Birth of Nucleolar Compartments: Phase Separation-Driven Ribosomal RNA Sorting and Processing

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The nucleolus is a phase-separated cell condensate where the initial steps of ribosome biogenesis take place. In this issue of *Molecular Cell*, Yao et al. (2019) report a super-resolution microscopy analysis of the internal structure of the nucleolus, revealing how nascent precursor ribosomal RNAs are initially partitioned and processed in this multilayered biocondensate.

Ribosomes are essential nanomachines responsible for translating mRNAs into proteins. In eukaryotes, they are composed of four ribosomal RNAs (rRNAs) and eighty ribosomal proteins (r-proteins). Their synthesis is initiated in the nucleolus, where three of the four rRNAs are synthesized by RNA polymerase I (Pol I) as a long precursor, which is then transported, processed, modified, and packaged with r-proteins into ribosomal subunits. This involves hundreds of ribosome assembly factors.

The nucleolus is a multilayered biocondensate formed by liquid-liquid phase separation that adopts the behavior/biophysics of immiscible liquids (Feric et al., 2016). Put simply, it's like when droplets of balsamic vinegar form in oil. Phase separation relies on multivalent weak interactions between RNA and protein cell constituents. Importantly, these interactions are driven by function. Proteins with intrinsically disordered regions or low-complexity sequences, which are particularly abundant in the nucleolus, are directly involved in such interactions.

In modern eukaryotes, the nucleolus consists of three subcompartments, nested one in the other like Russian dolls. The fibrillar center (FC), at the core, is surrounded by the dense fibrillar component (DFC), and both are immersed within the granular component (GC) (Thiry and Lafontaine, 2005). FC and DFC form a functional unit present in multiple copies in a large body of GC. These compartments are detectable by fluorescence and electron microscopy. Ribosomal RNA synthesis occurs at the interface between the FC and DFC, with the nascent transcripts radiating into the DFC for initial maturation.

How intranucleolar compartments are phase-separated from one another and how they are maintained in cells are only starting to be understood. Remarkably, mixing together two *in vitro* purified nucleolar proteins, one from the DFC (fibrillarin) and another from the GC (nucleophosmin), is sufficient to recapitulate a multilayered organization similar to that observed in the nucleolus in cells (Feric et al., 2016). Stiffening the nucleolus with light-activated optotags or disrupting it by infiltration of small charged polypeptides (such as those produced in patients suffering from neurodegenerative diseases) impacts nucleolar biochemistry, including RNA processing (Kwon et al., 2014; White et al., 2019; Zhu et al., 2019). How nascent precursor rRNAs are partitioned in the right direction, from the FC/DFC interface to the DFC, and how RNA cleavage is triggered have remained unclear.

Using super-resolution microcopy, Yao et al. have gained a fresh outlook on the multilayered nucleolar organization of live cells (Yao et al., 2019). First, they show that the cortex of the FC is rich in Pol I subunits (Figure 1), in agreement with the largely consensual view that RNA synthesis occurs at the FC/DFC interface. Second, they have quantified various nucleolar elements with unprecedented precision, including the number of FC/DFC units per cell (from several dozen to over 100, which is constant for a particular cell type, but which varies between cells types, making it a biomarker) and the number of transcriptionally active rDNAs per FC/DFC unit (2–3). Third, they reveal the spatial distribution of ribosome assembly factors, showing that fibrillarin and about half a dozen other tested proteins form a spherical network of 18–24 regularly spaced "beads" that assemble within the DFC around the FC (Figure 1). Fibrillarin is known to be involved in 2'-O methylation of precursor rRNAs, being a core protein of the box C/D small nucleolar RNPs, and also in pre-rRNA processing. Each assembly factor bead is ~133 nm in diameter. For reference, in yeast the size of an early-maturation-stage pre-ribosome is ~25 × 35 nm. It should be a bit larger in human cells. Each bead thus contains multiple maturing pre-ribosomes. The distance between two adjacent beads is 180 nm. Each DFC region has an outer diameter of ~628 nm and an inner diameter of ~362 nm. A similar spatial arrangement has been observed for the helicase DDX21, which regulates Pol I function together with the lncRNA SLERT (Xing et al., 2017).

By performing a candidate-based screen for factors driving the 5′ end of nascent pre-rRNA transcripts (the so-called 5′ external transcribed spacer, or 5′ ETS) out of the FC/DFC interface, these investigators have identified fibrillarin as playing an important role. They show that fibrillarin binds cotranscriptionally to the 5′ ETS and that its amino-terminally

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located self-associating glycine-arginine-rich (GAR) domain, containing intrinsically disordered regions (IDRs), promotes the phase separation of the RNA, leading to DFC formation and initial RNA processing (Figure 1). While both the methyltransferase domain of fibrillarin, conferring RNA binding capacity, and its GAR domain, with its self-associating IDRs, are important for RNA sorting and processing, its catalytic activity is dispensable for both processes. What is important is the presence of IDRs of a certain length, rather than their sequence, as swapping the GAR domain with unrelated IDRs or shuffling its amino acid composition does not affect the function. *In vitro*, fibrillarin condenses into droplets that recruit the 5′ ETS if it is properly folded. Thus, fibrillarin-mediated phase separation partitions nascent rRNA transcripts from the FC/DFC interface to the DFC, and this is required for initial RNA cleavage and DFC nucleation. In addition to fibrillarin, other ribosome assembly factors are likely involved in 5′ ETS recruitment to the DFC, as the screen performed by Ling-Ling Chen's team has indeed shown.

The size, shape, and number of nucleoli per cell nucleus are well known to vary in disease, particularly cancer (Derenzini et al., 2009). Counting individual FCs/DFCs and other nucleolar elements and measuring precisely their size might thus be exploited in disease diagnostics and prognostics. This will complement the arsenal of tools for the quantitative and qualitative evaluation of nucleolar morphology, such as the recently described iNo scoring method (Nicolas et al., 2016; Stamatopoulou et al., 2018). The nucleolus is a target for anticancer intervention, and several Pol I inhibitors have successfully entered clinical trials. By interfering with fibrillarin binding to the 5′ ETS (through tethering of an RNA-guided inactive Cas13d at the site of interaction), Yao et al. have extended the potential therapeutic avenues to inhibition of rRNA sorting.

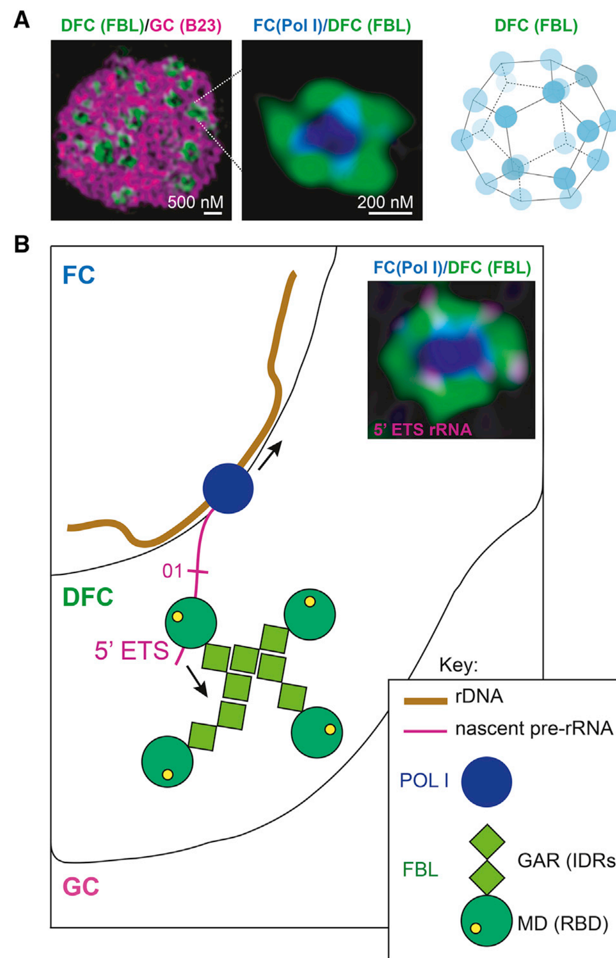

Figure 1. Phase Separation at the Nucleolar Core, Triggering Nascent Ribosomal RNA Sorting and Processing
(A) At left, structured illumination microscopy (SIM) analysis of FC (Pol I, blue), DFC (fibrillarin/FBL, green), and GC (nucleophosmin/B23, purple) nucleolar subcompartments. At right, spatial distribution of fibrillarin in the DFC: mathematical modeling of a spherical network-like structure of regularly spaced fibrillarin beads.
(B) Ribosomal RNA synthesis occurs at the FC/DFC interface, enriched in Pol I subunits. While the upstream part of the 5′ ETS has already been translocated to the DFC, its downstream part is still being synthesized at the FC/DFC interface. Fibrillarin consists of an amino-terminal region containing a glycine-arginine-rich (GAR) domain with self-associating intrinsically disordered regions (IDRs) and a carboxy-terminal methyltransferase domain (MD) containing an RNA-binding domain (RBD). The 5′ end of nascent transcripts (5′ ETS) is bound cotranscriptionally by fibrillarin, which self-associates via its GAR domain, promoting phase separation. Phase separation drives the nascent transcripts out of the FC/DFC interface, leading to DFC nucleation and initial pre-rRNA processing (01 site cleavage). To be functional in RNA sorting and cleavage, the GAR domain must contain IDRs of a certain length. The GAR domain acts in conjunction with the MD domain, which confers RNA-binding capacity. The catalytic activity of fibrillarin (represented as a yellow circle) is dispensable for both processes.

In conclusion, this work uncovers a novel principle: phase separation coupled to nascent RNA sorting and processing. The presence, in the DFC, of ribosome factor assembly beads, which are themselves phase-separated subdroplets, implies that there are many more interfaces within the nucleolus than anticipated. It is tempting to speculate that during evolution, other important nucleolar biochemistry reactions and ribosome biogenesis steps have converged onto these numerous subnucleolar interfaces, as they display force-generating surface tension.

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Watch Out for Those Terrible Twos! Dinucleotide Accumulation Dysregulates Mitochondrial Transcription

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In this issue of *Molecular Cell*, Nicholls et al. (2019) show that the oligoribonuclease REXO2 degrades mitochondrial RNA dinucleotides to prevent RNA-primed transcription at non-canonical sites in the mitochondrial genome, shedding new light on the importance of complete RNA degradation for transcriptional integrity.

The ancient proto-bacterial origin of the mitochondria is reflected in one of many ways by its mechanism of transcription. Mitochondrial DNA (mtDNA) is transcribed by a bacteriophage T7-like RNA polymerase (POLRMT) and comprises a circular genome totaling 16.5 kb, encoding 2 rRNAs, 13 mRNAs, and 22 tRNAs (Anderson et al., 1981), yet the genome only contains two main sites of transcription initiation: the heavy-strand promoter (HSP) and the light-strand promoter (LSP). Each of these promoters encodes polycistronic transcripts spanning almost the whole genome, but careful analysis of their sequence shows no, or very little, space between each gene. How are gene boundaries delineated? The explanation is known as the "tRNA punctuation" model and arose from the observation that tRNA genes are intercalated between mRNA and rRNA genes in mtDNA (Ojala et al., 1981). Since tRNAs reach their full maturation after being cleaved by specific 5′ and 3′ end ribonucleases, the mitochondria use these tRNA-specific cleavages to generate the individual transcripts from the remaining RNAs. Strikingly, while this would suggest that an equal number of mature RNAs would form, the recently published mitochondrial transcriptome reports disproportionate amounts of individual mRNAs, tRNAs, and rRNAs (Mercer et al., 2011), indicating the important role played by post-transcriptional RNA processing and degradation. A paper by Nicholls et al. (2019) in this issue of *Molecular Cell* now comprehensively illustrates this point.

Degradation of RNA is an integral part of gene regulation, and ribonucleases are necessary for RNA quality control and for attaining the balance between individual transcripts. During the degradation process, endonucleases make the initial incisions along the RNA, followed by the 3′-5′ activity of exonucleases. In bacteria, exonucleases are typically unable to degrade RNAs shorter than 5 nucleotides (nanoRNAs) and require the activity of a specialized oligoribonuclease, named Orn. Without the *orn* gene, bacteria accumulate nanoRNAs, which ultimately causes growth arrest and death, indicating an essential function of Orn in RNA metabolism (Ghosh and Deutscher, 1999). Intriguingly, the loss of Orn activity results in gene expression changes that are likely caused by nanoRNAs mediating transcription priming (Goldman et al., 2011). REXO2 (RNA exonuclease 2), the human homolog of Orn, has DNA and RNA nuclease activity and localizes primarily to the mitochondria (Nguyen et al., 2000; Bruni et al., 2013). In their paper, Nicholls and colleagues first use biochemical assays to demonstrate that REXO2 preferentially degrades DNA and RNA oligos composed of only two nucleotides—longer oligos requiring several-fold more enzymes—and that mutation of any of the four residues in the DEDD catalytic domain of REXO2 abrogates its nuclease activity. Nicholls et al. also obtained the crystal structures of both free and RNA-associated REXO2 and compared them with the structures of other known ribonucleases, allowing the authors to suggest that key residues near the active site of REXO2 create a steric barrier to oligos longer than two nucleotides, thus providing a compelling structural explanation for the catalytic activity of REXO2 being biased toward dinucleotides.

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