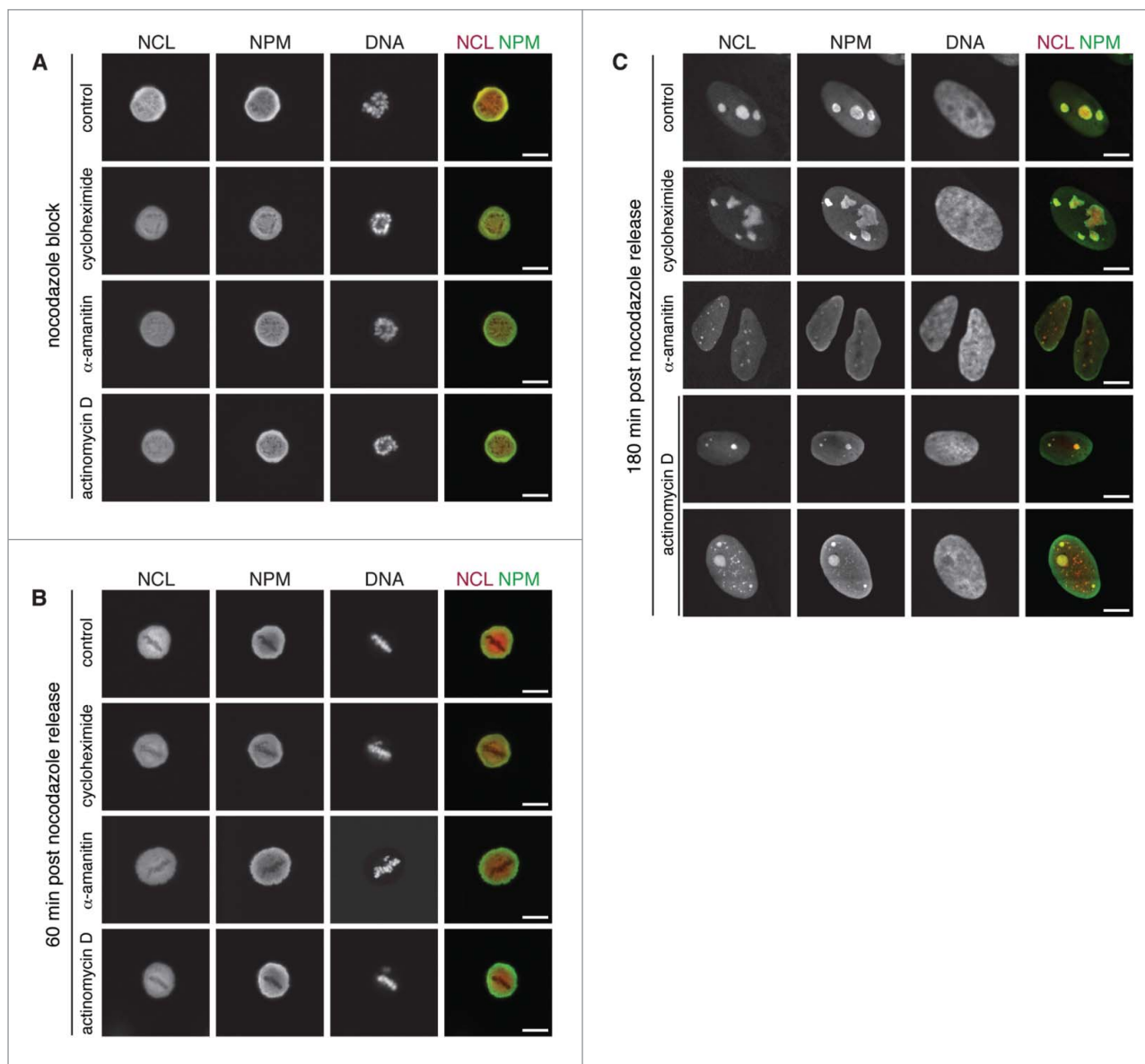


Figure 2. Impaired post mitosis nucleolus assembly through Pol II inhibition. (A) CLSM images showing NCL (red, stably expressed RFP-NCL), NPM (green, immunofluorescence) and DNA (DAPI) in U2OS cells treated with nocodazole (100 ng/ml) and either actinomycin D (50 ng/ml), α -amanitin (50 μ g/ml) or cycloheximide (50 μ g/ml) for 5 h. (B) Cells 60 min after they were released from the nocodazole block. (C) Cells 180 min after they were released from the nocodazole block. Scale bars, 10 μ m.

Regulation of RNA-driven liquid-liquid phase separation

For a phase separation process, the concentration of the various components is a critical parameter as illustrated by studies showing that droplet-like assembly only occurred above a critical concentration.^{10,23,24,45} This parameter could be regulated in the cell by nucleocytoplasmic transport processes and cellular turnover of the macromolecules involved and potentially competing binding partners. For RNA-driven liquid demixing phase separation systems, the interaction of

RNA transcripts with specific protein partners might be required to reach critical phase separation conditions *in vivo*, e.g., to allow for droplet formation and assembly at physiological protein concentrations lower than those needed in *in vitro* studies. In such a system, any perturbation of RNA-protein interaction would result in the dispersion of the droplets, their dissolution and redistribution of the liquid phases.

At the onset of mitosis, the nuclear envelope breaks down, nucleoplasm and cytoplasm mix rapidly and nuclear factors become diluted. Furthermore, Pol II

transcription is strongly reduced during mitosis.⁴⁶ Thus, the concentration of nuclear protein and RNA components might drop below the critical concentration required for a liquid-liquid phase separation process. In addition, nuclear proteins become more accessible and can be bound at their nuclear localization signal⁴⁷ (NLS) by proteins of the importin family.⁴⁸ It is well established that interaction of importins with the NLS part of a protein can interfere with the function of the protein⁴⁹ and possibly also with its RNA-binding ability. As the nuclear envelope reassembles, NLSs are released from importins in the nucleus and proteins are no longer inhibited from performing their nuclear functions. In support of this view, the nucleolus assembly correlates with post-mitotic nuclear envelope formation.⁵⁰

Another factor that could influence droplet formation and assembly are posttranslational modifications of the proteins involved. Phosphorylation, for example, has been shown to regulate the interaction of intrinsically disordered domains with RNA^{13,51} and to influence the ability of intrinsically disordered protein to phase separate.^{16,52} Moreover, other posttranscriptional modifications like acetylation, methylation or sumoylation could alter the RNA binding potential of a protein⁵³ or modify its localization.⁵⁴ Such a regulatory mechanism might apply also for the nucleolus. It was proposed that phosphorylation of an abundant nucleolar protein may drive the cell cycle nucleolus (dis-)assembly dynamics.⁵² The major cell cycle switch at the onset of mitosis is the activation of the cyclin-dependent kinase CDC2.⁵⁵ Interestingly, both NCL and NPM are strongly regulated via phosphorylation and are phosphorylated by CDC2 at the onset of mitosis.⁵⁶⁻⁵⁸ Furthermore, it was reported that phosphorylation controls the localization of NCL with translocation of mitotic phosphorylated NCL to the cytoplasm⁵⁹ and that phosphorylation of NPM prevents the protein to interact with RNA.⁵⁷ This suggests that mitotic phosphorylation of these proteins by CDC2 could inhibit nucleolar assembly by either acting negatively on the ability of NCL and NPM to associate to RNA or by blocking further association of these proteins when bound to RNA.

Model for RNA-dependent nucleolar assembly

A model for an *alu*RNA-dependent mechanism that drives changes of nucleolar structure including the disassembly at mitosis and reassembly at interphase is

depicted in Figure 3. At the onset of mitosis, the nucleolus disassembles, which correlates with the arrest of Pol I transcriptional activity. However, the absence of Pol I activity during mitosis and the restart of rRNA production at the end of cell division are not sufficient to explain these structural changes. Indeed, nucleoli assembly can occur in the presence of Pol I specific inhibitors as shown in Figure 2A and as previously reported.⁴² This process is strongly inhibited by treatment with Pol II inhibitors¹⁷ and during mitosis (Fig. 2C) where the assembly stops at the stage of nucleolar droplets. For the nucleation of such droplets, rRNA may be crucial as suggested in recent studies.^{10,60} However, these relatively small prenucleolar bodies containing NCL and NPM appear to require the association with Pol II *alu*RNA transcripts as the “glue” to assemble them into larger domains. In the absence of the latter RNAs the coalescence step required to achieve the liquid-liquid phase separation does not occur (Fig. 3A) and prevents proper post-mitotic reassembly of the nucleoli (Fig. 2C). Accordingly, we propose that the interaction of nucleolar proteins as for example NCL and NPM with RNA Pol II transcripts drives nucleolar assembly in a cell cycle-dependent manner (Fig. 3B). This process could involve phosphorylation-dependent RNA-protein interactions.

Concluding remarks

Increasing evidence points to RNA as an important factor for proper formation of nuclear subcompartments and intact chromatin structure.⁶¹⁻⁶³ The assembly of an RNA-dependent nuclear scaffold induces the local enrichment of effector molecules and their associated activities for efficient and controlled reactions in the nucleus.⁶⁴ The concept of (dis)assembly of the nucleolus driven by RNA interaction with intrinsically disordered domains of nucleolar proteins might also apply to other nuclear bodies.^{39,65,66} Modulating these RNA-protein interactions during the cell cycle appears to be a regulation principle that is relevant not only for the nucleolus but also in other systems: (i) Phosphorylation affects coilin protein activity and its association with RNA in Cajal bodies differently in mitosis as compared to interphase.^{67,68} (ii) The assembly of histone locus bodies occurs in an RNA-dependent manner and changes from G1 to S phase as reviewed recently.⁶⁹ (iii) Phosphorylation of the PRC2 (Polycomb Repressive Complex 2) component EZH2 is cell cycle-regulated and up-regulates

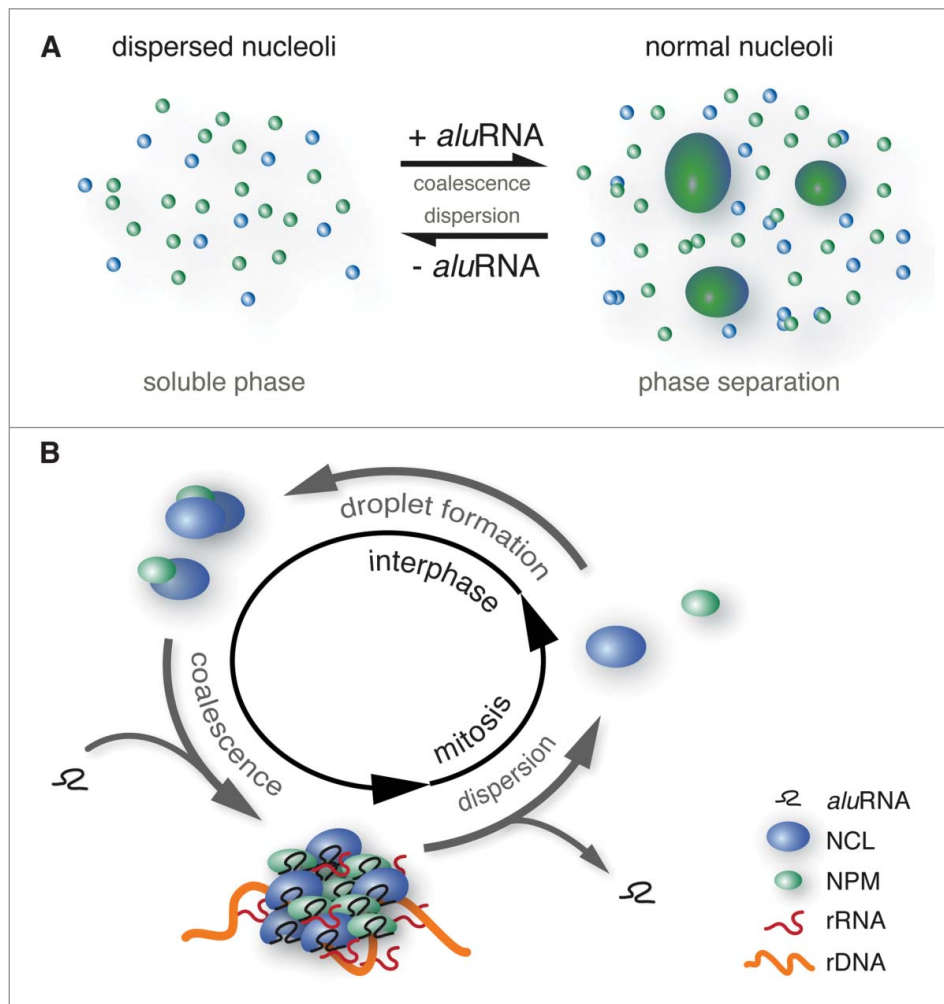


Figure 3. Model for RNA and cell cycle-dependent (dis)assembly of the nucleolus. (A) Scheme of RNA-driven liquid-liquid phase separation of the nucleolus. The interaction of nucleolar proteins like NCL or NPM (blue and green respectively) with *aluRNAs* might trigger a conformational change of their unstructured regions that drives the assembly of nucleolar subdomains via a liquid-liquid phase separation process into larger domains representing functional nucleoli. Depletion of *aluRNAs* induces a change of this equilibrium back into the dispersed state with small nucleolar droplets. (B) As illustrated in the enclosed scheme, assembly and disassembly of the nucleolus are dependent on the interactions between RNA transcripts (*aluRNAs*) and proteins (NCL, NPM). The RNA-protein assemblies form nucleolar domains that associate with rDNA and efficiently support Pol I transcriptional activity. Protein-RNA interactions could be dependent on the cell cycle state, which affects posttranslational modifications of the proteins, changes in their concentration and/or exposition to competing interacting species, like for example binding of importins to their NLS. Thereby, the formation of RNA-protein assemblies could be coupled to specific cell cycle phases.

its ability to bind RNA.⁷⁰ This could be relevant for the formation and composition of distinct subnuclear foci containing PRC complexes and referred to as polycomb bodies.⁷¹

In addition, the RNA-dependent composition of nuclear subcompartments can be modulated independently of the cell cycle. Indeed, external stimuli like for example growth signals as demonstrated previously by Yang et al.⁷² can have similar effects. In the latter study it was found that the activity of polycomb bodies and interchromatin granules/SC35-containing splicing domains is controlled by the RNA-binding specificities of the

polycomb complex Pc2 protein in dependence of its methylation state. The switch of Pc2 interactions between the TUG1 and MALALT1/NEAT2 non-coding RNAs affected the three-dimensional location of transcription units and the coordinated regulation of gene expression programs.

Deregulation of nuclear subcompartment organization is also linked to pathological phenotypes including cancer and neurodegenerative disorders.^{6,73,74} For example, highly proliferating tumor cells harbor larger and more active nucleoli for increased rRNAs and ribosome production.⁷⁵ On the other hand, cells from patients

suffering from neurodegenerative diseases often present less active nucleoli with structural aberrations.⁷⁶ Accordingly, there is a renewed interest for using the nucleolus as a potential target for therapeutic treatments of cancer and other diseases.^{77,78} It becomes clear that further elucidating the regulation of RNA-protein interactions is crucial to understand the mechanisms underlying the assembly dynamics and the activities of the nucleolus as well as those of other nuclear bodies. Thus, further work in this direction will allow making progress in the identification of key regulators, risk factors and potential therapeutic targets.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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