Supplemental Experimental Procedures

X. laevis oocyte collection

Frogs were anesthetized with 0.1% MS-222 solution for 15 minutes, and oocytes were surgically removed from adult female *X. laevis* frogs following an IACUC approved protocol as previously described (Feric and Brangwynne, 2013). Oocytes were incubated at 18°C in OR2 solution. To remove the follicular layer, the oocytes were first mechanically separated and then incubated for 1 hour and 20 minutes in 2 mg/ml collagenase (Sigma). Stage V-VI oocytes of diameter of 1-1.3 mm were used for all experiments and identified using a Zeiss stereoscope (Dumont, 1972).

DNA and mRNA constructs for *X. laevis*

Vector pCS2+ backbones were used for all fluorescent fusion constructs. The granular component was visualized either with NPM1::GFP, NPM1::RFP, or NPM1::Cerulean; the dense fibrillar component was visualized with FIB1::GFP or FIB1::RFP, and the fibrillar component was visualized with mCherry::POLR1E or GFP::POLR1E. The nuclear actin network was visualized with a Lifeact::GFP construct (Feric and Brangwynne, 2013).

Purification of nucleolar proteins

Recombinant versions of FIB1::GFP protein with a N-terminal 6×-His tag and NPM1 with a N-terminal GST tag were purified using the *E. coli* expression system, BL21(DE3) cells. For FIB1::GFP, cells were lysed in resuspension buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole, 14 mM β-mercaptoethanol, and 10% (vol/vol) glycerol) containing 1 mg/mL lysozyme and a protease inhibitor mixture (Roche Diagnostics). FIB1::GFP was captured with Ni-NTA agarose (Qiagen), washed well with Ni-Wash buffer (20 mM Tris·HCl, pH 7.5, 500

mM NaCl, 14 mM β-ME, 10% (vol/vol) glycerol, and 25 mM imidazole), and eluted with Ni-Elution buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 14 mM β-mercaptoethanol, 10% (vol/vol) glycerol, and 250 mM imidazole). Furthermore, elution from Ni-NTA was loaded onto a HiTrap Heparin column (GE) after being diluted in heparin binding buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 1% (vol/vol) glycerol, and 2 mM DTT) and eluted in 20 mM Tris, pH 7.4, 1 M NaCl, 1% (vol/vol) glycerol, and 2 mM DTT. Glycerol was added to 10% (vol/vol), and aliquots were flash frozen in liquid nitrogen and stored at −80°C.

For NPM1, cells were lysed in resuspension buffer (20 mM Tris, 300 mM NaCl, 10 mM β-mercaptoethanol, protease inhibitors (Sigma-FAST), 1 mM EDTA, pH 7.5) containing Benzonase (Millipore, 20U/uL). GST-NPM1 was captured using GSH beads, washed well with wash buffer (20 mM Tris, 300 mM NaCl, 1 mM EDTA, pH 7.5), and eluted with elution buffer (20 mM Tris, 300 mM NaCl, 10 mM BME, 10 mM reduced L-glutathiol, 1 mM EDTA, pH 7.5). Eluted protein was dialyzed in the presence of Turbo3C/HRV3C/PreScission protease (Biovision, cat #. 9206-1) against 10 mM Tris, 0.15 M NaCl, 2 mM DTT, pH 7.5 overnight at 4°C. Furthermore, HPLC was performed and eluent was lyophilized before storing in -20°C.

Phase separation *in vitro*

For *in vitro* experiments, frozen FIB1 aliquots were thawed at room temperature and buffer exchanged (Amicon; 0.5 mL, 3–10k) into freshly made high salt buffer (20 mM Tris, pH 7.5, 1 M NaCl, and 1 mM DTT) to inhibit droplet formation. Similarly, lyophilized NPM1 was resuspended in Guanidine-HCl and refolded via dialysis in 20 mM Tris, 0.15 M NaCl, 2 mM DTT, pH 7.5 overnight. Protein solutions were subsequently mixed with high purity wheat germ rRNA (BioWorld) and varying volumes of salt buffer (20 mM Tris, pH 7.5, and 1 mM DTT with

varying NaCl concentration) to obtain final rRNA concentrations of 5 μg/mL for FIB1 and 100 μg/mL for NPM1 and desired protein/salt concentrations. NPM1 labeled with Dylight 594 NHS Ester (ThermoFisher Scientific) was added in trace amount to visualize NPM1 droplets in imaging-based assays. Samples were prepared in imaging chambers using silicone wells (Grace BioLabs) and observed under a microscope to score for phase behavior after incubation of 30 minutes onward. For three-phase assays, FIB1::GFP and NPM1 were phase separated with 5 μg/mL and 100 μg/mL of rRNA respectively at 150 mM NaCl buffer, mixed together after 5 minutes, and incubated for 30 minutes prior to imaging.

Effect of fluorescent tag on *in vitro* phase separation of FIB1 protein

Since the main FIB1 construct used is GFP tagged, whether the GFP tag alters the phase separation behavior and physical properties of the *in vitro* droplets was investigated. The phase boundary and fusion dynamics of untagged FIB1 droplets (visualized with sparsely labeled RNA), as well as droplets of FIB1 tagged with a GFP mutant (A206K) which is known to exist as a monomer (Zacharias et al., 2002) were explored (Fig. S2B). The various constructs do exhibit somewhat different behavior. For example, the GFP-tag shifts the phase boundary, requiring roughly 2-fold less protein to phase separate as compared to the untagged GFP. The inverse capillary velocity of FIB1 is also impacted roughly 2-fold by the GFP tag (Fig. S2B). Nonetheless, the impact of various means of tagging proteins is insignificant compared to the more than 10-fold difference in properties of FIB1 versus NPM1 droplets.

Microinjection of mRNA & protein constructs and nuclei dissection

Nuclei were microinjected with a Narishige micromanipulator and PicoPump PV820 as previously described (Feric and Brangwynne, 2013). mRNA constructs were microinjected into the cytoplasm, and oocytes were allowed to incubate overnight at 18°C. Proteins were injected directly into the nucleus, and oocytes were allowed to incubate for at least 2 hours before imaging. Nucleolar proteins NPM1 conjugated with dylight and FIB1::GFP were microinjected directly into the nucleus at an initial concentration of 32 μM and 2 μM, respectively, in 150 mM NaCl, 10 mM Tris pH 7.5, and 20 mM DTT. Oocytes were allowed to recover and were subsequently imaged at least 2 hours after microinjection. For all experiments, nuclei were manually dissected using forceps and a hair loop in mineral oil under *in vivo* conditions (Gall and Wu, 2010).

Actin disruption and coarsening

To disrupt actin, latrunculin A (Lat-A, Sigma) treatment was performed for 1-2 hours at 2 μg/ml at constant rotation. After Lat-A treatment, nuclei were dissected in mineral oil and placed in an imaging chamber consisting of a glass coverslip and glass coverslide separated by a silicone well (Grace Biolabs) as previously described (Feric and Brangwynne, 2013). Movies capturing all three nucleolar compartments consisted of 10-15 μm z-stacks with 1-2 μm step size and were acquired for several hours. Maximum intensity projections were made in each channel, and fusion events were analyzed in time for the granular and dense fibrillar components. The aspect ratio of each nucleolar phase was determined as $A.R. = \ell_{long}/\ell_{short}$ as a function of time, where ℓ_{long} and ℓ_{short} represent the lengths of the long and short axes of the nucleolar phase. The data was fit to an exponential to determine the relaxation time: $A.R. = a + b \cdot \exp\left(-t/\tau_f\right)$,

and the characteristic length scale, ℓ, of the nucleolar compartment was the measured radius. The time scale for fusion, τ_f, is expected to be directly proportional to the characteristic length scale, ℓ, of droplets according to the relation: $\tau_f \approx (\eta/\gamma) \cdot \ell$ (Brangwynne et al., 2011). Here, the inverse capillary velocity is the ratio of the viscosity of the droplet, η, to surface tension, γ, which underlies the spherical shape of droplets.

Surface tension measurements in *X. laevis* nuclei

After actin disruption by Lat-A, dissected nuclei were placed in glass bottom petri dishes (MatTek) filled with mineral oil. A 0.5 mm right angle prism (Edmund Optics) was manually placed adjacent to nuclei to visualize the XZ dimension. For experiments of the steady-state shape profile, nuclei were imaged after 2-3 hours of incubation in the petri dish. For experiments involving the relaxation of nucleolar shape, the oocytes were left to sit overnight after actin disruption, so that the nucleoli had time to sediment and fuse into one massive nucleolus (R>15 μm). The next day, those oocytes were rotated to cause the massive nucleolus to round into a sphere for several hours. Finally, nuclei were dissected and immediately imaged to capture the rapid deformation under gravity.

Using a right angle prism imaging approach to avoid imaging artifacts along the optical axis (Z), we examined the steady-state shape profile in the XZ dimension (Fig. 1J). The XZ shape profile is a balance between surface tension, which will promote rounder droplets, and gravitational forces, F_g, (along the optical Z-axis), which will tend to flatten droplets. The shape profile of the brightest XZ frame was obtained from custom image analysis and two length scales were obtained: R, which is the distance from the center to the widest point and H, which is the height

of the droplet (Fig. 2b inset). The surface tension was obtained as $\gamma = \Delta\rho g H^2/B \approx \Delta\rho g H^2/4.308[1 - H/R]$, where $\Delta\rho$ is the known density difference between the nucleolus and the surrounding nucleoplasm (Feric and Brangwynne, 2013), g is the acceleration due to gravity, and B is the empirically determined shape factor, which is a function of H/R ratio (Hansen, 1993).

The preceding analysis focuses on the steady state shape of large nucleoli deformed under the force of gravity. However, the timescale over which the nucleolus deforms to this shape can also yield insights into its properties. From dimensional analysis, the time scale for this shape relaxation is given by $\tau_g \approx (\eta/\gamma^2) \cdot F_g \approx (\eta/\gamma^2) \cdot \Delta\rho \cdot g \cdot \ell^3$, where g is the gravitational acceleration. We measured relaxation times on the order of 10-30 minutes for large nucleoli of diameter >30 microns (Fig. 1L, Supplemental Movie 4). Solving for viscosity, we obtain values of 30 ± 10 Pa·s (mean ± s.e.m). From these measurements of surface tension and viscosity, we obtain an independent estimate of the ratio of surface tension to viscosity: $\eta/\gamma \approx 50 \pm 10$ s/μm (mean ± s.e.m) (Fig. 1L inset). These measurements are consistent with those made from fusion relaxation experiments (Fig. 1H).

Surface tension measurements of nucleolar proteins *in vitro*

Purified nucleolar proteins (NPM1-dylight) were allowed to phase separate with RNA and were gently centrifuged to form large droplets. Small volumes from the phase separated solution were pipetted into a glass bottom dish filled with mineral oil (Sigma). Glass bottom dishes (MatTek) were previously treated with sigma cote (Sigma) for NPM1 droplets to create non-wetting conditions. XZ shape profiles were imaged by using a 0.5 mm prism (Edmund optics) and

analyzed as described in the previous section. By analyzing the sedimentation rate of NPM1 droplets (Feric and Brangwynne, 2013), we obtained a density difference between NPM1 droplets and the surrounding low concentration solution of $\Delta\rho = 60 \pm 20\ kg/m^3$, which was used along with the shape profile to estimate the surface tension as described above.

Wettability of protein droplets

Coverslips were treated with 1% Pluronic F-127 solution (Sigma Aldrich) to make the surface hydrophilic or with Sigmacote (Sigma Aldrich) to make the surface hydrophobic. Solutions of Pluronic or Sigmacote were placed on coverslips for approximately 10 min and washed off with DI water (Millipore) and dried with nitrogen gas. Protein droplets were placed in imaging chambers containing the treated coverslips. 3-D volume stacks were acquired and projected in XZ to obtain the shape profile. Contact angles were measured in Image-J as the angle between the line tangent to the drop and contact line interior of the drop.

Microrheology of protein droplets

Microrheology was performed in FIB1 and NPM1 droplets by adding R=50 nm fluorescent polystyrene microspheres (Invitrogen) to protein solutions inside an imaging chamber. Using spinning disk confocal microscopy, time-lapse movies were acquired 1-2 μm above the coverslip with a 100 ms interval and an exposure time less than one-fifth of the acquisition interval. Images were analyzed using particle-tracking algorithms as previously described (Feric and Brangwynne, 2013), and the two-dimensional mean-squared displacement (MSD) was calculated as a function of lag-time. We fit the data to obtain the diffusive exponent and the diffusion coefficient; using the Stokes-Einstein equation, we obtained the viscosity. The noise floor was

obtained by performing similar experiments on R=50 nm fluorescent polystyrene microspheres dried onto a glass coverslip.

Handling and imaging of mammalian cells

NIH 3T3 fibroblast cells were maintained in DMEM media supplemented with 10% FBS, 1% Penicillin/Streptomycin, and 1% Glutamax 100X. For maintenance, cells were trypsinized and passaged when they reached 70-80% confluence. For imaging, cells are plated on fibronectin-coated glass bottom dishes in HBSS/2% FBS and imaged using a 37°C heating stage. All images are taken with a Nikon A1 laser scanning confocal using a 60X, 1.4 NA oil immersion objective.

ATP depletion in mammalian cells

Cells were incubated for 30 min in 2 mM sodium azide and 10 mM deoxyglucose in fibronectin-coated glass bottom dishes in HBSS/2% FBS at 37°C and were imaged directly after incubation (Brangwynne et al., 2007).

Expression in mammalian cells

NPM1-mCherry cell lines were expressed by performing a lentiviral transfection of a cloned SFFV-NPM1-mCherry construct (cloned using In-Fusion HD Cloning Kit from Clontech to insert NPM1 into a SFFV-mCherry vector). FIB1::GFP expression was performed using FuGENE to transiently transfect 3T3 cells with a CMV-eGFP-FIB1 construct that was a gift from Sui Huang (Addgene plasmid #26673) (Chen and Huang, 2001). RPA194::GFP expression was also performed by transiently transfecting 3T3 cells with a CMV-eGFP-RPA194 construct (a gift from Tom Misteli, Addgene plasmid #17660) (Dundr et al., 2002).

C. elegans strain maintenance and imaging

C. elegans lines were maintained at 20°C on NGM plates seeded with OP50 bacteria. Adult hermaphrodites were then anesthetized with 1% levamisole hydrochloride in M9 and imaged on M9-agarose pads using a spinning disk confocal with a 100X/NA 1.4 oil immersion objective.

Fluorescence recovery after photobleaching (FRAP) of *X. laevis* nucleoli

Nuclei were injected with mRNA for each nucleolar component, dissected the next day in mineral oil, and placed in an imaging chamber as described above. To deplete ATP, nuclei were injected with 2 mg/mL Apyrase (Sigma) 1-2 hours before dissection. Each nucleolar component was photobleached with a spot 1 μm in diameter and the recovery of fluorescence intensity within the region of interest was obtained for each experiment. Intensity recovery curves were normalized and corrected for photobeaching (Phair et al., 2003). To determine the relaxation timescale, τ_f, the recovery curves were fit to the following expression: $I = a - b \cdot e^{-t/\tau_f}$, where a and b are also fit parameters.

FRAP of nucleolar proteins *in vitro*

In vitro droplets were photo-bleached with a spot 1 μm in diameter and the recovery of fluorescence intensity within the region of interest was obtained for each experiment. Intensity traces were corrected for photo-bleaching, normalized, and fit to an exponential function as above. For aging experiments, FRAP was performed on *in vitro* droplets at different time points as indicated.

FRAP of mammalian cells

3T3 cells expressing NPM1::mCherry and FIB1::GFP were photo-bleached with a spot ~1 μm in diameter and the recovery of fluorescence intensity within the region of interest was obtained for each experiment. Intensity traces were corrected for photo-bleaching, normalized, and fit to the exponential function above. For ATP depletion experiments, cells expressing NPM1::mCherry and FIB1::GFP were incubated in 2 mM sodium azide/10 mM deoxyglucose in HBSS/2% FBS for 30 minutes previous to imaging. Cells were imaged using a heating stage at 37°C.

FRAP of *C. elegans*

C. elegans line expressing intestinal FIB1::GFP were maintained at 20°C on NGM plates seeded with OP50 bacteria. L2-L3 larvae or adults were anesthetized with 1% levamisole hydrochloride in M9, placed on M9-agarose pads, and FRAP experiments were performed.

Preparation of non-biological multiphase droplets

Three immiscible liquids were used: DI water, Crisco oil, and silicone oil (viscosity 1,000 cSt, Sigma). For visualization, biotin-4-fluorescein (Biotium) was added to water at 0.1 mg/ml, and Oil Red O (Sigma) was added to Crisco oil at 1 mg/ml. Solutions were made to have a ratio of 5:1:1 of water:silicone oil:Crisco oil. To create multiphase droplets, solutions were vigorously vortexed and pipetted into imaging chambers containing silicone wells (Grace BioLabs).

Microscopy

Experiments for coarsening, surface tension, and *in vivo* characterization of nucleoli of *X. laevis*; *in vitro* experiments with nucleolar proteins, and non-biological multiphase droplets were

performed on an inverted Zeiss spinning-disc confocal microscope with Slidebook software as previously described (Feric and Brangwynne, 2013). Images of Lifeact::GFP network and FRAP experiments were performed on an inverted Nikon laser scanning confocal microscope with a 60X oil immersion objective.

Image Analysis

Custom built software was created in Matlab to perform quantitative image analysis. Code was also adapted from Matlab Multiple Particle Tracking Code (see http://physics.georgetown.edu/matlab/index.html) (Crocker and Grier, 1996) to apply band pass filters, link nucleoli in three dimensions from volume stacks, and/or track nucleoli in time. ImageJ was used to pseudocolor all images, apply filters, and prepare maximum intensity z-projections.

Design and Implementation of Coarse-Grained Simulations

The lattice-based computer simulations were performed using a coarse-grained description for each molecule and an interaction matrix that defines the effective strengths of inter-module interactions. In keeping with their modular architectures, we modeled FIB1 and rRNA as linear polymers of interaction modules. The pentameric NPM1 was modeled as a branched polymer with five arms. Each arm has three interaction modules anchored to a pre-pentamerized OD (Fig. 6A). Each interaction module is represented as a bead (see Fig. 6A) and occupies a lattice site such that no two beads can occupy the same site at the same time. The connected architecture is enforced through a linker between beads with a 3D-infinite square well potential. For FIB1 and rRNA, a square well distance of 4 lattice sites was used, and for NPM1, a distance of 2 lattice

sites was used. The total number of modules divided by the total number of lattice sites specifies the concentration of modules on the lattice, which has 115 sites to a side. The ternary system comprising of 900 of each of the three polymers starts out in the dispersed phase and is evolved by a collection of $5x10^{10}$ Monte Carlo moves. In the simulations, a bond can form between pairs of modules that occupy adjacent lattice sites. Parameters of the interaction matrix specify if a bond will form between a pair of interaction modules. This matrix also specifies the effective free energy to be assigned to a given bond. For a pair of modules, the parameters of the interaction matrix are governed by the overall competition between a) the interactions of each module in the pair with the solvent and b) the interactions involving the pair of interest and all other modules in the system. If these two classes of interactions are equivalent in free energy, then no bond will form. If there is an effective preference for the interaction between a pair of modules, then a favorable, negative free energy is assigned to the bond. The configurations of molecules and their positions and orientations with respect to one another were evolved using a Monte Carlo sampling strategy that combines a set of moves including the making and breaking of bonds between modules, pivot moves, crankshaft motions, reptations, and cluster moves of molecules. Pivot moves relocate an end module to a position within its linker length. Crankshaft motions relocate a central module to a position within both its linker lengths. Reptations advance all modules forward like a snake. Cluster moves translate all proteins that are bound together through interactions. Moves that lead to more than one module per lattice site are rejected. The standard Metropolis criterion was used to accept or reject the new configurations that result from bond breaking / making moves. The acceptance criterion for pivot and crankshaft moves was of the form: $\min\{1, N_p N_c^{-1} \exp(-\Delta E)\}$, where N_p and N_c are the number of possible interacting partners, given one to one binding, in the proposed (p) and current (c) positions respectively, and

ΔE is the change in energy associated with the proposed move. For reptation moves the acceptance criterion is $\min\{1,(N_p V_p)(N_c/V_c)^{-1}\exp(-\Delta E)\}$, where N_p and N_c are again the number of possible interacting states in the proposed and current states respectively, and V_p and V_c are the total number of conformations the module could be placed in the proposed state and current state, respectively. These modifications to classical Metropolis Monte Carlo acceptance ensure the preservation of microscopic reversibility. Cluster moves do not make or break interactions, nor do they change the internal structure within clusters. Instead, they displace clusters with respect to one another. Cluster moves are always accepted if the move does not engender steric overlap.

Supplemental References

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