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# The nucleolus as a multiphase liquid condensate

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Abstract | The nucleolus is the most prominent nuclear body and serves a fundamentally important biological role as a site of ribonucleoprotein particle assembly, primarily dedicated to ribosome biogenesis. Despite being one of the first intracellular structures visualized historically, the biophysical rules governing its assembly and function are only starting to become clear. Recent studies have provided increasing support for the concept that the nucleolus represents a multilayered biomolecular condensate, whose formation by liquid–liquid phase separation (LLPS) facilitates the initial steps of ribosome biogenesis and other functions. Here, we review these biophysical insights in the context of the molecular and cell biology of the nucleolus. We discuss how nucleolar function is linked to its organization as a multiphase condensate and how dysregulation of this organization could provide insights into still poorly understood aspects of nucleolus-associated diseases, including cancer, ribosomopathies and neurodegeneration as well as ageing. We suggest that the LLPS model provides the starting point for a unifying quantitative framework for the assembly, structural maintenance and function of the nucleolus, with implications for gene regulation and ribonucleoprotein particle assembly throughout the nucleus. The LLPS concept is also likely useful in designing new therapeutic strategies to target nucleolar dysfunction.

Protein *trans*-acting factors
Proteins important for ribosomal subunit biogenesis that interact only transiently with maturing ribosomal subunits; they are not found on mature ribosomes.

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Among numerous microscopically visible nuclear substructures, the nucleolus is the most prominent and represents a functionally and biophysically distinct body or compartment. The nucleolus is, in fact, so prominent that it drew the attention of early biologists over 200 years ago, in light microscopy studies by Fontana, Valentin and Wagner[1]. Following these early descriptions came the understanding of the functional importance of the nucleolus, including its primary role as the site for the initial steps of ribosome biogenesis, including RNA polymerase I (Pol I)-driven transcription, processing and modification of ribosomal RNA (rRNA) and the assembly of rRNA-containing complexes[2] (FIG. 1). These processes involve several hundred protein *trans*-acting factors and small nucleolar RNAs[3], which serve to guide the specificity of rRNA chemical modifications, pre-rRNA folding and cleavage. Once precursor subunits are released from the nucleolar structure, they undergo further maturation in the nucleoplasm and cytoplasm prior to becoming fully functional ribosomal subunits, ready to engage in translating mRNA into protein. This nucleolar function is accompanied by organization of the nucleolus into distinct subcompartments. In mammalian cells, nucleoli display three layers, nested like Russian dolls, where successive steps of ribosome production take place, starting at the inner core where rRNA transcription occurs and proceeding towards the periphery (FIG. 1).

In addition to the ribosome, other ribonucleoprotein particles are assembled, at least in part, in the nucleolus[2]. This is notably the case for the signal recognition particle, involved in protein secretion and targeting to the endoplasmic reticulum of transmembrane proteins, and for telomerase, required for the maintenance of chromosome ends[4]. There have also been reports of precursors of tRNAs, and even of mRNAs, transiting through the nucleolus[1]. Although it is not entirely clear why non-rRNAs transit through the nucleolus during their life cycle, it is largely assumed that they do so to benefit from the abundant assembly and modification machineries present there.

The nucleolus is physically separated from the rest of the nuclear space, yet is accessible for dynamic exchange due to the absence of a delimiting membrane. Hence, it is an ideal locale for fine regulation of cell homeostasis, for example, through the transient sequestration of key regulators of the cell cycle[5]. The nucleolus also plays important roles in sensing diverse stresses, including genotoxic and oxidative stress, heat shock, nutrient deprivation, oncogene activation and viral infection[6]. Although these diverse nucleolar functions have been

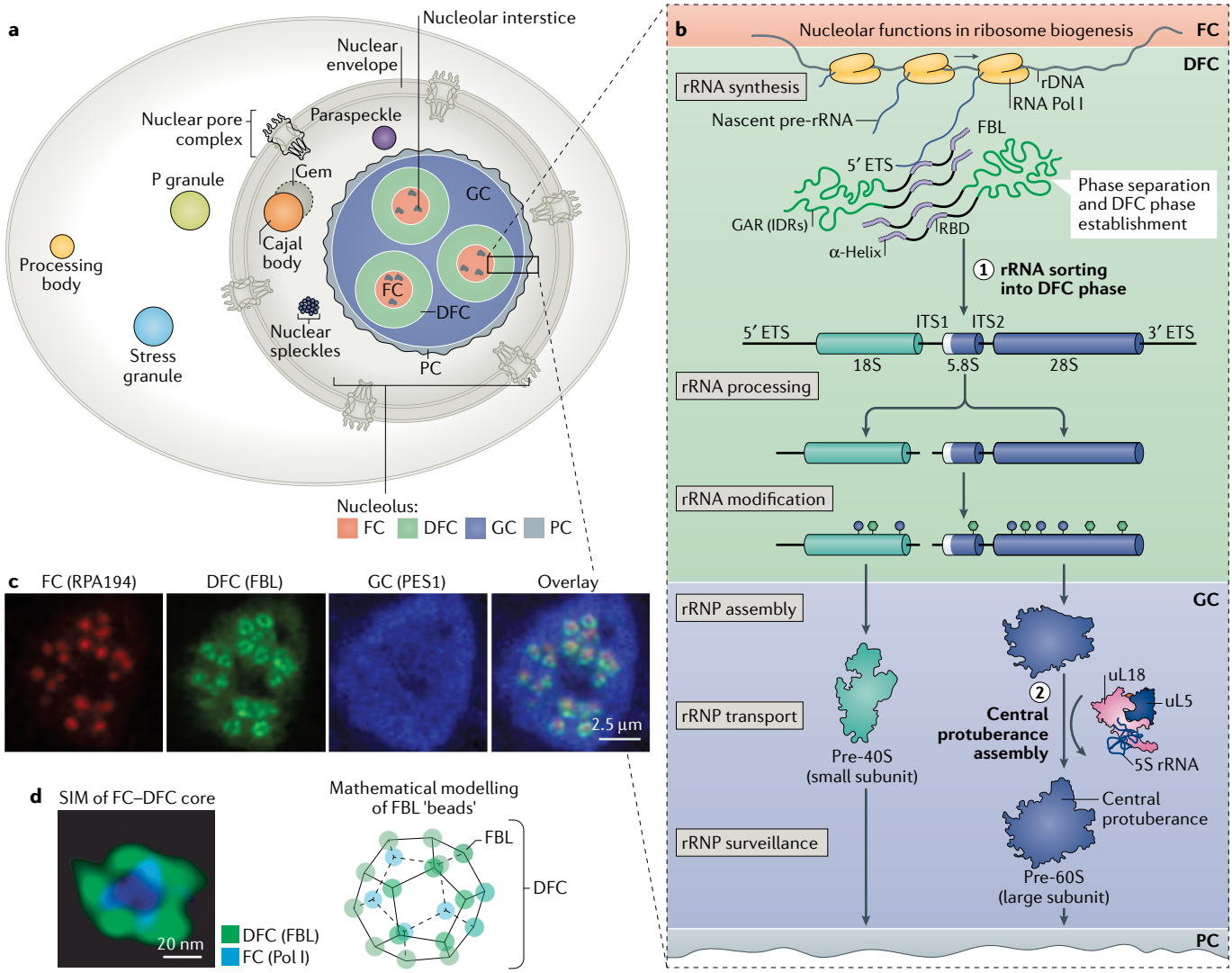



Fig. 1 | **Nucleolar organization and its role in ribosome biogenesis.** **a** | Schematic of a eukaryotic cell with representative cytoplasmic and nuclear condensates important for gene expression regulation. The nucleolus is represented with its three subcompartments: the fibrillar centre (FC), the dense fibrillar component (DFC) and the granular component (GC). **b** | Coordination of the nucleolar substructure with its roles in ribosome biogenesis. The main functions of the nucleolus in ribosome biogenesis are indicated in boxes. In cells, these steps are largely concomitant. The importance of the liquid–liquid phase separation model in understanding the connections between nucleolar functions and phase behaviour is illustrated with two examples: nascent pre-ribosomal rRNA (pre-rRNA) sorting (step 1)[23] and central protuberance assembly (step 2)[81]. (1) As nascent transcripts emerge from the FC–DFC interface where they are synthesized, they are bound by the RNA binding domain (RBD) of fibrillarin (FBL). FBL self-associates through its Gly–Arg-rich domain (GAR domain), which consists of intrinsically disordered regions (IDRs). This promotes establishment of the DFC phase, sorting of the nascent transcript from the FC–DFC interface to the DFC and initial pre-rRNA processing. Note that whereas the RBD of FBL is located in its methyltransferase domain, this methylation function is not required either for rRNA sorting or for processing. (2) A complex consisting of the ribosomal proteins uL5 and uL18 and the 5S rRNA (produced in the nucleoplasm by RNA polymerase III, not shown) is assembled late on the maturing pre-60S subunits to form the central protuberance, a structure essential for ribosome function. Inhibition of central protuberance assembly by depletion of uL5, uL18 or factors important for their assembly severely disrupts the nucleolar phase (see FIG. 6a). Interestingly, depletion of the vast majority of the other 78 ribosomal proteins, in particular those belonging to the small ribosomal subunit, has no, or little, effect on nucleolar phase behaviour. **c** | The three subnucleolar compartments detected by immunofluorescence in confocal microscopy: the FC detected with RNA polymerase I (Pol I) subunit RPA194, the DFC with FBL and the GC with the protein Pescadillo ribosomal biogenesis factor 1 (PES1). **d** | Each human nucleolus consists of several dozen FC–DFC modules. Interestingly, the number of these modules is relatively constant within a particular cell type but varies considerably between cell types, making it a powerful biomarker for cell classification. Each FC–DFC module contains two or three transcriptionally active ribosomal DNAs (rDNAs) at the FC–DFC interface where Pol I complexes are enriched and where transcription occurs (left panel; image of FC–DFC interface in a HeLa cell obtained with structured illumination microscopy (SIM)). Ribosome assembly factors, such as FBL (present at roughly 3,600 copies per FC–DFC unit), form a spherical network of 18–24 regularly spaced 'beads' ~130 nm in diameter that assemble into the DFC around the FC (right panel; 3D in silico model of the clustered FBL distribution in the DFC). ETS, external transcribed spacer; ITS, internal transcribed spacer; PC, perinucleolar chromatin; rRNP, ribosomal ribonucleoprotein. Images in part **c** are unpublished and a courtesy of D.L.J.L. and R.B. Part **d** adapted with permission from REF.[23], Elsevier.

**Small nucleolar RNAs**
Antisense small RNAs involved in RNA modification, pre-rRNA folding and processing.

**Phase behaviour**
The collection of biophysical features exhibited by materials capable of undergoing phase transitions, including the conditions under which phase separation occurs and the interfacial properties (for example, surface tension) defining wetting and coarsening behaviour.

**Liquid–liquid phase separation**
(LLPS). The process by which a single, uniform liquid phase demixes into two compositionally distinct liquid phases. Oil and water mixtures are an everyday example of phase-separated mixtures.

**Coacervate**
A term often used in the chemistry field to describe a condensed phase arising from a type of polymeric phase transition.

**Fluorescence recovery after photobleaching**
(FRAP). A technique that utilizes intense laser illumination to irreversibly deactivate fluorescent molecules locally, after which the return of a fluorescence signal within that region is indicative of biomolecular exchange with non-bleached fluorescent molecules from the surrounding areas of the sample.

**Ribosomal proteins**
Proteins that are structurally integrated into the ribosome. The fully assembled human ribosome contains 80 ribosomal proteins, most of which are assembled together with the four ribosomal RNAs within the nucleolus, with a notable exception of the acidic proteins forming the P stalk, which are assembled in the cytoplasm.

**Non-equilibrium**
A system that is not within a global thermodynamic free-energy minimum. Although equilibrium approaches may be applicable in some cases, biological systems are fundamentally out of equilibrium, due to their continuous consumption of energy and production and degradation of molecules.

largely perceived as uncoupled from biophysical models of nucleolar assembly, they are increasingly being revisited through the lens of nucleolar phase behaviour, as discussed further below.

Consistent with the active, multifunctional nature of the nucleolus, nucleolar components are highly dynamic, exhibiting continual flux within the nucleolus and exchange with the surrounding nucleoplasm. Yet studies over the past several decades have revealed that the nucleolus is denser than the surrounding nucleoplasm (BOX 1) and can be separated biochemically[7,8]. Moreover, it exhibits a well-defined, often roughly spherical shape, even in the absence of a surrounding membrane, making the nucleolus a paradigmatic membraneless organelle. Interestingly, however, the nucleolar shape, size and number per cell nucleus change upon stress[6], and the morphology can be profoundly remodelled in disease. Upon viral infection, for example by flaviviruses such as Zika virus or Dengue virus, the nucleolar morphology may undergo substantial remodelling, as viral proteins reach deep inside the nucleolus to interact with nucleolar antigens whereas nucleolar proteins redistribute throughout the cell, with implications for viral replication and assembly[9–11]. As another example, cancer cells often display nucleolar abnormalities[9], in addition to other cancer-associated nuclear changes (nuclear shape, size and chromatin texture[12]). Nucleolar structure has also been linked to ageing and lifespan regulation[10]. Hence, nucleolar morphology, which reflects its function, is a potent yet so far largely underrated disease biomarker[13,14].

There is now substantial evidence that assembly of the nucleolus is driven by liquid–liquid phase separation (LLPS; BOX 2), and studies of its biophysical nature (FIG. 2) have uncovered key principles of this biomolecular condensate[15–24] — a term increasingly adopted for describing large-scale membraneless assemblies[25,26]. In fact, owing to its large size and functional importance to the cell, the nucleolus is one of the most prominent of dozens of different types of liquid-like condensates found throughout the nucleus and cytoplasm. The LLPS model is consistent with some of the earliest cell biology studies on the nucleolus, which noted nucleolar coalescence events wherein two nucleoli would partially fuse into a roughly spherical shape (FIG. 2a) and discussed the possibility that the dynamics of nucleoli could reflect their coacervate nature[27].

Modern light microscopy approaches enabled new insights into the physicochemical properties of nucleoli, in particular their rapid molecular exchange and permeability[28,29]. Molecular dynamics into and out of the nucleolus has been extensively probed using fluorescence recovery after photobleaching (FRAP) (FIG. 2b), a technique readily available and easy to implement with commercial fluorescence microscopes. FRAP experiments with nucleolar proteins (and other nuclear condensates) showed that they exhibit a remarkable degree of exchange with the surrounding nucleoplasm: individual biomolecules typically only spend 10–20 s within the nucleolus before moving to the nucleoplasm[28,30]. These seemingly incongruous features of the nucleolus — rapid molecular turnover and liquid-like dynamics yet coherent shape — are reconciled by the LLPS model, which provides a predictive biophysical framework for understanding of these and many other nucleolar features. Importantly, there is strong evidence that the layered architecture of the nucleolus arises through multiphase liquid immiscibility[16], providing a mechanistic biophysical explanation for how the nucleolus can remain a highly structured and coherent body, even when most of its components are constantly turning over.

Despite the increasing support for the LLPS model as a principle of nucleolar organization, it must be recognized that nucleoli are dynamic structures that are built around a steady-state flux of both substrates (for example, nucleotides and ribosomal proteins) and products (mature pre-ribosomal particles). In contrast to the more equilibrium conditions often characterizing non-living matter or the typical in vitro reconstitution of biological structures using purified biological components, cells are fundamentally active, non-equilibrium forms of complex soft matter. Much of this non-equilibrium behaviour arises from energy-dependent activity, which, in the context of the nucleolus, orchestrates the structural remodelling of maturing pre-ribosomes, as well as numerous other intracellular processes from protein synthesis and degradation to cell-cycle progression. Reconciling this non-equilibrium behaviour with the simplest version of the LLPS model remains a major outstanding challenge, not only for the nucleolus but also the entire condensate field[31].

In this Review, we highlight how the LLPS model has begun to shed light on the rich structure and functional aspects of nucleolar biology, particularly its role in ribosome biogenesis; we note that there are several other aspects of nucleolar function described in detail elsewhere[2], but their links to LLPS remain largely unexplored. Here, we focus on how the LLPS model has begun maturing to incorporate the realities of nucleolar function in ribosome biogenesis, including the highly multicomponent and non-equilibrium nature of the process. We also underscore key outstanding questions and the extent of work that remains to be done for a complete biophysical picture, particularly given the incredible complexity of RNA processing reactions taking place within the nucleolus. Notwithstanding these remaining challenges, the LLPS model has begun to move the field of nucleolar biology away from largely descriptive cataloguing of molecular players and processes towards a framework based on quantitative physical principles. Moreover, as the prototypical nuclear condensate, these insights into the structure and function of the nucleolus will continue to shed light on the nature of the biophysical forces driving functional compartmentalization within living cells, and the role of intracellular condensates in physiology and disease.

## Nucleolar structure

The nucleolus of interphase cells displays a particular internal organization comprising functionally and compositionally distinct subcompartments. Most eukaryotes have only two subnucleolar compartments[32]. This is notably the case for the widely used budding yeast eukaryotic model. During evolution, a third nucleolar

subcompartment emerged at the transition between anamniotes and amniotes, within the class Reptilia, and this tripartite organization has been retained in all modern eukaryotes inspected[33,34]. Mammalian cell nucleoli, which are by far the best studied, display three internal phase-separated subcompartments: the fibrillar centre (FC), the dense fibrillar component (DFC) and the granular component (GC). The FC and the DFC form functional units present in multiple copies immersed in a single GC, which consists of maturing ribosomal subunits (FIG. 1). The FC–DFC modules correspond to the sites of rRNA synthesis and initial ribosomal subunit maturation steps (see below). The single fibrillar compartment of ancient eukaryotes diverged into the modern FC and DFC subcompartments in a transition that coincides with an extensive expansion of the ribosomal DNA (rDNA) spacers, the sequence that separates the rDNA units[32].

The three internal layers of the nucleolus are well documented morphologically, and immunofluorescence microscopy has identified many protein components enriched in each subcompartment. Moreover,

Box 1 | **The nucleolus by numbers**

Multiple studies on the nucleolus have characterized key parameters of nucleolar structure and function. A list of these are outlined below (the figure schematically shows the parameters).

**Nucleolar assembly**

- The number of nucleolar organizer regions (NORs): 10 in human, 12 in mouse. This includes both active and inactive NORs.
- The number of active nucleolar organizer regions: ~6 in HeLa cells[105].

**Nucleolar organization and structure**

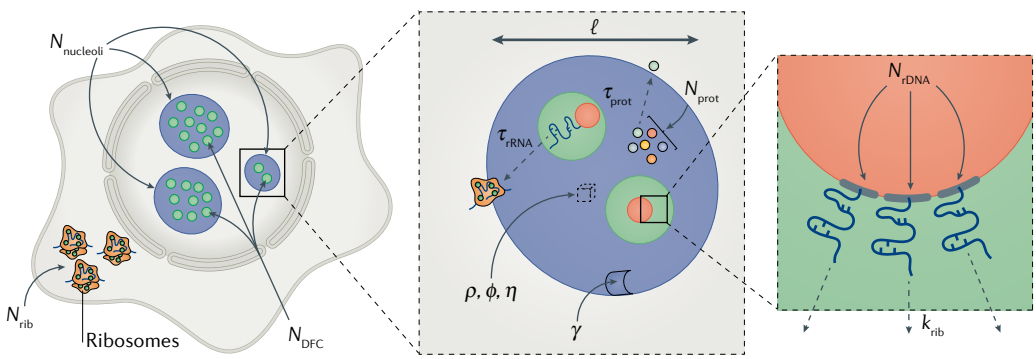
- The average number of nucleoli per cell nucleus ($N_{nucleoli}$): ~2–5 in HeLa cells[106].
- The size (diameter) of the nucleolus ($\ell$): ~1–5 µm in HeLa cells, ~1–10 µm in *Xenopus laevis*[15].
- The number of fibrillar centre (FC)–dense fibrillar component (DFC) modules per nucleolus ($N_{DFC}$): several dozen in human cells. This number is relatively constant within a particular cell type but varies considerably between cell types, making it a powerful biomarker[23].
- The size of the DFC: outer diameter ~630 nm and inner diameter ~360 nm in human cells[23].
- The number of different nucleolar proteins ($N_{prot}$): >1,300 in human cells[35].
- The average residency time of nucleolar protein in the mammalian nucleolus ($\tau_{prot}$): ~10–100 s[28].
- The percentage of disorder in nucleolar proteins: the median fraction of disordered residues per protein in human cells is 14% (12% in mouse), 20% (22% in mouse) and 36% in cytosolic-associated, nucleolar-associated and perichromosomal region-associated proteins, respectively[35].

**Biophysical properties**

- The viscosity ($\eta$): ~10 Pa·s for the granular component (GC)[16]. For reference, the viscosity of water is $10^{-3}$ Pa·s.
- The surface tension (GC–nucleoplasm interface) ($\gamma$): ~$10^{-6}$ N/m[16,107]. For reference, the surface tension of an air–water interface is 0.07 N/m.
- The density (average) ($\rho$): 1.155 g/ml[29], estimates from the *X. laevis* germinal vesicle.
- The volume fraction of biomolecules (average) ($\phi$): 0.16 (REF.[29]). For reference, for equal-sized spheres, the highest possible density of their packing in a given volume is represented by a volume fraction of 0.74.

**Functional characteristics**

- The number of transcriptionally active ribosomal DNAs per FC–DFC module in human cells ($N_{rDNA}$): 2–3 (REF.[23]).
- The ribosomal RNA transcription rate ($k_{rib}$): ~$10^3$–$10^4$ transcripts/min[108–110].
- The average nucleolar residency time of ribosomal RNA ($\tau_{rRNA}$): ~30–60 min[74].
- The number of ribosomes per cell ($N_{rib}$): ~$10^5$ in yeast and ~$10^6$–$10^7$ in animal cells[110,111].
- The organization of ribosomal assembly factors in the DFC: in human cells, DFCs establish a spherical network of 18–24 regularly spaced 'beads' of ~130 nm in diameter, with a distance between two adjacent beads of ~180 nm (see FIG. 1d). Considering that early maturation-stage yeast pre-ribosomes are ~25 nm × 30 nm and human counterparts are likely a bit larger, each bead may correspond to multiple maturing pre-ribosomes.



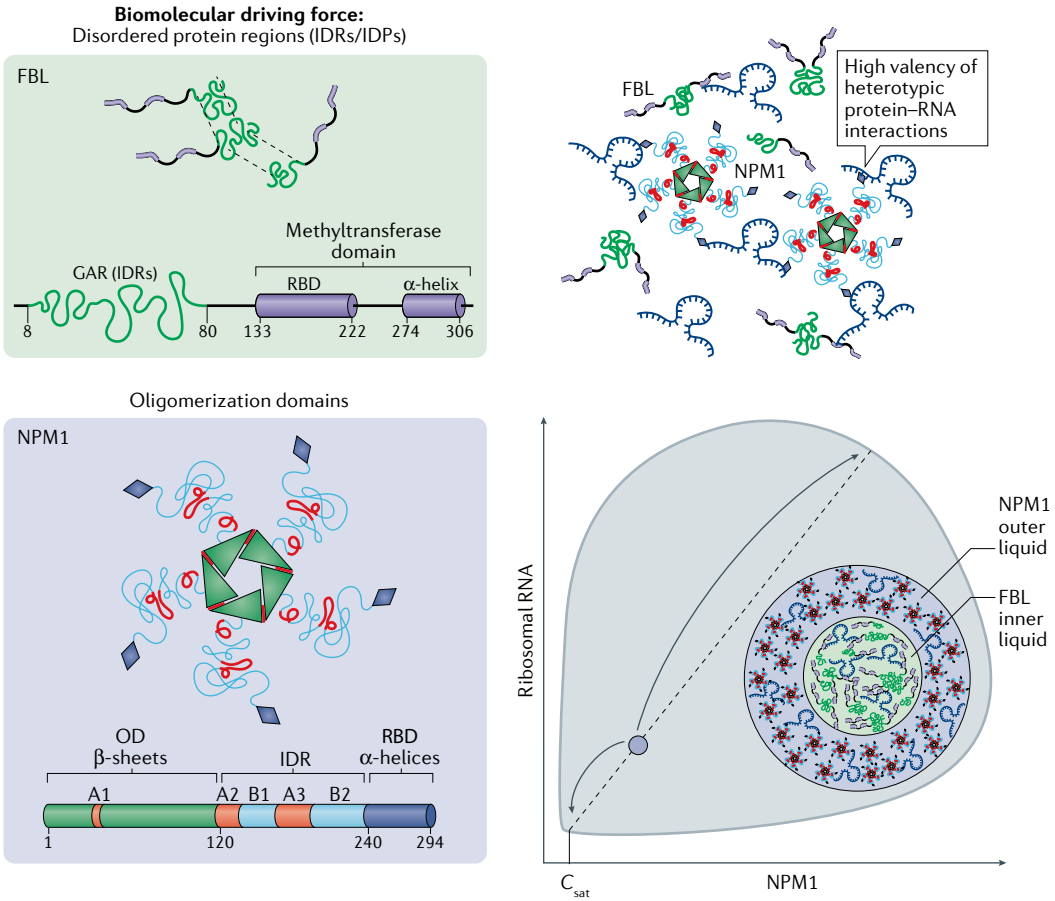
Box 2 | **Molecular biophysics of liquid–liquid phase separation**

Liquid–liquid phase separation (LLPS) is now well-established as a key process driving the formation of various intracellular condensates. LLPS is a simple yet powerful physical mechanism that occurs even in non-living systems, for example in oil–water mixtures or synthetic polymeric materials. LLPS results in the 'demixing' of an initially homogeneous system, such that a specific set of favourably interacting molecules concentrate together. Such behaviour is governed by a saturation concentration ('threshold') of biomolecules, $C_{sat}$, above which they condense into the liquid form. This behaviour can be represented schematically in a phase diagram (see the figure), which is derived from the thermodynamic free-energy landscape of interacting biomolecules. In the simplest scenario of a single-component solution, phase separation results in fixed $C_{sat}$; in other words, even if the total system concentration changes, the concentration inside and outside condensates is fixed. By contrast, for multicomponent mixtures like those found in endogenous cell condensates, $C_{sat}$ varies as a function of total component concentration — the phase diagram shown here represents an example of such behaviour.

Intrinsically disordered regions (IDRs), which are particularly abundant in nucleolar proteins[35], have received much attention as key motifs driving LLPS. In vitro, many IDRs have been shown to undergo LLPS, with substantial in vivo support for the role of IDR-containing proteins as building blocks for the liquid meshwork of numerous condensates[42,112]. Additional means to provide increased interaction valency, such as protein–protein and RNA–protein interactions, are important for promoting phase separation in cells. Accordingly, at sufficiently high valency and concentration, mixtures of proteins and RNA substrates can undergo LLPS. The resulting condensates are thought to allow control over the local molecular environment and to facilitate rapid exchange between components in and out of the condensate.

Nucleolar proteins strongly implicated in driving LLPS include the dense fibrillar component constituent, fibrillarin (FBL), and the scaffold protein of the granular component, nucleophosmin (NPM1). FBL displays a Gly–Arg-rich domain (GAR domain) that contains IDRs. In NPM1, there is a succession of acidic (A1–A3) and basic (B1 and B2) tracts in the IDR region. The interaction valency can be amplified when substrate binding domains, also referred to as nucleic acid or RNA binding domains (RBDs), are coupled to oligomerization domains (ODs), such as the pentamerization domain in NPM1.

Recent work has revealed that in addition to protein composition (presence of IDRs, Arg repeats and so on) and concentration, other parameters are important to drive LLPS. These include pH, temperature, protein post-translational modifications and the presence of an RNA seed (to promote nucleation and/or lower the saturation concentration)[113–115].
IDP, intrinsically disordered protein.




**Surfactant**
A 'surface-acting' molecule that tends to localize at the interface of two phases, reducing surface tension; amphiphilic molecules are classic surfactants, due to their ability to simultaneously interact with both water and relatively hydrophobic structures. Ki-67 protein has been proposed to act as a surfactant in the context of the perichromosomal region.

**Perinucleolar chromatin**
Condensed chromatin lining the nucleolus during interphase and enriched in specific genes to react to the environment and others (stress, sensory and so on).

a recent systematic survey identified dozens of proteins exhibiting a 'rim-like' co-localization at the nucleolar surface, which may represent a distinct quasi-2D nucleolar surfactant[35]. In addition, the nucleolus is often surrounded by a ring of condensed chromatin, known as the perinucleolar chromatin (FIG. 1a), corresponding mainly to inactive chromosomal regions enriched in genes important for sensory perception and development[36].

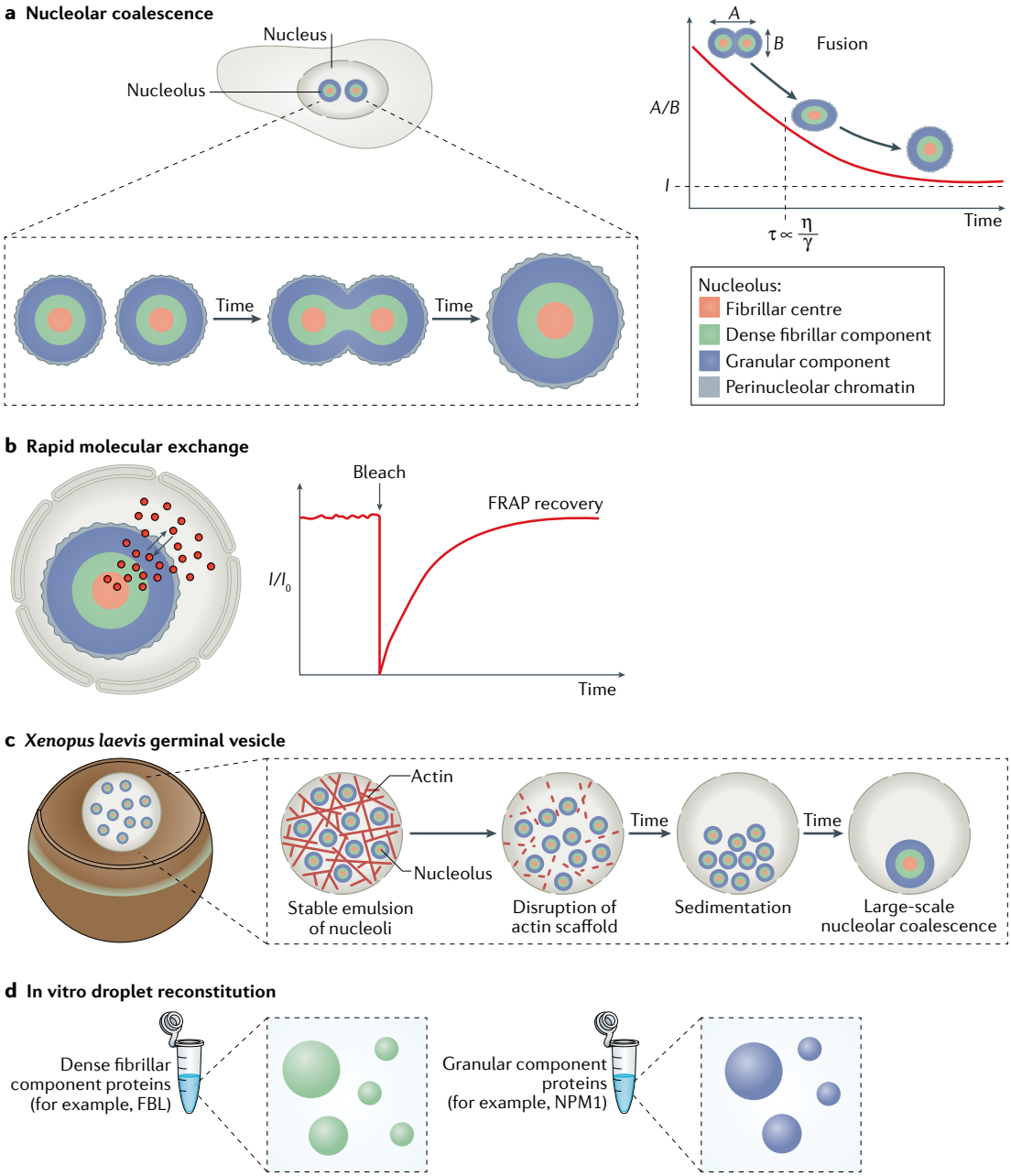



Fig. 2 | **Support for the liquid-phase nature of nucleoli. a** | Nucleoli will spontaneously coalesce with one another upon contact, in a manner similar to conventional liquids. This is mediated by surface tension, which seeks to minimize the total surface area. The timescale of coalescence (τ) can be determined by plotting the aspect ratio of coalescing nucleoli as a function of time, to determine the ratio of effective viscosity (η) and surface tension (γ). **b** | Nucleoli, like many other liquid condensates, exhibit rapid molecular exchange of component biomolecules. The dynamics can be monitored using fluorescence recovery (that is, the ratio of current integrated fluorescence intensity to initial intensity, $I/I_0$) after photobleaching (FRAP), which for typical nucleolar proteins reveals full molecular exchange with the surrounding nucleoplasm on timescales of tens of seconds. **c** | The *Xenopus laevis* oocyte contains a nucleus — the germinal vesicle — that is unusually large and contains hundreds of amplified extrachromosomal nucleoli, which represent a nucleolar emulsion stabilized within an elastic actin network. Upon actin disruption, nucleoli sediment under gravity and coalesce with one another, resulting in a single extremely large nucleolus. **d** | Purified nucleolar proteins such as fibrillarin (FBL) and nucleophosmin (NPM1) will readily phase separate in vitro, forming condensed liquid droplets that exhibit biophysical features similar to those of intact nucleoli.

Sometimes, projections of the perinucleolar chromatin may reach deeply inside the structure of the nucleolus, where they appear as nucleolar interstices or vacuoles (FIG. 1a).

Electron microscopy, and more recently super-resolution microscopy, has highlighted this rich substructure and linked it to nucleolar function. Indeed, super-resolution approaches recently revealed that each

Intrinsically disordered regions
(IDRs). Protein regions that exhibit considerable conformational heterogeneity. The biased amino acid sequences of IDRs encode an intrinsic preference for conformational disorder and an inability to fold into singular well-defined 3D structures under physiological conditions.

Partitioning
The preference of a component for one of two distinct phases, quantified as the ratio of concentrations in the two phases (defining the partition coefficient, for example $K = C_{nucleolus} / C_{nucleoplasm}$) or as the free energy of transfer ($\Delta G_{transfer} = -RT\log(K)$).

P granule
A perinuclear condensate implicated in germ cell lineage maintenance in *Caenorhabditis elegans*. P granules may serve similar functions to polar granules or nuage, which regulate germ cell biology across animal cells.

Emulsions
Liquid–liquid phase-separated solutions in which droplets of one liquid phase are dispersed throughout another immiscible continuous liquid phase; for example, droplets of vinegar in oil.

Ostwald ripening
The process by which larger droplets grow at the expense of smaller droplets, as a result of droplet surface tension (Laplace pressure) causing a higher chemical potential within the smaller droplet.

Viscoelastic
A viscoelastic material is one whose response to applied stresses is intermediate between that of a purely elastic solid and that of a purely viscous liquid; striking but common examples include silly putty and corn starch/water mixtures.

Saturation concentration
The concentration of a structurally key biomolecule above which liquid–liquid phase separation occurs. Note that for multicomponent systems governed by heterotypic interactions, the saturation concentration may not be identical to the concentration outside the condensate.

human nucleolus consists of several dozen FC–DFC modules, the number of which can vary between cell types[37], and confirmed that rRNA transcription occurs at the FC–DFC interface where Pol I complexes are enriched (FIG. 1d), with each FC–DFC module containing two or three transcriptionally active rDNAs[23]. Ribosome assembly factors, such as the box C/D small nucleolar RNA-associated methyltransferase fibrillarin (FBL or FIB1) and others, form a spherical network of 18–24 regularly spaced, nanoscale (100–200 nm) 'beads' that assemble into the DFC around the FC (FIG. 1d); such spatial distribution of ribosome assembly factors was previously noted for the helicase DDX21, which together with the long non-coding RNA SLERT regulates Pol I function[38]. Why ribosome assembly factors are arranged into such nanosized 'beads' is not yet clear, but it suggests that individual ribosomal maturation steps may occur in distinct 'reaction chambers', spatially segregated from others in order to increase the efficacy and selectivity of each step.

## Nucleolus as an LLPS-driven body

Many nucleolar proteins harbour intrinsically disordered regions (IDRs), often including charged domains, which have been highlighted as key drivers of phase separation (see BOX 2). It has long been known that many nucleolar proteins, including fibrillarin and rRNA transcription factor nucleolin, exhibit Gly–Arg-rich domains, sometimes referred to as GAR domains[39]. Within these GAR domains, the Arg residues in particular appear key, as together with similarly positively charged Lys residues they have been long appreciated as central to nucleolar localization sequences[40]. Additionally, there is an enrichment of negatively charged acidic tracts (that is, Glu and/or Asp-rich IDRs) that can interact with basic tract-containing proteins in vitro[18]. Consistent with the importance of these charged residues, another nucleolar methyltransferase, TGS1, was earlier shown to contribute to the coalescence and steady-state dynamics of the nucleolus, through multiple weak and transient interactions mediating its self-association and interaction with Lys and/or Arg-rich KKD/E domain-containing small nucleolar RNA ribonucleoproteins[41]. Despite these tantalizing hints, how these IDRs contribute to driving nucleolar assembly and partitioning through LLPS has only started to become clear, in large part through recent in vitro reconstitution studies.

***Evidence from in vitro reconstitution approaches.*** As with many intracellular structures, approaches towards 'bottom-up' reconstitution have been fruitful for understanding the molecular and biophysical principles governing the assembly and function of nucleoli. The first reconstitution approaches focused on the key nucleolar proteins fibrillarin and nucleophosmin.

Fibrillarin is a prominent protein within the DFC (FIG. 1) and in humans contains a disordered N-terminal Gly–Arg-rich region (BOX 2), much like that found in known phase-separating proteins such as the P granule helicase Laf1 (REF.[42]), among others[43]. This region contributes to fibrillarin phase separation in vitro, in a protein and salt concentration-dependent manner[16,23,44]; the nucleolar protein GAR1, named for its two Gly–Arg-rich domains, exhibits a similar tendency to phase separate[45].

Phase-separated emulsions coarsen over time, meaning that the average size of droplets grows through either direct coalescence of droplets upon contact or Ostwald ripening. Both mechanisms predict that the average droplet size, $\langle R \rangle$, will increase with time, $t$, according to $\langle R \rangle \sim t^{1/3}$. The growth dynamics of in vitro nucleolar protein condensates, as well as nucleoli observed in developing *Caenorhabditis elegans* embryos, exhibits such $t^{1/3}$ coarsening dynamics; interestingly, emerging evidence suggests that the surrounding viscoelastic chromatin can decrease the scaling exponent for coarsening nuclear condensates[46]. Evidence of a saturation concentration, below which nucleolar phases do not assemble (BOX 2) and that was shown to be important for several nucleolar proteins, provides additional quantitative support for the LLPS model in nucleolar organization[20,44]. Importantly, for fibrillarin, the saturation concentration also depends on the presence of RNA — higher RNA concentrations decrease the saturation concentration (that is, greater tendency to phase separate) — which led to a model whereby the preferential condensation of fibrillarin around actively transcribing rDNA arises from the high RNA concentration there[44].

Nucleophosmin resides predominantly in the GC (FIG. 1) and is known to have a central role in nucleolar structure[47,48]. Nucleophosmin exhibits a domain architecture characteristic of many phase separation-prone proteins (for example, G3BP, PGL1 and others): an N-terminal oligomerization domain, an intervening disordered region and a C-terminal substrate (RNA) binding domain (BOX 2). The N-terminal domain of nucleophosmin drives pentamerization, whereas the disordered linker region displays multiple acidic tracts that can interact with basic proteins, particularly Arg-rich proteins, which are known to have high nucleolar partitioning properties. In vitro experiments showed that nucleophosmin can phase separate either together with Arg-rich proteins, such as the GC scaffold protein SURF6 or ribosomal proteins such as RPL23a, or together with RNA[17,18] (BOX 2). In addition to heterotypic LLPS, nucleophosmin can undergo homotypic LLPS in vitro, which is mediated by weak basic tracts linking the acidic tracts and its pentamerization domains[17]. The phase behaviour of nucleophosmin can potentially be regulated by phosphorylation of its oligomerization domain, which is dependent on its subcellular localization and cell cycle phase. Specifically, hyperphosphorylation promotes oligomerization domain unfolding that impedes pentamerization[49], and thereby likely weakens LLPS of nucleophosmin, in a manner similar to that seen upon truncation of the oligomerization domain[16].

***In vivo insights.*** Key insights into the biophysical properties of nucleoli in living cells have emerged from studies in the frog *Xenopus laevis*. Mature *X. laevis* oocytes contain a nucleus, commonly referred to as the germinal vesicle, that is roughly 600 μm in diameter and contains hundreds of nuclear bodies, primarily nucleoli, that assemble around actively transcribing

extrachromosomal rDNA. An interferometric imaging technique was utilized to show that Cajal bodies and nucleoli in the germinal vesicle are of surprisingly low density, allowing their penetration by fluorescent dextran molecules in a molecular weight-dependent manner[29]. This low-density structure was referred to as 'sponge-like', but, at least in the case of Cajal bodies, their highly spherical nature was described as potentially reflecting a 'semi-fluid' nature (they behave somewhat like liquids).

Mature *X. laevis* germinal vesicle nucleoli are indeed remarkably spherical, and can be observed to spontaneously coalesce and round up upon contact, and can also be pushed together with a microneedle, exhibiting the same fusion behaviour[15] (FIG. 2a). Consistent with spontaneous coalescence events occurring throughout oogenesis, the nucleolar volume, $V$, was found to exhibit a broad size distribution, well-characterized by a power law: $P(V) \sim V^{-3/2}$ (corresponding to the presence of many smaller nucleoli and few large nucleoli). This −3/2 exponent is in quantitative agreement with simulation and theory predictions from a slowly coarsening emulsion model[15]. The nucleolar emulsion was subsequently shown to require stabilization by a nuclear actin scaffold[16,50,51], which upon destabilization, for example using actin depolymerizing drugs, gives rise to a small number of extremely large and spherical nucleoli[50] (FIG. 2c). These findings provide further support for a physical picture in which nucleoli can be thought of as RNA and protein-rich liquid condensates, which readily fuse within one another but are kinetically stabilized against large-scale coalescence. In the case of the *X. laevis* germinal vesicle, an unusual filamentous actin network stabilizes the emulsion by hindering nucleolar motion, whereas in more typical somatic cells, the surrounding chromatin would play a similar role[21]. Indeed, the shape of the nucleolus may be impacted by numerous factors, particularly the surrounding and the integrated chromatin. When considering HeLa cells versus stem cells versus embryos, changes in nucleolar shape may reflect differences in chromatin density and compaction state — the *X. laevis* germinal vesicle, for example, has extremely low chromatin density, whereas one expects HeLa cells to exhibit a higher degree of dense, compacted chromatin relative to stem cells. Thus, extrinsic factors particular to different cell types may contribute to nucleolar shape and size.

## Nucleolus as a multiphase condensate

As discussed above, nucleoli exhibit quantitative signatures consistent with the model that LLPS is a primary driver of nucleolar assembly. However, nucleoli exhibit a complex internal substructure. The recent proposal that this substructure may represent multiple coexisting, immiscible liquid phases[16] may be surprising in the light of the simple LLPS model, given that we are mostly familiar with the phase behaviour of simple oil and water mixtures, with only two distinct phases and no such multilevel layering. However, it is well known that more complex mixtures of non-biological liquids — such as water and oils with different properties (for example, vegetable oil and silicone oil) — can give rise to more complex phase behaviour, including richly structured multiphase assemblies[16,52] (FIG. 3a,b). Remarkably, it was found that key nucleolar proteins of the DFC (fibrillarin) and GC (nucleophosmin) subcompartments, which were known to individually undergo in vitro phase separation, have disfavourable mutual interactions that can drive similar multiphase immiscibility[16] (see also next section). Moreover, the resulting immiscible subphases recapitulate the 'core–shell' architecture of nucleoli, with fibrillarin forming a 'core' surrounded by a nucleophosmin-rich liquid 'shell', recapitulating the organization into the DFC and GC phases observed in cells (BOX 2; FIG. 3c,d). Although the molecular origins of the subcompartment immiscibility are still not well understood, findings in this study suggest that the IDRs within fibrillarin and nucleophosmin may be fully miscible. Instead, it appears that the RNA binding domains of these two proteins, which provide substrate specificity, encode the formation of mutually incompatible liquid phases — a conclusion that is echoed in a recent study on the molecular origins of stress granule–P body immiscibility[53]. Interestingly, however, it is known that the relative organization of two or more immiscible phases is dictated by the values of the surface tension between the phases[52]. Nevertheless, our understanding of the biomolecular determinants of condensate surface tensions, and the potential role of any surfactant-like biomolecules in modulating these interfacial properties, is extremely limited.

## Nucleolar material properties

In addition to its interfacial material properties, the bulk mechanical properties (for example, elasticity and viscosity) of the nucleolus are thought to be important for its structure and function (see BOX 1). For macroscopic materials, characterization of their mechanical properties is often achieved by measuring their relationship between stress, or the normalized force applied to a material, and the resulting strain, or the normalized displacement. For a solid, the measured stress is proportional to strain (as with a Hookian spring, for which the force is proportional to the displacement), whereas for a liquid the stress is proportional to the rate of change of the strain (FIG. 3e). Materials that exhibit intermediate properties are referred to as viscoelastic; viscoelasticity is common in complex fluids containing macromolecular (for example, polymeric) components. Although measurements of microscopic materials are more difficult, these material properties and their impact on structure and molecular dynamics can be determined using specialized techniques (FIG. 3f,g). Measuring viscoelasticity and its link to biological function is still a largely unexplored aspect of nucleolar biology, but intriguing initial results are beginning to emerge.

The stability of a complex fluid's viscoelastic properties over time should not be taken for granted, as many polymeric liquids exhibit metastability, with properties that can change with time, for example undergoing a liquid-to-gel transition[54] (a process also known as maturation or hardening). This transition towards more solid-like material properties results in decreased fusion rates and decreased molecular exchange between

**Cajal bodies**
(Also known as coiled bodies). Nuclear condensates containing coilin and survival of motor neuron protein (SMN). Cajal bodies are enriched in U snRNAs and share some protein components with the nucleolus, such as fibrillarin.

**Stress granule**
Cytoplasmic condensates that form in response to stress (for example, oxidative stress and heat stress).

**P body**
Cytoplasmic condensates involved in mRNA degradation.

**Surface tension**
A material parameter characterizing the energy per unit area associated with an interface between two distinct phases. Liquids tend to round up into spheres as spheres minimize the surface area and thus minimize the interfacial energy imposed by surface tension. As nucleoli exhibit multiple subphases, each interface between them will have its own surface tension.

**Complex fluids**
Typically soft, liquid-like materials that often contain multiple macromolecular components such as polymers or colloidal particles and that usually exhibit viscoelastic properties; an emulsion is a type of complex fluid.

**Metastability**
A physical system may be said to be metastable if it resides in a local thermodynamic minimum, which over longer periods may begin transitioning into a different state associated with an even lower (global) energy minimum.

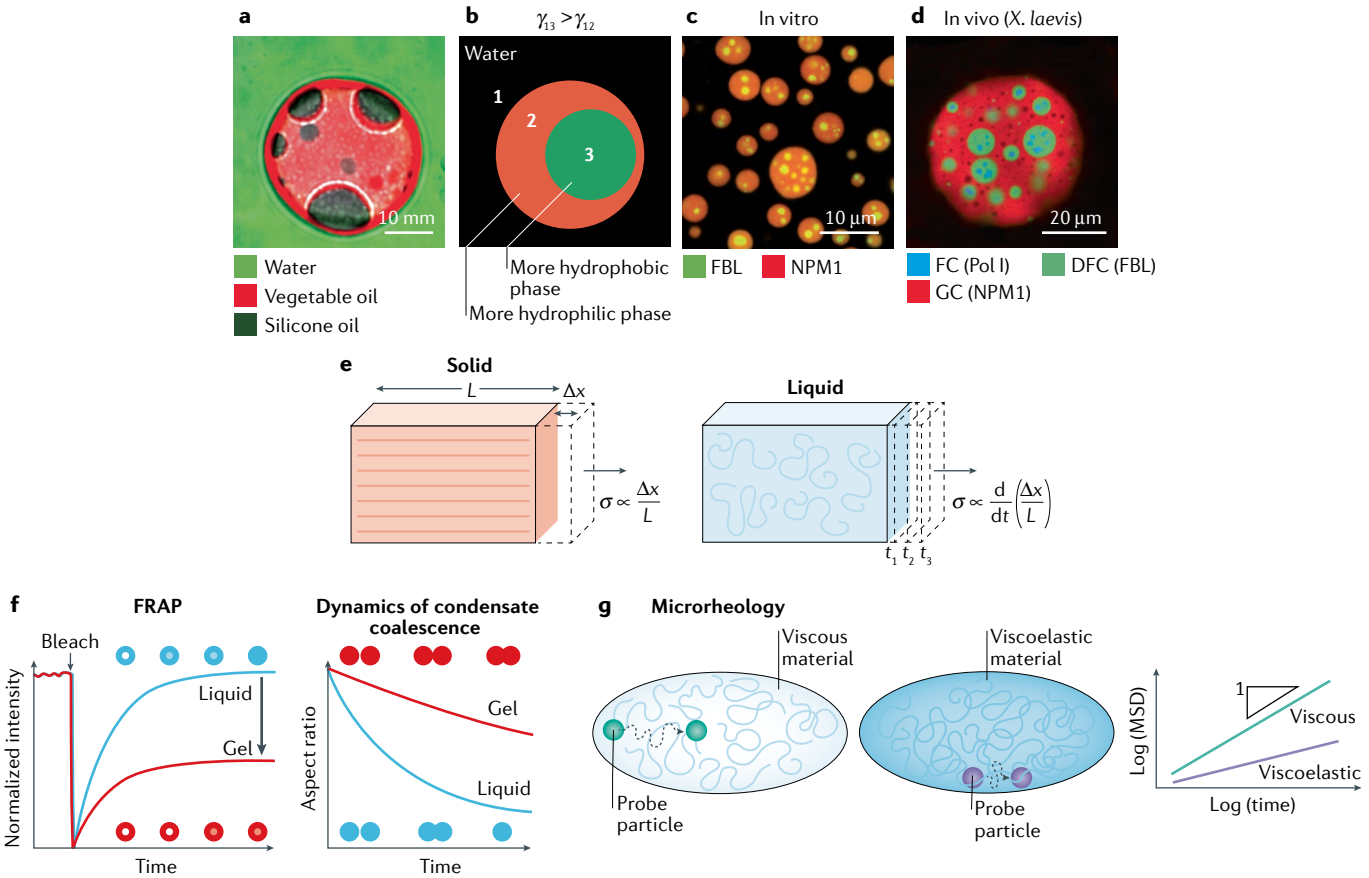



Fig. 3 | **Understanding nucleolar substructure and material properties.** **a** | Non-biological multiphase droplets established by mixing water, vegetable oil and silicone oil. These non-biological liquids spontaneously form multiphase droplets due to their material properties, providing an illustrative example of how similar physical self-assembly rules may be at play in the nucleolus and other multiphase biomolecular condensates. **b** | Schematic organization of immiscible multiphase droplets. The more effectively hydrophobic liquid (green; 3) has a higher surface tension with water (1) than the more effectively hydrophilic liquid (red; 2) ($\gamma_{13} > \gamma_{12}$), thus driving the multiphase arrangement shown, as this organization minimizes the free energy of the system. **c** | Condensates formed from the nucleolar proteins nucleophosmin (NPM1; scaffold protein of the granular component (GC)) and fibrillarin (FBL; component of the dense fibrillar component (DFC)) appear to be relatively immiscible with one another, with NPM1-rich condensates forming a 'liquid shell' around FBL-rich condensates. **d** | Example of a large coalesced nucleolus in the *Xenopus laevis* germinal vesicle. The fibrillar centre (FC), DFC and GC are indicated. Note how the coalesced DFC regions are highly spherical, underscoring their physical nature as a liquid subphase within the GC. **e** | Solid-like materials are often probed by applying a defined stress (force per unit area, $\sigma$) or strain (displacement, $\Delta x$, normalized by sample size, $L$) and monitoring the other. Solids exhibit a relationship in which the stress is directly proportional to the strain. By contrast, liquid-like materials exhibit a stress that is directly proportional to the rate of change of the strain. **f** | Several techniques are increasingly implemented to probe the material state of nucleoli and other condensates. Full recovery of the signal in fluorescence recovery after photobleaching (FRAP) experiments (blue) is consistent with liquid-like properties, whereas incomplete FRAP signal recovery (red) is consistent with more solid-like properties (left). Nevertheless, the dynamics must be carefully interpreted[103]. Quantifying the dynamics of condensate coalescence can also provide insight into bulk material properties: liquids tend to fully round up due to surface tension, whereas solid-like particles tend to exhibit arrested coalescence (right). **g** | Passive microrheology consists of tracking Brownian motions/thermal fluctuations of probe particles embedded within soft materials to determine the viscoelasticity. The graph shows the mean-squared displacement (MSD) of particle position, which is suppressed in materials with higher viscosity, and exhibits a characteristic time-dependence (slope on log–log plot, indicated by the triangle; a slope of 1 characterizes Brownian motion within a simple viscous liquid) that can indicate viscoelasticity[104]. Pol I, polymerase I; *t*, time. Figure adapted with permission from REF.[16], Elsevier.

the phases (decreased molecular dynamics). A large number of studies using techniques such as FRAP, droplet coalescence or microrheology (FIG. 3f,g) suggest that ribonucleoprotein condensates can indeed often become more solid-like over time, which could underlie pathology, prominently including many neurodegenerative diseases[55–58] (see also below). Consistent with this possibility for the nucleolus, droplets assembled from purified fibrillarin have been observed to undergo rapid gelation[16], losing nearly all molecular rearrangement (on the second timescale) after an hour. Experiments on large nucleoli in the *X. laevis* germinal vesicle confirmed many of the same liquid properties for the fibrillarin-rich DFC core and the nucleophosmin-rich GC shell, including a relatively stable and low-viscosity fluidity of the GC, with apparently more viscoelastic and potentially time-dependent changes in the DFC properties[16].

Studies in the *X. laevis* germinal vesicle suggest that nucleolar fluidity requires active maintenance. As described above, when the surrounding actin network is disrupted, the nucleolar emulsion is destabilized, nucleoli begin contacting one another and two or more touching nucleoli coalesce into a single, larger spherical condensate. From studies in fluid dynamics and soft

Microrheology
A technique used to measure the rheological properties of a microscopic material. Passive microrheology consists of tracking Brownian motions/thermal fluctuations of probe particles embedded within soft materials to determine viscoelasticity. Active microrheology utilizes externally applied stresses (for example, through optical or magnetic tweezers) and is particularly attractive for studying non-equilibrium systems where fluctuating motion may not be purely thermal (for example, ATP-dependent fluctuations).

Nucleolar breakdown
The process of nucleolar disassembly at the onset of mitosis.

Nucleolar genesis
The process of nucleolar (re) assembly at the end of mitosis.

Spinodal decomposition
The process by which phase separation occurs spontaneously without any nucleation barriers, due to the negative curvature of the free-energy landscape.

Heterogeneous nucleation
A process by which nucleation and growth of one phase within another is facilitated by nucleation on a favourable pre-existing surface (such as nascent pre-ribosomal RNAs in the case of nucleolar genesis).

Precursor–product relationship
A relationship between A and B if the production of B depends upon the disappearance of A. This applies, for example, to RNA processing (between upstream and downstream intermediates) and to the successive intermediate condensates formed during nucleolar genesis.

Perichromosomal region
Liquid-like condensate made of nucleolar proteins forming a 'sheath' around the compacted mitotic chromosomes (absent at the centromeres).

Nucleolar-derived foci
Condensates consisting of nucleolar proteins and formed during mitosis after nuclear envelope breakdown.

matter[59], it is known that the timescale for fusion is set by a ratio of viscosity to surface tension (inverse capillary velocity; $\tau \sim \eta/\gamma$, where $\tau$ is the timescale of coalescence, $\eta$ is the ratio of effective viscosity and $\gamma$ is the surface tension). This study found that the values of $\eta/\gamma$ for coalescing nucleoli depend on ATP, as ATP depletion with the enzyme apyrase led to an increase of this value by over an order of magnitude, which was interpreted as an ATP-dependent viscosity. Similar approaches have also been recently utilized in human cells (HeLa), finding that the behaviour of the nucleoplasmic–nucleolus interface is ATP-dependent and sensitive to changes in chromatin structure[21]. These collective findings raise the possibility that cells can use active processes to tune nucleolar dynamics and biophysical properties, including the interface between the nucleolus and other nuclear components.

## Non-equilibrium nucleolar dynamics

In addition to having complex, multiphase organization, nucleoli also exhibit active, non-equilibrium features — with components subject to active modification and flux between the nucleolus and the rest of the nucleus. This contrasts with the simple picture of equilibrium phase-separated liquid mixtures, which exhibit molecular exchange between the liquid and surrounding phase but are otherwise chemically inert, with no steady-state component flux. As increasingly sophisticated modelling approaches begin building from the simplest LLPS framework, new insights will emerge into how cells dynamically regulate the biomolecular interactions that give rise to the emergent liquid-like organization.

***Nucleolar dynamics throughout mitosis.*** Using different cell biology and biochemistry techniques[28,60–62] it has been documented that the nucleolar structure and biomolecular dynamics change throughout the cell cycle, representing a dramatic manifestation of underlying non-equilibrium activity of the nucleolus. During interphase, nucleolar proteins are in constant flux into and out of the nucleolus, undergoing rapid exchange with the surrounding nucleoplasm. Importantly, however, residence of proteins within the nucleolus can occur on a timescale that may be tuned by actively modulating the weak, multivalent interactions at the heart of LLPS.

One of the best illustrations of the non-equilibrium aspect of nucleolar homeostasis is the major redistribution of nucleolar proteins that takes place during mitosis. At the onset of mitosis, the nucleolus undergoes rapid nucleolar breakdown, concomitant with the shutdown of Pol I activity, followed by slow nucleolar genesis at mitotic exit, which coincides with the resumption of Pol I function[63–65] (FIG. 4a,b). Of note, in modern eukaryotes with open mitosis, not only the nucleolus but, indeed, most condensates disassemble and reassemble as the nucleus itself breaks down and reforms during each cell cycle[66]. Thus, whereas it is not entirely clear how nucleolar disassembly is promoted, nuclear envelope breakdown likely contributes, in part, through dilution of nucleolar components in the nucleoplasm.

From numerous studies in non-living matter, the formation of a new liquid phase typically occurs through a process known as nucleation and growth (and possibly through spinodal decomposition). Classical nucleation theory describes how a new phase must overcome the energetic barrier associated with the creation of a new interface (an energetic cost parametrized by surface tension)[67]. These barriers can be lowered by nucleation on energetically favourable surfaces (that is, those with low surface tension), but it remains unclear whether such heterogeneous nucleation represents an accurate description of nucleolar genesis. In fact, the underlying biophysics of nucleolar genesis appears to be considerably more complicated than what can be captured using classical nucleation theory (FIG. 4c). For example, the dynamics of nucleolar breakdown and nucleolar genesis highlights a rich interdependence of nucleolar substructures. Over the course of ~5 min during nucleolar breakdown, the FC is dissolved first, then the DFC and then the GC, whereas during ~30 min of nucleolar genesis the FC reforms first, then the DFC and then the GC[63–65] (FIG. 4a). After nucleolar breakdown, nucleolar constituents are incorporated into successive intermediate condensates that form at the expense of one another (often referred to as the precursor–product relationship): the perichromosomal region is the first to form, then the nucleolar-derived foci and, finally, the prenucleolar bodies (FIG. 4a,b). Towards the end of mitosis, nucleolar proteins are redistributed from the prenucleolar bodies to nucleolar organizer regions, which serve as nucleation sites or 'seeds' for nucleolus reformation[64,68]. Nucleolar organizer regions are rDNA-bearing segments, which are located on the short arms of the five acrocentric chromosomes (human chromosomes 13, 14, 15, 21 and 22). Thus, diploid human cells contain ten nucleolar organizer regions, but they are never all transcriptionally active at the same time (see BOX 1).

How the formation of intermediate nucleolar condensates is regulated is not known. The chromosome surfactant protein Ki-67 was shown to be essential for perichromosomal region formation[35,69–71], a process that is itself thought to be sequential[35], involving at least two steps. The role of the intermediate condensates formed during nucleolar genesis is also unclear. In the case of the perichromosomal region, which outlines the condensed chromosomes like a 'sheath', it has been proposed to help partition equal amounts of nucleolar proteins between the two daughter cells ('chromosome passenger' model). Of interest, nucleolar proteins in general, and particularly mitotic chromosome-associated ones (perichromosomal region), have significantly higher levels of disorder per residue than cytosolic proteins, suggesting that the perichromosomal region could also be liquid-like[35] (BOX 1).

Several studies utilizing *C. elegans* and *Drosophila melanogaster* embryos demonstrated behaviours of nucleolar components during nucleolar reformation that are generally consistent with classical phase separation theory, but also highlighted the limitations of the equilibrium framework for describing complex nucleolar dynamics[19,44,72]. The precursor structures were shown to be liquid-like — and referred to as 'extranucleolar droplets' — and were shown to either coalesce with nucleolar organizer regions or dissolve. Notably,

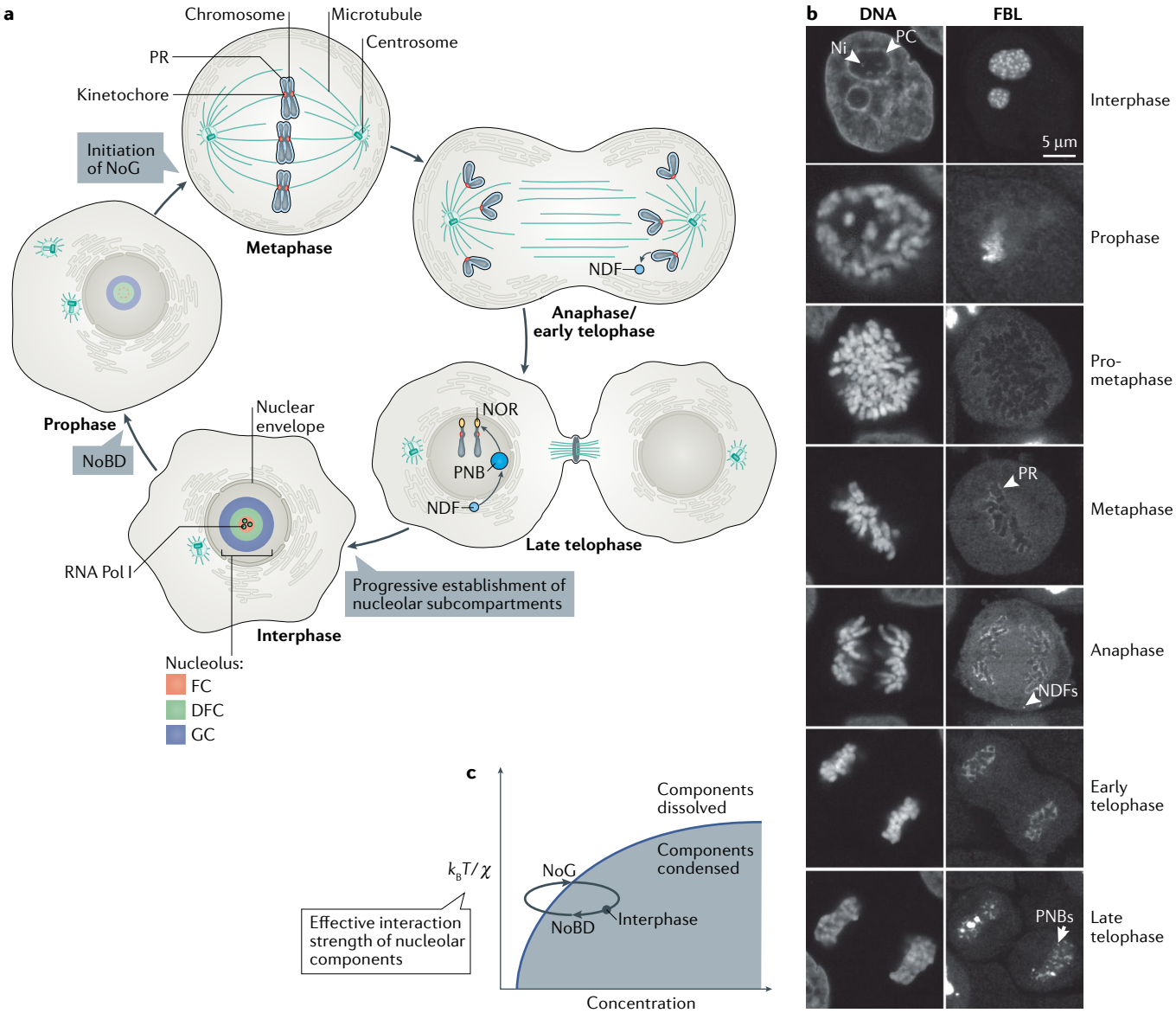



Fig. 4 | **Nucleolar dynamics in mitosis. a** | Nucleolar breakdown (NoBD) is first apparent at prophase, when polymerase I (Pol I) function is shut down, with the fibrillar centre (FC) being the first subnucleolar compartment to be dissolved. This is soon followed by nuclear envelope disassembly (occurring at 2 min after the onset of NoBD), then by dissolution of the dense fibrillar component (DFC; at 3 min after initiation of NoBD) and, finally, by disappearance of the granular component (GC; at 5 min after initiation of NoBD) (details not shown). Nucleolar reformation (nucleolar genesis (NoG)) starts shortly after its dissolution. At metaphase, the nucleolar proteins concentrate around the chromosomes forming the liquid-like perichromosomal region (PR). During anaphase, nucleolar proteins relocalize to small foci termed nucleolar-derived foci (NDFs). At telophase, as the nuclear envelope reforms, nucleolar proteins are relocalized from NDFs to nuclear foci, the prenucleolar bodies (PNBs). Pol I is reactivated, and the nucleolar proteins are redistributed from the PNBs to the nucleolar organizer regions (NORs), corresponding to the rDNA units encoded on the short arms of the five acrocentric chromosomes (human chromosomes 13, 14, 15, 21 and 22) and acting as a nucleation site for nucleolar assembly. The FC is the first nucleolar compartment to form at actively transcribed NORs (details not shown), and the nuclear envelope integrity is then restored, followed by formation of the DFC (at 9 min after FC assembly) and that of the GC (27 min after FC assembly) (see also FIG. 5b). **b** | Example images of NoBD and NoG using confocal microscopy of HeLa cells. The nucleoplasm (DNA) was stained with DAPI, the DFC with fibrillarin (FBL). **c** | Schematic illustration of the dynamics of nucleoli during the cell cycle, interpreted within the liquid–liquid phase separation framework. *y* axis represents normalized inverse interaction strength, $k_BT/\chi$, and *x* axis represents the concentration of key nucleolar components. The value $k_BT/\chi$ (where $k_B$ is the Boltzmann constant, *T* is temperature and $\chi$ is the Flory–Huggins miscibility parameter) defines an effective interaction strength of nucleolar components, with lower values (that is, lower *T* and/or greater $\chi$) implying a stronger propensity for phase separation. Interphase nucleoli represent a cell state well within the two-phase region of an effective nucleolar phase diagram (shaded region). Upon changes in component concentration (for example, through nuclear envelope breakdown) and effective interaction strength (for example, through post-translational modifications), the cell moves into the one phase region of the phase diagram and NoBD is accelerated. Concomitant with reformation of the nuclear envelope, the concentration of nucleolar components again increases, and their interactions become more favourable, leading to NoG. Ni, nucleolar interstices; PC, perinucleolar chromatin. Images in part **b** are unpublished and a courtesy of D.L.J.L. and R.B.

Prenucleolar bodies
Nuclear condensates consisting of nucleolar protein formed during mitosis at the time of nuclear membrane reassembly.

Alu RNAs
RNAs encoded in Alu repeats, which are the most abundant repetitive genetic elements found in primates, representing up to 10% of the human genome.

RNA exosome
A 3′–5′ exoribonucleolytic and endonucleolytic multi-protein complex involved in RNA 3′ end formation, RNA turnover and RNA surveillance (quality control).

these extranucleolar droplets would appear to form randomly throughout the nucleus (ostensibly unseeded, formed via homogeneous nucleation), whereas nucleoli were seeded at nucleolar organizer regions and exhibited lower saturation concentration requirements for their formation than extranucleolar droplets. Studies in *D. melanogaster* examined the temperature dependence of nucleolar formation, revealing that some nucleolar proteins exhibit enhanced condensation at decreased temperature, whereas others exhibit the opposite behaviour, suggesting a role for active processes in nucleolar assembly, outside the simplest LLPS paradigm[19]. Studies in *D. melanogaster*[72] and *C. elegans*[44] both demonstrate that nucleolar organizer regions at which nucleoli are reformed during development showed active rDNA transcription, and rRNA (or, rather, pre-rRNA) appears to act as a 'seed' for nucleolus formation (FIG. 5a,b). In addition to the clear importance of rRNA for nucleating functional nucleoli, other non-rRNAs have also been shown to be important for regulating nucleolar homeostasis. These include the nucleolar-enriched Alu RNAs encoded in pre-mRNA introns, whose depletion disrupts nucleolar structure[73]. Overall, these studies reveal that nucleolar assembly requires active non-equilibrium processes, particularly rRNA transcription, which appear to effectively stabilize the biomolecular interactions underlying phase separation.

***Nucleolar compositional fluxes.*** Perhaps the most interesting feature of the non-equilibrium nature of nucleoli is the steady-state flux of components into and out of them. In particular, rRNA at different stages of processing progresses through the nucleolus and precursor ribosomal subunits constantly exit the nucleolus to continue their assembly in the nucleoplasm and cytoplasm[74]; many ribosome assembly *trans*-acting factors show similarly high dynamics, constantly shuttling into and out of the nucleolus. This is often described as a directed process, also referred to as 'vectorial' or an 'assembly line'. Although this language invokes mechanical imagery that belies the inherent stochasticity of biological processes, some steps in ribosome biogenesis (for example, transcription of precursor rRNA) must occur before others (for example, RNA modification and folding, or binding by ribosomal proteins and ribosome assembly factors), echoing the sequential formation of nucleolar subcompartments at mitotic exit (see above). Although the mechanistic details describing how ribosome assembly occurs in the nucleolus are far from being fully understood, recent studies have shed light on how a directional flux through the nucleolus may be achieved through driving forces inherent to the LLPS mechanism.

One recent study has shown that, as it emerges from the FC–DFC interface where it is synthesized, the 5′ end of the nascent rRNA transcript is bound co-transcriptionally by fibrillarin via its RNA binding domain[23]. Data support a model in which the directional sorting of nascent transcripts towards the DFC and its initial processing are promoted by phase separation, resulting from the self-association of fibrillarin through its GAR domain[23] (BOX 2; FIG. 1b) (of note, fibrillarin constructs lacking their GAR domain and the methyl transferase domain, which confers RNA binding, do not promote DFC formation). The RNA sorting mechanism does not require the methyltransferase activity of fibrillarin, which is also dispensable for RNA cleavage[23]. Thus, it appears that the phase separation capacity of fibrillarin mediates the partitioning of nascent transcripts from the FC–DFC interface to the DFC and this is required for initial rRNA processing and DFC nucleation.

Another recent study utilized a simple biophysical approach to extract thermodynamic properties of the interactions among nucleolar components, which provide insight into the physical forces underlying LLPS and partitioning of nucleolar components[75] (BOX 2). These results indicated a strongly heterotypic nature of the underlying biomolecular interactions, which provide the cohesivity holding together nucleolar components. Particularly important for nucleolar structure are those interactions between nucleophosmin and nascent rRNA (FIG. 5c,d), as well as other nucleolar scaffold proteins, such as SURF6, further highlighting the importance of nascent rRNA for nucleolar assembly. The progressive assembly of ribosome subunits leads to increasingly large ribosomal particles, which progressively contribute less and less to the network of heterotypic interactions required for phase separation and favourable partitioning into nucleoli. As a consequence, partitioning into nucleoli of assembled pre-ribosome subunits is disfavoured, potentially explaining how these subunits can preferentially be expelled out of the nucleolus[75].

Overall, the findings discussed in this section support a physical picture in which nucleoli assemble 'on demand': nascent rRNA drives nucleolar condensation through LLPS, and fully assembled pre-ribosomal particles are thermodynamically expelled out of the nucleolus (FIG. 5d). Thus, the physical nature of the nucleolus is tightly coupled to its function. Additional aspects of this coupling between structure and function will be further discussed below.

## Interplay of structure and function

The ribosome is perhaps the most complex macromolecular machine in the cell, and thus it should not be surprising that the assembly of ribosomal subunits within the nucleolus is a highly sophisticated process that entails thousands of reactions[3,76]. This complexity is daunting and presents a challenge for any biophysical modelling effort attempting to link the nucleolar structure and its function in facilitating ribosome biogenesis. Below, we suggest ways in which the LLPS model provides a fruitful starting point for understanding these links between nucleolar structure and function.

***Nucleolar function supports its structure.*** The myriad ribosome biogenesis reactions that take place within the nucleolus encompass broadly five distinct processes: production of three out of four rRNAs by Pol I (the fourth rRNA, 5S, is produced in the nucleoplasm by Pol III); pre-rRNA processing; pre-rRNA modification; assembly of precursor ribosomal subunits; and quality control mechanisms, which involve the RNA exosome and cofactors that in budding yeast act in a specialized nucleolar condensate termed the 'No-body' (nucleolar body) that

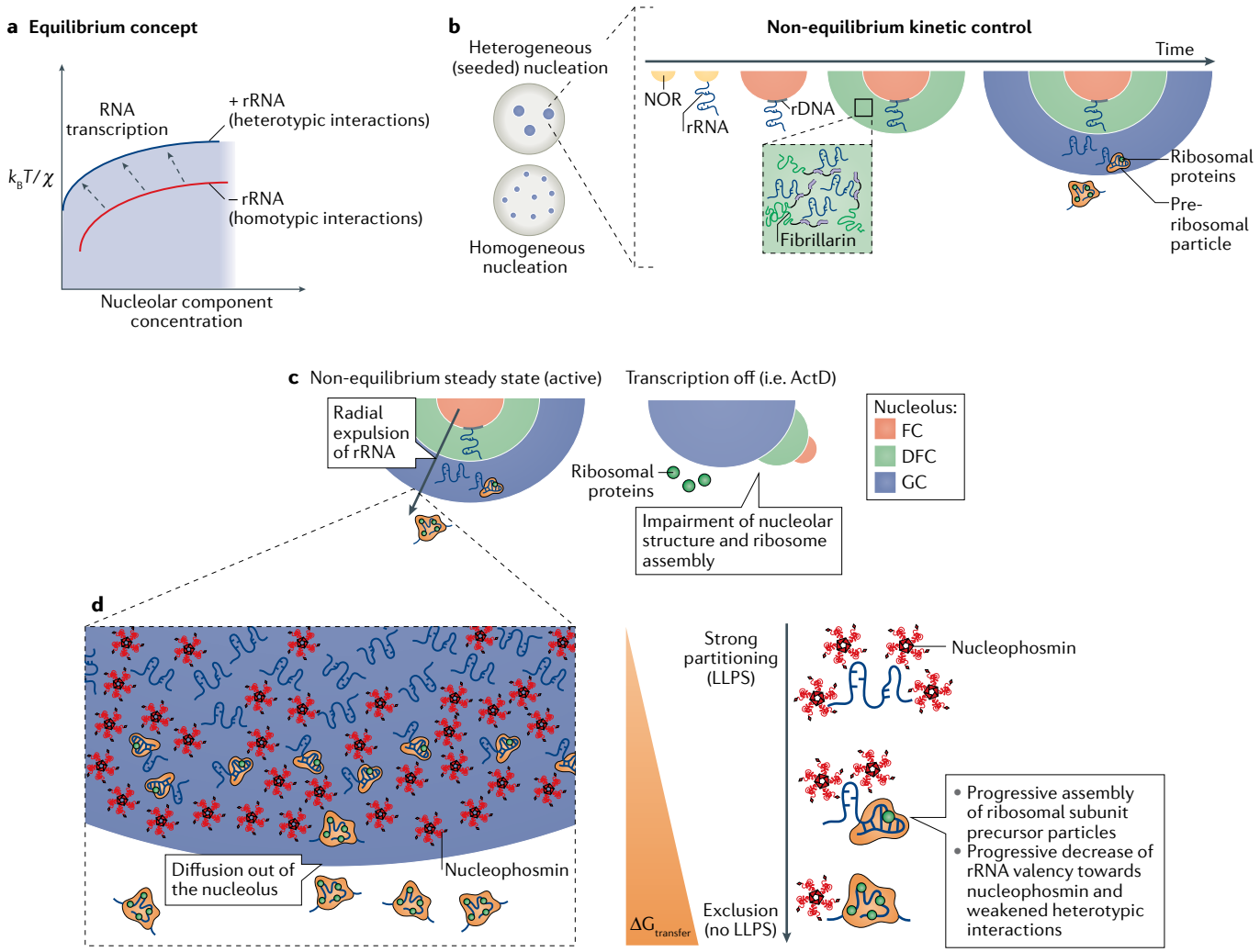
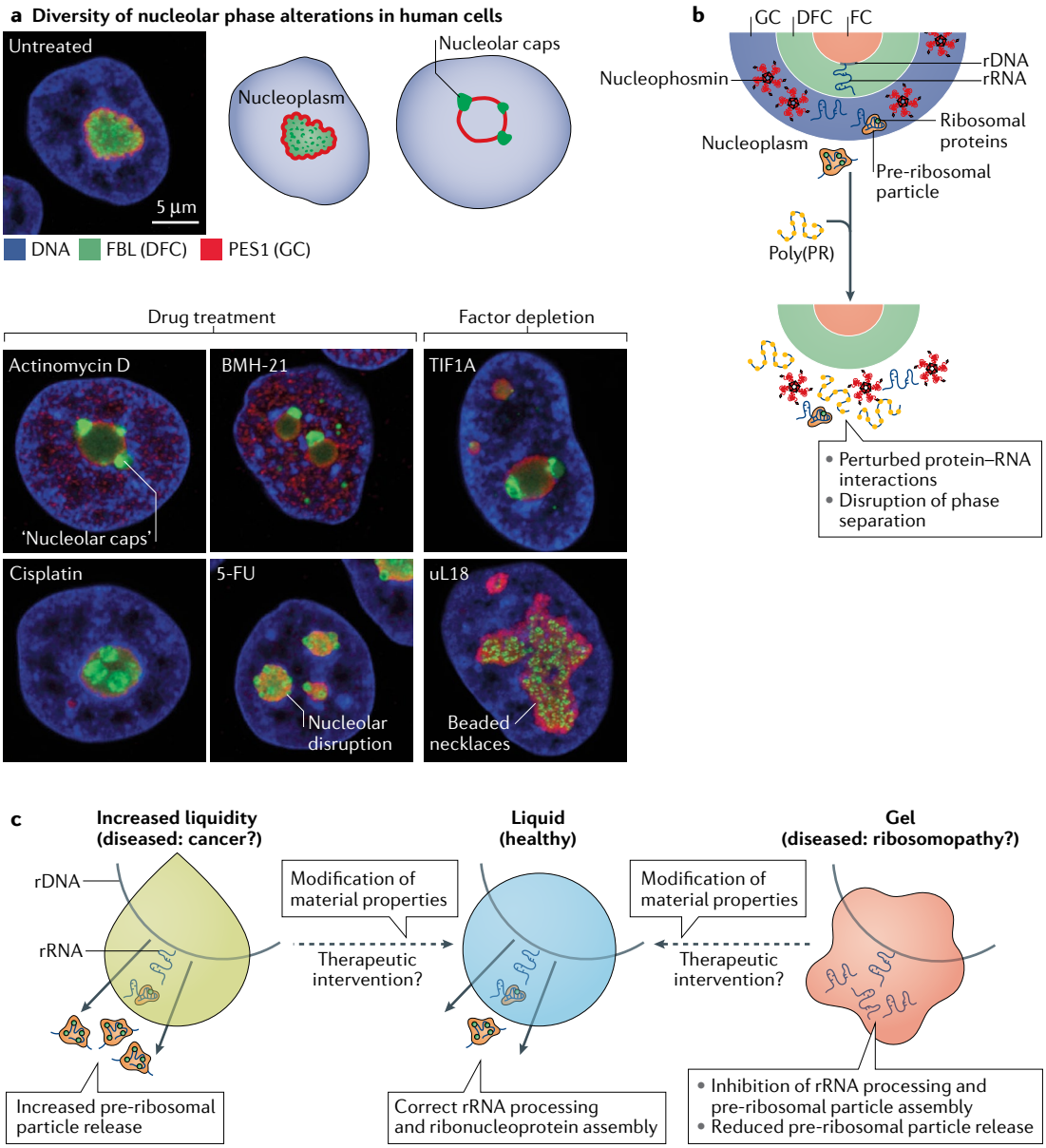



Fig. 5 | **Dynamics of ribosome biogenesis in the nucleolus. a** | Changes in the local concentration of ribosomal RNA (rRNA) appear to strongly change the location of the threshold for condensation of nucleolar components. This schematic phase diagram (binodal boundary) shows how a shift in the boundary may facilitate 'on-demand' assembly of nucleoli, by promoting nucleation specifically at transcriptionally active nucleolar organizer regions that are enriched in rRNA and support active transcription. **b** | Nucleoli appear to form via heterogeneous (seeded) nucleation promoted by transcription of rRNA at nucleolar organizer regions (NORs) (rather than homogeneous nucleation that would lead to nucleolar genesis randomly throughout the nucleus). Nucleoli form in a 'sequential' fashion, starting with the fibrillar centre (FC), followed by the dense fibrillar component (DFC) and finishing with the granular component (GC). Inset: interaction of fibrillarin with nascent transcripts has recently been shown to be important for establishment of the DFC[23] (see also FIG. 1b). **c** | Maintenance of nucleoli requires continuous active production of rRNA and supply of ribosomal proteins resulting in a directional flux: rRNA is radially expelled as it is transcribed, processed, modified and folded with proteins into ribosomal subunits (left). Disruption of active processes — that is, upon inhibition of polymerase I-mediated transcription (for example, by treatment with actinomycin D (ActD)) — results in a radically altered phase structure, with prominent nucleolar 'caps' forming (right, and see FIG. 6a). **d** | The radial organization of nucleoli may reflect a fundamentally non-equilibrium energetic landscape, with a gradient in the effective thermodynamic free energy of partitioning of key nucleolar components. Recent findings suggest that as rRNA assembles with ribosomal proteins into ribosomal subunits, the valence (or number) of contacts of an rRNA with the liquid–liquid phase separation (LLPS) GC meshwork decreases, leading to more unfavourable (that is, larger free energy of transfer and thus lower solubility in the GC) interactions within the nucleolus, giving rise to selective 'exclusion' of fully processed and assembled subunits. $\Delta G_{transfer}$, free energy of transfer; $k_B$, Boltzmann constant; $\chi$, Flory–Huggins miscibility parameter; rDNA, ribosomal DNA; $T$, temperature. Part **d** adapted from REF.[75], Springer Nature Limited.

is enriched in defective ribosomal subunit precursors destined for degradation[77,78]. There is ample evidence for an intimate connection between these processes and the structure of the nucleolus. For example, there has been general consensus that rRNA synthesis, performed by Pol I, occurs at the interface between the FC and the DFC (see above and FIG. 1b–d). Nascent transcripts are believed to radiate into the DFC, where they undergo initial maturation steps, such as post-transcriptional chemical modifications, including methylation and pseudouridylation, before proceeding into the GC for further maturation. Given this intimate link between pre-ribosomal particle biogenesis and nucleolar structure, inhibiting this nucleolar function can have considerable consequences for nucleolar morphology.

The most emblematic case of nucleolar phase alteration is the treatment of cells with low doses of actinomycin D, which inhibits nucleolar RNA synthesis (Pol I function),




leading to 'nucleolar segregation', whereby DFCs segregate from the GC, forming 'nucleolar caps'[79] (FIG. 6a). When nucleolar subcompartments are segregated, they are no longer embedded within each other like nested Russian dolls but, instead, are spatially juxtaposed, in a manner highly reminiscent of the multiphase organization seen with Cajal bodies in the *X. laevis* germinal vesicle[29] or stress granules studded with P bodies[53]. Given that multiphase organization of condensates is generally thought to reflect relative surface tension values, the structural reorganization of immiscible nucleolar subphases may reflect their rRNA transcription-dependent surface tensions. Knockdown of TIF1A, which is important for Pol I function, leads to a strikingly similar segregation of nucleolar components (FIG. 6a). Treating cells with other small-molecule inhibitors that target rRNA synthesis, pre-rRNA processing or yet to be identified ribosome biogenesis steps[80] produces a wide range of highly specific disruption phenotypes where nucleolar components adopt novel spatial distributions (FIG. 6a), again suggesting still poorly understood changes to relative surface tensions between nucleolar phases.

In an effort to understand which cell components are important for nucleolar structure maintenance, the 80 ribosomal proteins and 625 abundant nucleolar proteins were depleted one by one in cells, and the nucleolar structure was carefully inspected[13,81]. Surprisingly, only very few ribosomal proteins were shown to be important for maintenance of the nucleolar structure, nearly all being late-assembling proteins of the large ribosomal subunit. Of these, uL5 (formerly RPL11) and uL18 (RPL5) appear to be the most important, with their depletion or the depletion of factors (RRS1 and BXDC1) regulating their assembly into maturing 60S subunits, leading to severe disruption of nucleolar organization (with nucleaolar markers appearing as 'beaded necklaces') (FIG. 6a). This disruption was suggested to be caused, at least in part, by loss of interactions between the Arginine-rich

◀ Fig. 6 | **Links between nucleolar structure and function. a** | Nucleolar condensate organization can be disrupted by approaches that perturb its function. Images show HeLa cells subject to various treatments and stained with DAPI (labelling DNA), fibrillarin (FBL; labelling the dense fibrillar component (DFC)) and Pescadillo ribosomal biogenesis factor 1 (PES1; labelling the granular component (GC)). Prominently, inhibition of ribosomal RNA (rRNA) transcription by actinomycin D treatment (0.2 μg/ml, 2 h), BMH-21 treatment (1 μM, 3 h) or knockdown (small interfering RNAs (siRNAs); 10 nM, 3 days) of the RNA polymerase I factor TIF1A leads to the formation of 'nucleolar caps', whereby the DFC segregates from the GC. Treatment with cisplatin (3 μg/ml, 24 h), which generally functions as a DNA synthesis inhibitor, is associated with redistribution of the rRNA transcription machinery, rRNA synthesis inhibition and cell death (not shown). Similarly, treatment with 5-fluorouracil (5-FU; 15 μM, 24 h), which blocks rRNA processing, causes considerable nucleolar disruption with an appearance of 'fragmented nucleoli'. Finally, depletion (siRNAs; 10 nM, 3 days) of one of the large ribosomal subunit proteins, uL18 — which is required for central protuberance assembly (see FIG. 1b) — leads to severe nucleolar phase disruption, whereby the nucleolar markers appear as 'unfolded beaded necklaces'. **b** | The expression of disease-associated peptides, including proline–arginine repeat proteins (poly(PR)s) such as those encoded in *C9ORF72* (implicated in neurodegenerative disorders)[95], may interfere with establishment of the nucleolar phase. Specifically, the liquid-like behaviour of the nucleolus is severely compromised, with changes in nucleolar shape and reduced protein mobility indicative of a transition to a hardened material state. The disease peptides interact with nucleolar components and appear to compete with normal interactions between nucleolar components. This, in consequence, inhibits nucleolar function in ribosome biogenesis and protein homeostasis. **c** | The liquid-like properties of nucleoli are likely important for enabling dynamic assembly of ribosomal subunits and their flux out of the nucleolus. Studies have begun to suggest that altered nucleolar properties could underlie nucleoli-associated diseases. For example, ribosomopathies, which result from the shortage of properly formed ribosomes, could potentially be caused by aberrant nucleolar hardening and decreased rRNA and ribonucleoprotein processing. By contrast, certain types of cancer that can be associated with increased ribosome biogenesis (that fuels active translation and cell proliferation) could stem from increased flux of rRNA through the nucleolus, which could in turn be promoted by increased nucleolar fluidity. Thus, modulation of nucleolar material properties could be explored as a strategy for the development of novel therapeutic approaches for the treatment of devastating human diseases. FC, fibrillar centre; rDNA, ribosomal DNA. Images in part **a** are unpublished and a courtesy of D.L.J.L. and R.B.

motifs of uL18 and nucleophosmin[13], which are known to be important for establishment of the GC by LLPS[18]. Interestingly, uL5 and uL18 are two ribosomal proteins important for nucleolar surveillance, a p53-dependent antitumour pathway that kills dysfunctional cells[82,83]. Their structural and functional importance for nucleolar organization may result from uL5 and uL18 being assembled together with the 5S rRNA into 60S to form the central protuberance (FIG. 1b), a remarkable architectural landmark of the ribosome essential to its function. Thus, formation of an essential ribosomal feature, the central protuberance, appears to be deeply rooted with the multiphase organization of the nucleolus.

On the whole, the fundamental significance of such observations is that inhibition of nucleolar function can dramatically alter its structure. Put simply, it is evident that nucleolar form relies on its function. The opposite is also the case — changes in nucleolar structure and material properties will impact its function, with considerable consequences for physiology. These aspects are discussed in the following subsections.

***Impact of nucleolar liquid phase on function.*** To probe the links between changes in the nucleolar liquid phase and function, optogenetic tools are increasingly being used to modulate the biomolecular interactions underlying condensates with light[84–88]. For example, a recent study fused nucleophosmin to a blue light-dependent oligomerization domain, which caused it to become primarily immobile in the nucleolus upon blue light activation[85]; this is expected to cause gelation if sufficiently strong and multivalent interactions are established[54]. Such a transition to a gel will change the effective viscoelasticity of phase-separated droplets, and will affect diffusion rates through the condensate, in a manner dependent on the dynamics and effective mesh size of the gel. Indeed, following blue light-induced nucleophosmin oligomerization, nucleoli showed decreased fusion rates and the diffusion of large molecules (>10 nm; for example, Pol I) within them was reduced, which is consistent with gelation. Importantly, this optogenetic gelation of the nucleolus had considerable phenotypic consequences for ribosome biogenesis, whereby unprocessed rRNA transcripts accumulated and processed rRNA decreased[85]. These phenotypic changes occurred in the timescale of a day, which is compatible with the time required to accumulate low-abundance, short-lived pre-rRNA species. Overall, this finding underscores how the nucleolar material state, and hence its structure, can impact its functional activity.

Beyond the impact on ribosome assembly, the liquid nature of nucleoli has also been suggested to have a role in preventing irreversible, pathological protein aggregation. Under stress, the nucleolus prevents formation of potentially toxic aggregates[22], which appears to be a direct result of the intrinsic fluidity of the condensed nucleolar phase. Upon proteotoxic stress, such as heat shock, metastable and misfolded nuclear proteins are transiently stored in the GC compartment of the nucleolus. Here, they interact with nucleophosmin, and possibly other nucleolar proteins, to avoid coaggregation and to allow access to and refolding by protein chaperones, such as HSP70 — which also accumulate in the nucleolus during stress — when stress is relieved[22]. Notably, during stress, changes in the nucleolar material state appear to lead to reduced mobility of nucleophosmin and the misfolded nuclear proteins, which is restored in an HSP70-dependent manner upon recovery from stress. Apparently, nucleolar proteins are also monitored by this mechanism, which is in line with the abundant presence of intrinsically disordered and low-complexity sequence-containing proteins in the nucleolus[35], which do not fold stably and tend to aggregate upon conformational stress[89]. Altogether, it can be expected that a thermally sensitive proteome of ~200 proteins is looked after by this surveillance mechanism, which could therefore have a substantial impact on overall protein homeostasis in the cell.

Interestingly, the capacity of the nucleolus to store unfolded proteins and prevent aggregation is limited as the system is saturable[22]. Indeed, upon prolonged exposure to high heat stress or after expression of polypeptides associated with neurodegenerative diseases, such as *C9ORF72*-encoded dipeptide repeats (see also below) in combination with thermal stress, the liquid-like behaviour of the nucleolus was severely compromised, with changes in nucleolar shape and reduced protein mobility indicative of a transition to a hardened material state. Importantly, this change was irreversible, and

Nucleolar surveillance
A p53-dependent antitumoural surveillance pathway triggered upon ribosomal assembly dysfunction and involving the sequestration of Hdm2 by a complex consisting of ribosomal proteins uL5, uL18 and the 5S ribosomal RNA.

Central protuberance
A major architectural landmark of the large ribosomal subunit (60S) essential for ribosomal function; it promotes transmission of allosteric information between the functional centres of the large ribosomal subunit, and between the small and large subunits during translation.

Ribosomopathies
Congenital or somatic tissue-specific diseases resulting from mutations in ribosomal proteins or ribosome biogenesis factors and leading to the shortage of mature ribosomes and, generally, to a hypo-proliferation phenotype. The blood and the brain are prime targets of ribosomopathies. Ribosomopathies often lead to cancer owing to secondary mutations.

Amyotrophic lateral sclerosis
A disease that causes the death of neurons controlling voluntary muscles.

Frontotemporal dementia
Diseases that affect mostly the frontal and temporal lobes of the brain associated with personality, behaviour and language.

the localization of misfolded proteins that entered the nucleolus upon stress was not restored upon recovery from stress. Thus, prolonged stresses can lead to nucleolar phase transition towards a more solid-like state, which largely compromises its function in mitigating the aggregation of disordered proteins[22]. That the role of the nucleolus in protein homeostasis relies on its liquid–liquid-like properties was further exemplified by the observation that disruption of the nucleolar structure by low doses of actinomycin D (see above and FIG. 6a) resulted in the formation of stable aggregates of stress-denatured proteins in the nucleoplasm; these aggregates were highly toxic as they sequestered bystander proteins[22].

***Disruption of nucleolar liquid phase in disease and ageing.*** The tight coupling between nucleolar material properties and function suggests that dysregulation of the nucleolar material state will inevitably lead to pathology (FIG. 6b,c). In the context of ribosome biogenesis, both excess and insufficient amounts of functional ribosomes can be detrimental, leading to cancer or ribosomopathies, respectively. Notably, both disease scenarios are associated with nucleolar morphology alterations, ranging, in the case of cancer, from an increased number of nucleoli per cell nucleus to increased nucleolar size and irregular nucleolar shape[90,91]. Moreover, the nucleolus is also strongly implicated in neurodegenerative diseases, such as amyotrophic lateral sclerosis and frontotemporal dementia, caused by hexanucleotide repeat (GGGGCC or $G_4C_2$) expansions in the *C9ORF72* gene. These expansions lead to the expression of charged dipeptides, which, as described above, interfere with the nucleolar phase, resulting in increased protein aggregation upon stress[22] and also inhibition of ribosome biogenesis. Specifically, it was shown that these dipeptides have strong affinity for rRNA, displacing nucleophosmin away from rRNA, causing its relocalization to the nucleoplasm and GC phase dissolution. This had important consequences for cell function, perturbing ribosome biogenesis and subsequent protein translation, and eventually leading to cell death[92–95] (FIG. 6b).

Furthermore, the nucleolar material state may be linked to organism ageing. Consistent with this possibility, FRAP experiments in non-dividing *C. elegans* intestinal cells revealed reduction of fibrillarin dynamics — indicative of gelation/hardening — in older worms as compared with larvae[16]; these findings paralleled time-dependent gelation of in vitro fibrillarin droplets, a phenomenon not observed with nucleophosmin droplets, whose lower viscosity does not change with time. Given that RNA can impact the fluidity of various model phase-separated condensates[42,57,96], maturation of the DFC to a gel-like state could potentially reflect changes in the flux of newly synthesized rRNA transiting through the DFC. Of relevance, other studies have shown that decreased expression of rRNA in *C. elegans* was associated with smaller nucleoli and longer-living animals[97]; knockdown of fibrillarin decreases the nucleolar size and increases longevity. These findings connecting the nucleolus and longevity are interesting in the context of earlier studies reporting that ageing yeast cells exhibit increasingly large and fragmented nucleoli, which are associated with accumulation of extrachromosomal rDNA circles[98,99]. It is tempting to speculate that each of these ageing-associated changes can be understood as alterations in nucleolar phase behaviour and material state.

Overall, the coupling between nucleolar structure and function opens up exciting new possibilities for novel biomedical strategies, for example utilizing the nucleolus as a disease biomarker[13] and, although still a prospect for the future, developing therapeutics targeting the nucleolar material state.

## Conclusions and perspective

Two hundred years after its initial discovery, the nucleolus still harbours many mysteries. It is a complex, multilayered structure, interwoven with chromatin and including thousands of protein and RNA components, which are constantly moving into and out of the surrounding nucleoplasm and interacting specifically to perform dozens of highly regulated reactions. This structural complexity is tightly connected to nucleolar function as a centre for ribosome biogenesis, as well as several other processes essential to maintain cell homeostasis. Despite this complexity, the LLPS model provides a simple and powerful conceptual framework for explaining a diverse set of experimental observations regarding nucleolar behaviour and has begun to pave the way for a fully predictive biophysical understanding of nucleolar structure and function. Nevertheless, there are still many open questions.

To start, we need a better understanding of the mechanisms driving LLPS in cells. One specific area is elucidating the dynamic regulation of the weak multivalent interactions that underlie LLPS, including the role of post-translational modifications (phosphorylation and others). Remarkably, the intracellular levels of the acetyl donor acetyl-CoA have recently been shown to regulate the internal distribution of nucleolar proteins, phase behaviour (fluidity) and function (rRNA synthesis), presumably through differential acetylation of nucleolar proteins, although the precise substrates responsible for this regulation remain to be characterized in detail[100].

Future studies will also be needed to better understand how the phases in the nucleolus, and the transfer of molecules within and between them, mechanistically coordinate the assembly of rRNA-containing complexes. For example, the observation that ribosome assembly factors form nanometric beads that may each correspond to 'reaction chambers' (FIG. 1d) suggests there may be more structural heterogeneity within the nucleolus than anticipated from the simple view of a tripartite (FC, DFC and GC) nucleolar organization. Such heterogeneity, in the form of gradients or highly localized nanophases that may be akin to that described for stress granules[101] and P granules[102], could potentially be critical for directing the order of ribosome biogenesis assembly reactions. Approaches that rely on live-cell super-resolution, together with structural information on fine nucleolar organization and associated ribosome assembly intermediates extracted from novel cryo-electron microscopy approaches, will both be key to linking nucleolar structure with the complexity of ribosome assembly reactions.

Perhaps most dauntingly, these and other non-equilibrium steady-state aspects of nucleolar biology require consideration of more refined versions of the LLPS model. The better understanding of the nucleolus as a multiphase liquid condensate will be important for developing new approaches that modulate the material properties of the nucleolus, and allow probing and controlling of its phase behaviour and function. These may include the use of novel chemical and optogenetic tools to change nucleolar viscoelasticity and thereby tune ribosome biogenesis, which are only beginning to emerge but likely have broad applications, for example in treating cancer and ribosomopathies. Although much work remains, this potential to modulate the biochemistry of nucleolar-based processes with material state-based therapeutics is an exciting direction for future research.

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1. Pederson, T. The plurifunctional nucleolus. *Nucleic Acids Res.* **26**, 3871–3876 (1998).
2. Boisvert, F. M., van Koningsbruggen, S., Navascues, J. & Lamond, A. I. The multifunctional nucleolus. *Nat. Rev. Mol. Cell Biol.* **8**, 574–585 (2007).
3. Lafontaine, D. L. J. Noncoding RNAs in eukaryotic ribosome synthesis and function. *Nat. Struct. Mol. Biol.* **22**, 11–19 (2015).
4. Iarovaia, O. V. et al. Nucleolus: a central hub for nuclear functions. *Trends Cell Biol.* **29**, 647–659 (2019).
5. Amon, A. A decade of Cdc14 — a personal perspective. Delivered on 9 July 2007 at the 32nd FEBS Congress in Vienna, Austria. *FEBS J.* **275**, 5774–5784 (2008).
6. Boulon, S., Westman, B. J., Hutten, S., Boisvert, F. M. & Lamond, A. I. The nucleolus under stress. *Mol. Cell* **40**, 216–227 (2010).
7. Andersen, J. S. et al. Directed proteomic analysis of the human nucleolus. *Curr. Biol.* **12**, 1–11 (2002).
8. Scherl, A. et al. Functional proteomic analysis of human nucleolus. *Mol. Biol. Cell* **13**, 4100–4109 (2002).
9. Salvetti, A. & Greco, A. Viruses and the nucleolus: the fatal attraction. *Biochim. Biophys. Acta* **1842**, 840–847 (2014).
10. Hetman, M. & Slomnicki, L. P. Ribosomal biogenesis as an emerging target of neurodevelopmental pathologies. *J. Neurochem.* **148**, 325–347 (2019).
11. Calle, A. et al. Nucleolin is required for an efficient herpes simplex virus type 1 infection. *J. Virol.* **82**, 4762–4773 (2008).
12. Zink, D., Fischer, A. H. & Nickerson, J. A. Nuclear structure in cancer cells. *Nat. Rev. Cancer* **4**, 677–687 (2004).
13. Stamatopoulou, V., Parisot, P., De Vleeschouwer, C. & Lafontaine, D. L. J. Use of the iNo score to discriminate normal from altered nucleolar morphology, with applications in basic cell biology and potential in human disease diagnostics. *Nat. Protoc.* **13**, 2387–2406 (2018).
14. Frankowski, K. J. et al. Metarrestin, a perinucleolar compartment inhibitor, effectively suppresses metastasis. *Sci. Transl Med.* **10**, eaap8307 (2018).
15. Brangwynne, C. P., Mitchison, T. J. & Hyman, A. A. Active liquid-like behavior of nucleoli determines their size and shape in *Xenopus laevis* oocytes. *Proc. Natl Acad. Sci. USA* **108**, 4334–4339 (2011).
**This paper shows that *X. laevis* nucleoli are highly spherical, and when pushed together with microneedles readily coalesce into a large sphere; the fusion dynamics can be used to determine the apparent viscosity, which is dependent on the ATP concentration**.
16. Feric, M. et al. Coexisting liquid phases underlie nucleolar subcompartments. *Cell* **165**, 1686–1697 (2016).
**This paper shows that *X. laevis* nucleolar subcompartments (DFC and GC) readily fuse with one another, whereas in vitro reconstitution of the DFC/GC core–shell architecture supports the concept that nucleolar subcompartments are immiscible liquids arranged by their relative surface tensions**.
17. Mitrea, D. M. et al. Self-interaction of NPM1 modulates multiple mechanisms of liquid–liquid phase separation. *Nat. Commun.* **9**, 842 (2018).
18. Mitrea, D. M. et al. Nucleophosmin integrates within the nucleolus via multi-modal interactions with proteins displaying R-rich linear motifs and rRNA. *eLife* **5**, e13571 (2016).
**This paper demonstrates that the GC meshwork protein nucleophosmin undergoes heterotypic LLPS in vitro with proteins containing Arg-rich tracts or rRNA, thus providing critical insight into the underlying biophysics driving nucleolar assembly and biomolecule localization to the nucleolus**.
19. Falahati, H. & Wieschaus, E. Independent active and thermodynamic processes govern the nucleolus assembly in vivo. *Proc. Natl Acad. Sci. USA* **114**, 1335–1340 (2017).
**This paper uses a microfluidic system to reveal that the tendency of nucleolar proteins to condense in living cells exhibits differential sensitivity to imposed temperature changes, suggesting that active, non-equilibrium processes contribute to nucleolar phase separation in living cells**.
20. Weber, S. C. & Brangwynne, C. P. Inverse size scaling of the nucleolus by a concentration-dependent phase transition. *Curr. Biol.* **25**, 641–646 (2015).
21. Caragine, C. M., Haley, S. C. & Zidovska, A. Nucleolar dynamics and interactions with nucleoplasm in living cells. *eLife* **8**, e47533 (2019).
22. Frottin, F. et al. The nucleolus functions as a phase-separated protein quality control compartment. *Science* **365**, 342–347 (2019).
**This paper demonstrates that the GC phase of the nucleolus plays a role in protein homeostasis by transiently storing metastable and potentially misfolded nuclear proteins during heat stress in order to prevent their aggregation**.
23. Yao, R. W. et al. Nascent pre-rRNA sorting via phase separation drives the assembly of dense fibrillar components in the human nucleolus. *Mol. Cell* (2019).
**This paper demonstrates that the box C/D-associated methyltransferase fibrillarin plays an essential role in initial pre-rRNA sorting and cleavage through establishment by phase separation of the DFC subcompartment of the nucleolus**.
24. Ferrolino, M. C., Mitrea, D. M., Michael, J. R. & Kriwacki, R. W. Compositional adaptability in NPM1–SURF6 scaffolding networks enabled by dynamic switching of phase separation mechanisms. *Nat. Commun.* **9**, 5064 (2018).
25. Shin, Y. & Brangwynne, C. P. Liquid phase condensation in cell physiology and disease. *Science* **357**, eaaf4382 (2017).
26. Banani, S. F., Lee, H. O., Hyman, A. A. & Rosen, M. K. Biomolecular condensates: organizers of cellular biochemistry. *Nat. Rev. Mol. Cell Biol.* **18**, 285–298 (2017).
27. Ehrenberg, L. Influence of temperature on the nucleolus and its coacervate nature. *Hereditas* **32**, 407–418 (1946).
28. Phair, R. D. & Misteli, T. High mobility of proteins in the mammalian cell nucleus. *Nature* **404**, 604–609 (2000).
29. Handwerger, K. E., Cordero, J. A. & Gall, J. G. Cajal bodies, nucleoli, and speckles in the *Xenopus oocyte* nucleus have a low-density, sponge-like structure. *Mol. Biol. Cell* **16**, 202–211 (2005).
30. Houtsmuller, A. B. et al. Action of DNA repair endonuclease ERCC1/XPF in living cells. *Science* **284**, 958–961 (1999).
31. Lee, C. F. Formation of liquid-like cellular organelles depends on their composition. *Nature* **581**, 144–145 (2020).
32. Thiry, M. & Lafontaine, D. L. J. Birth of a nucleolus: the evolution of nucleolar compartments. *Trends Cell Biol.* **15**, 194–199 (2005).
33. Lamaye, F., Galliot, S., Alibardi, L., Lafontaine, D. L. J. & Thiry, M. Nucleolar structure across evolution: the transition between bi- and tri-compartmentalized nucleoli lies within the class Reptilia. *J. Struct. Biol.* **174**, 352–359 (2011).
34. Bartholome, O. et al. Relationships between the structural and functional organization of the turtle cell nucleolus. *J. Struct. Biol.* **208**, 107398 (2019).
35. Stenström, L. et al. Mapping of the nucleolar proteome reveals spatiotemporal organization related to intrinsic protein disorder. Preprint at *bioRxiv* https://doi.org/10.1101/2020.01.30.923003 (2020).
36. Nemeth, A. et al. Initial genomics of the human nucleolus. *PLoS Genet.* **6**, e1000889 (2010).
37. Lafontaine, D. L. J. Birth of nucleolar compartments: phase separation-driven ribosomal RNA sorting and processing. *Mol. Cell* **76**, 694–696 (2019).
38. Xing, Y. H. et al. SLERT regulates DDX21 rings associated with Pol I transcription. *Cell* **169**, 664–678.e16 (2017).
39. Girard, J. P. et al. GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. *EMBO J.* **11**, 673–682 (1992).
40. Scott, M. S., Boisvert, F. M., McDowall, M. D., Lamond, A. I. & Barton, G. J. Characterization and prediction of protein nucleolar localization sequences. *Nucleic Acids Res.* **38**, 7388–7399 (2010).
41. Colau, G., Thiry, M., Leduc, V., Bordonne, R. & Lafontaine, D. L. J. The small nucle(ol)ar RNA cap trimethyltransferase is required for ribosome synthesis and intact nucleolar morphology. *Mol. Cell. Biol.* **24**, 7976–7986 (2004).
42. Elbaum-Garfinkle, S. et al. The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics. *Proc. Natl Acad. Sci. USA* **112**, 7189–7194 (2015).
43. Chong, P. A., Vernon, R. M. & Forman-Kay, J. D. RGG/RG motif regions in RNA binding and phase separation. *J. Mol. Biol.* **430**, 4650–4665 (2018).
44. Berry, J., Weber, S. C., Vaidya, N., Haataja, M. & Brangwynne, C. P. RNA transcription modulates phase transition-driven nuclear body assembly. *Proc. Natl Acad. Sci. USA* **112**, E5237–E5245 (2015).
**This paper shows that the dynamics of nucleolar assembly behaviour is consistent with the classical dynamics of coarsening liquid droplets, albeit in which classical phase separation behaviour is locally amplified by transcription of rRNA at nucleolar organizer regions**.
45. Taylor, N. et al. Biophysical characterization of organelle-based RNA/protein liquid phases using microfluidics. *Soft Matter* **12**, 9142–9150 (2016).
46. Lee, D. S. W., Wingreen, N. S. & Brangwynne, C. Chromatin mechanics dictates subdiffusion and coarsening dynamics of embedded condensates. Preprint at *bioRxiv* https://doi.org/10.1101/2020.06.03.128561 (2020).
47. Amin, M. A., Matsunaga, S., Uchiyama, S. & Fukui, K. Depletion of nucleophosmin leads to distortion of nucleolar and nuclear structures in HeLa cells. *Biochem. J.* **415**, 345–351 (2008).
48. Grisendi, S., Mecucci, C., Falini, B. & Pandolfi, P. P. Nucleophosmin and cancer. *Nat. Rev. Cancer* **6**, 493–505 (2006).
49. Mitrea, D. M. et al. Structural polymorphism in the N-terminal oligomerization domain of NPM1. *Proc. Natl Acad. Sci. USA* **111**, 4466–4471 (2014).
50. Feric, M. & Brangwynne, C. P. A nuclear F-actin scaffold stabilizes ribonucleoprotein droplets against gravity in large cells. *Nat. Cell Biol.* **15**, 1253–1259 (2013).
51. Feric, M., Broedersz, C. P. & Brangwynne, C. P. Soft viscoelastic properties of nuclear actin age oocytes due to gravitational creep. *Sci. Rep.* **5**, 16607 (2015).
52. Mao, S., Kuldinow, D., Haataja, M. P. & Kosmrlj, A. Phase behavior and morphology of multicomponent liquid mixtures. *Soft Matter* **15**, 1297–1311 (2019).
53. Sanders, D. W. et al. Competing protein–RNA interaction networks control multiphase intracellular organization. *Cell* **181**, 306–324 (2020).
54. Rubinstein, M. & Colby, R. *Polymer Physics* 199–305 (Oxford Univ. Press, 2003).
55. Molliex, A. et al. Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. *Cell* **163**, 123–133 (2015).
56. Patel, A. et al. A liquid-to-solid phase transition of the ALS protein FUS accelerated by disease mutation. *Cell* **162**, 1066–1077 (2015).

57. Zhang, H. et al. RNA controls PolyQ protein phase transitions. *Mol. Cell* **60**, 220–230 (2015).
58. Murakami, T. et al. ALS/FTD mutation-induced phase transition of FUS liquid droplets and reversible hydrogels into irreversible hydrogels impairs RNP granule function. *Neuron* **88**, 678–690 (2015).
59. Eggers, J., Lister, J. R. & Stone, H. A. Coalescence of liquid drops. *J. fluid Mech.* **401**, 293–310 (1999).
60. Andersen, J. S. et al. Nucleolar proteome dynamics. *Nature* **433**, 77–83 (2005).
61. Lam, Y. W., Evans, V. C., Heesom, K. J., Lamond, A. I. & Matthews, D. A. Proteomics analysis of the nucleolus in adenovirus-infected cells. *Mol. Cell. Proteomics* **9**, 117–130 (2010).
62. Moore, H. M. et al. Quantitative proteomics and dynamic imaging of the nucleolus reveal distinct responses to UV and ionizing radiation. *Mol. Cell. Proteomics* **10**, M111 009241 (2011).
63. Dundr, M., Misteli, T. & Olson, M. O. The dynamics of postmitotic reassembly of the nucleolus. *J. Cell Biol.* **150**, 433–446 (2000).
64. Leung, A. K. et al. Quantitative kinetic analysis of nucleolar breakdown and reassembly during mitosis in live human cells. *J. Cell Biol.* **166**, 787–800 (2004).
65. Hernandez-Verdun, D. Assembly and disassembly of the nucleolus during the cell cycle. *Nucleus* **2**, 189–194 (2011).
66. Rai, A. K., Chen, J. X., Selbach, M. & Pelkmans, L. Kinase-controlled phase transition of membraneless organelles in mitosis. *Nature* **559**, 211–216 (2018).
67. Kashchiev, D. *Nucleation* 17–42 (Elsevier, 2020).
68. Sirri, V., Hernandez-Verdun, D. & Roussel, P. Cyclin-dependent kinases govern formation and maintenance of the nucleolus. *J. Cell Biol.* **156**, 969–981 (2002).
69. Booth, D. G. et al. Ki-67 is a PP1-interacting protein that organises the mitotic chromosome periphery. *eLife* **3**, e01641 (2014).
70. Sobecki, M. et al. The cell proliferation antigen Ki-67 organises heterochromatin. *eLife* **5**, e13722 (2016).
71. Cuylen, S. et al. Ki-67 acts as a biological surfactant to disperse mitotic chromosomes. *Nature* **535**, 308–312 (2016).
72. Falahati, H., Pelham-Webb, B., Blythe, S. & Wieschaus, E. Nucleation by rRNA dictates the precision of nucleolus assembly. *Curr. Biol.* **26**, 277–285 (2016).
73. Caudron-Herger, M. et al. Alu element-containing RNAs maintain nucleolar structure and function. *EMBO J.* **34**, 2758–2774 (2015).
74. Thiry, M., Cheutin, T., O'Donohue, M. F., Kaplan, H. & Ploton, D. Dynamics and three-dimensional localization of ribosomal RNA within the nucleolus. *RNA* **6**, 1750–1761 (2000).
75. Riback, J. A. et al. Composition-dependent thermodynamics of intracellular phase separation. *Nature* **581**, 209–214 (2020).
**This paper establishes that most intracellular condensates have a non-fixed saturation concentration stabilized by heterotypic biomolecular interactions, which provides a thermodynamic basis for 'on-demand' nucleolar assembly in the presence of nascent rRNA, whereas assembled ribosomal subunits are expelled into the nucleoplasm**.
76. Bohnsack, K. E. & Bohnsack, M. T. Uncovering the assembly pathway of human ribosomes and its emerging links to disease. *EMBO J.* **38**, e100278 (2019).
77. Dez, C., Houseley, J. & Tollervey, D. Surveillance of nuclear-restricted pre-ribosomes within a subnucleolar region of *Saccharomyces cerevisiae*. *EMBO J.* **25**, 1534–1546 (2006).
78. Lafontaine, D. L. J. A 'garbage can' for ribosomes: how eukaryotes degrade their ribosomes. *Trends Biochem. Sci.* **35**, 267–277 (2010).
79. Hernandez-Verdun, D., Roussel, P., Thiry, M., Sirri, V. & Lafontaine, D. L. J. The nucleolus: structure/function relationship in RNA metabolism. *Wiley Interdiscip. Rev. RNA* **1**, 415–431 (2010).
80. Burger, K. et al. Chemotherapeutic drugs inhibit ribosome biogenesis at various levels. *J. Biol. Chem.* **285**, 12416–12425 (2010).
81. Nicolas, E. et al. Involvement of human ribosomal proteins in nucleolar structure and p53-dependent nucleolar stress. *Nat. Commun.* **7**, 11390 (2016).
**This paper is a systematic investigation of the role of the 80 human ribosomal proteins in nucleolar structure, pre-rRNA processing, mature rRNA production and p53 homeostasis, revealing the importance of the central protuberance assembly in nucleolar phase establishment (see companion www.ribosomalproteins.com)**.
82. Donati, G., Peddigari, S., Mercer, C. A. & Thomas, G. 5S ribosomal RNA is an essential component of a nascent ribosomal precursor complex that regulates the Hdm2–p53 checkpoint. *Cell Rep.* **4**, 87–98 (2013).
83. Sloan, K. E., Bohnsack, M. T. & Watkins, N. J. The 5S RNP couples p53 homeostasis to ribosome biogenesis and nucleolar stress. *Cell Rep.* **5**, 237–247 (2013).
84. Shin, Y. et al. Spatiotemporal control of intracellular phase transitions using light-activated optodroplets. *Cell* **168**, 159–171.e14 (2017).
85. Zhu, L. et al. Controlling the material properties and rRNA processing function of the nucleolus using light. *Proc. Natl Acad. Sci. USA* **116**, 17330–17335 (2019).
86. Mann, J. R. et al. RNA binding antagonizes neurotoxic phase transitions of TDP-43. *Neuron* **102**, 321–338.e8 (2019).
87. Kilic, S. et al. Phase separation of 53BP1 determines liquid-like behavior of DNA repair compartments. *EMBO J.* **38**, e101379 (2019).
88. Wei, M.-T., Chang, Y.-C., Shimobayashi, S. F., Shin, Y. & Brangwynne, C. P. Nucleated transcriptional condensates amplify gene expression. Preprint at *bioRxiv* https://doi.org/10.1101/737387 (2020).
89. Galea, C. A. et al. Large-scale analysis of thermostable, mammalian proteins provides insights into the intrinsically disordered proteome. *J. Proteome Res.* **8**, 211–226 (2009).
90. Derenzini, M., Montanaro, L. & Trere, D. What the nucleolus says to a tumour pathologist. *Histopathology* **54**, 753–762 (2009).
91. Farley-Barnes, K. I., Ogawa, L. M. & Baserga, S. J. Ribosomopathies: old concepts, new controversies. *Trends Genet.* **35**, 754–767 (2019).
92. Kwon, I. et al. Poly-dipeptides encoded by the C9orf72 repeats bind nucleoli, impede RNA biogenesis, and kill cells. *Science* **345**, 1139–1145 (2014).
93. Wen, X. et al. Antisense proline–arginine RAN dipeptides linked to C9ORF72-ALS/FTD form toxic nuclear aggregates that initiate in vitro and in vivo neuronal death. *Neuron* **84**, 1213–1225 (2014).
94. Lee, K. H. et al. C9orf72 dipeptide repeats impair the assembly, dynamics, and function of membrane-less organelles. *Cell* **167**, 774–788.e17 (2016).
**This paper shows that dipeptide repeat polypeptides produced by non-ATG templated translation from the neurodegenerative disease-causing gene *C9ORF72* penetrate the nucleolus, where they interfere with establishment of multivalent weak interactions important for its phase behaviour, leading to loss of function and cell death**.
95. White, M. R. et al. C9orf72 poly(PR) dipeptide repeats disturb biomolecular phase separation and disrupt nucleolar function. *Mol. Cell* **74**, 713–728.e6 (2019).
96. Boeynaems, S. et al. Spontaneous driving forces give rise to protein–RNA condensates with coexisting phases and complex material properties. *Proc. Natl Acad. Sci. USA* **116**, 7889–7898 (2019).
97. Tiku, V. et al. Small nucleoli are a cellular hallmark of longevity. *Nat. Commun.* **8**, 16083 (2017).
98. Sinclair, D. A. & Guarente, L. Extrachromosomal rDNA circles — a cause of aging in yeast. *Cell* **91**, 1033–1042 (1997).
99. Sinclair, D. A., Mills, K. & Guarente, L. Accelerated aging and nucleolar fragmentation in yeast sgs1 mutants. *Science* **277**, 1313–1316 (1997).
100. Houston, R. et al. Acetylation-mediated phase control of the nucleolus regulates cellular acetyl-CoA responses. Preprint at *bioRxiv* https://doi.org/10.1101/2020.01.24.918706 (2019).
101. Jain, S. et al. ATPase-modulated stress granules contain a diverse proteome and substructure. *Cell* **164**, 487–498 (2016).
102. Putnam, A., Cassani, M., Smith, J. & Seydoux, G. A gel phase promotes condensation of liquid P granules in *Caenorhabditis elegans* embryos. *Nat. Struct. Mol. Biol.* **26**, 220–226 (2019).
103. Taylor, N. O., Wei, M. T., Stone, H. A. & Brangwynne, C. P. Quantifying dynamics in phase-separated condensates using fluorescence recovery after photobleaching. *Biophys. J.* **117**, 1285–1300 (2019).
104. Gardel, M. L. & Weitz, D. A. *Microrheology* 1–50 (Springer, 2005).
105. Roussel, P., Andre, C., Comai, L. & Hernandez-Verdun, D. The rDNA transcription machinery is assembled during mitosis in active NORs and absent in inactive NORs. *J. Cell Biol.* **133**, 235–246 (1996).
106. Farley, K. I., Surovtseva, Y., Merkel, J. & Baserga, S. J. Determinants of mammalian nucleolar architecture. *Chromosoma* **124**, 323–331 (2015).
107. Caragine, C. M., Haley, S. C. & Zidovska, A. Surface fluctuations and coalescence of nucleolar droplets in the human cell nucleus. *Phys. Rev. Lett.* **121**, 148101 (2018).
108. Huang, S. Building an efficient factory: where is pre-rRNA synthesized in the nucleolus? *J. Cell Biol.* **157**, 739–741 (2002).
109. Lewis, J. D. & Tollervey, D. Like attracts like: getting RNA processing together in the nucleus. *Science* **288**, 1385–1389 (2000).
110. Warner, J. R. The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* **24**, 437–440 (1999).
111. Duncan, R. & Hershey, J. W. Identification and quantitation of levels of protein synthesis initiation factors in crude HeLa cell lysates by two-dimensional polyacrylamide gel electrophoresis. *J. Biol. Chem.* **258**, 7228–7235 (1983).
112. Nott, T. J. et al. Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles. *Mol. Cell* **57**, 936–947 (2015).
113. Brangwynne, C., Tompa, P. & Pappu, R. V. Polymer physics of intracellular phase transitions. *Nat. Phys.* **11**, 899–904 (2015).
114. van der Lee, R. et al. Classification of intrinsically disordered regions and proteins. *Chem. Rev.* **114**, 6589–6631 (2014).
115. Choi, J. M., Holehouse, A. S. & Pappu, R. V. Physical principles underlying the complex biology of intracellular phase transitions. *Annu. Rev. Biophys.* **49**, 107–133 (2020).



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