Independent active and thermodynamic processes govern the nucleolus assembly in vivo

Hanieh Falahati[a] and Eric Wieschaus[a,b,1]

[a]Lewis–Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544; and [b]Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, Princeton, NJ 08544

Edited by Geraldine Seydoux, Johns Hopkins University School of Medicine, Baltimore, MD, and approved December 23, 2016 (received for review September 14, 2016)

Membraneless organelles play a central role in the organization of protoplasm by concentrating macromolecules, which allows efficient cellular processes. Recent studies have shown that, in vitro, certain components in such organelles can assemble through phase separation. Inside the cell, however, such organelles are multicomponent, with numerous intermolecular interactions that can potentially affect the demixing properties of individual components. In addition, the organelles themselves are inherently active, and it is not clear how the active, energy-consuming processes that occur constantly within such organelles affect the phase separation behavior of the constituent macromolecules. Here, we examine the phase separation model for the formation of membraneless organelles in vivo by assessing the two features that collectively distinguish it from active assembly, namely temperature dependence and reversibility. We use a microfluidic device that allows accurate and rapid manipulation of temperature and examine the quantitative dynamics by which six different nucleolar proteins assemble into the nucleoli of *Drosophila melanogaster* embryos. Our results indicate that, although phase separation is the main mode of recruitment for four of the studied proteins, the assembly of the other two is irreversible and enhanced at higher temperatures, behaviors indicative of active recruitment to the nucleolus. These two subsets of components differ in their requirements for ribosomal DNA; the two actively assembling components fail to assemble in the absence of ribosomal DNA, whereas the thermodynamically driven components assemble but lose temporal and spatial precision.

liquid–liquid phase separation | intracellular phase transition | membrane-less organelle | RNA granule | *Drosophila* nucleologenesis

Membraneless organelles are highly concentrated assemblies of proteins and RNAs that provide specialized microenvironments for particular cellular functions (1). Recent studies suggest that such organelles may form via a liquid–liquid phase separation (LLPS) process, in which the constituent components spontaneously assemble on reaching a critical concentration at a given temperature (2–5). LLPS provides an attractive energy-efficient mechanism for cells to organize different biochemical reactions spatially, whereas the liquid nature of the emerging organelles, such as P granules and nucleoli (2, 6), allows for rapid exchange of molecules. The role of LLPS has been supported by studies in which the purified RNA binding proteins that localize to such subcellular bodies in vivo also self-assemble in vitro (3–11). However, because of the complexity of living cells, our current understanding of the role of LLPS in membraneless organelle assembly is by far limited to in vitro studies. Particularly, membraneless organelles are multicomponent, and the interactions between different components can enhance or diminish the ability of individual proteins to phase separate inside living cells (12). Therefore, the behavior of the individual components in the simplified in vitro systems are not necessarily predictive of their behavior in vivo with all native interacting partners. Such limitations of in vitro systems underline the necessity for a rigorous in vivo assessment of the LLPS model.

An alternative mechanism for the formation of membraneless organelles in vivo is active assembly. Based on this model, an enzymatic reaction couples an energy source, such as ATP, to a reaction, which results in the formation of membraneless organelles. Several studies suggest that active transport of components can drive the formation of high-concentration assemblies in vivo. For example, the formation of stress granules and the growth of P bodies in response to stress rely on motor proteins (13, 14). Similarly, transport of AMPA receptors to high-concentration synaptic puncta, which resemble membraneless organelles, is dependent on Kinesin-1 (15). Many active processes occur constantly in living cells and also, within membraneless organelles, which could also contribute to the formation of high-concentration assemblies. For example, several membraneless organelles, such as nucleoli and histone locus bodies, form at active sites of transcription that would provide high concentrations of nascent RNA. Any protein that can bind to the nascent RNA would thus be enriched at these sites of transcription without requiring any other mechanism.

Finally, the LLPS and active recruitment models for the formation of membraneless organelles are not mutually exclusive. For instance, the presence of actively transcribed RNA can modulate the demixing behavior of certain phase-separating proteins (7, 16). In addition, posttranslational modifications, such as phosphorylation of proteins, can regulate their localization to membraneless organelles, potentially by changing intermolecular interactions that govern LLPS (17–19). Nevertheless, in the absence of an in vivo assay for evaluation of the LLPS model, it is not quite clear to what extent the formation of membraneless

Significance

The role of thermodynamically driven processes in inherently active biological systems has daunted scientists for decades. One such conundrum emerges in the formation of membraneless organelles that, according to in vitro studies, assemble via thermodynamically driven phase separation but harbor numerous active processes within themselves. Disentangling the role of thermodynamic and active processes in their formation is, however, impossible in minimal in vitro systems of individual constituent components. In this work, we introduce a microfluidics-based temperature assay to address this question at its full complexity in vivo and use it to study the assembly of six nucleolar proteins in *Drosophila* embryos. Although four of these proteins follow the phase separation model, the recruitment of others to the nucleolus is active.

Author contributions: H.F. and E.W. designed research; H.F. performed research; H.F. analyzed data; and H.F. and E.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. Email: efw@princeton.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1615395114/-/DCSupplemental.

organelles is through an LLPS and to what extent it is driven by active reactions.

Here, we introduce an in vivo temperature-based assay to test the LLPS model for the assembly of membraneless organelles and use it to study the mechanism by which six different nucleolar proteins (Fig. 1*A* and Table S1) localize to the nucleolus in *Drosophila melanogaster* cleavage-stage embryos. Development in *Drosophila* embryos starts without a nucleolus and proceeds through 13 rapid nuclear divisions followed by a pause at the interphase of nuclear cycle 14 (NC14). Transcription of ribosomal DNA (rDNA) begins at NC11 (16). To follow the formation of a visible nucleolus, we used six fluorescently tagged nucleolar proteins involved in different steps of ribosomal biogenesis and localized to different subcompartments of the nucleolus (16, 20) (Fig. 1*A* and Table S1). For comparisons, one of the proteins, Fibrillarin, was tagged with the red fluorescent protein, TagRFP (hereafter RFP-Fib) and always coexpressed with the other five EGFP-tagged proteins. At room temperature, all six proteins show a similar temporal pattern of accumulation into the nucleolus at 5–8 min into the interphase of NC13, with the median emergence times for assemblies being 5 ± 0.5 min for nucleolar phosphoprotein 140 (Nopp140); 6 ± 0.5 min for Fib, Modulo (Mod), and Rpl135 [also known as RNA polymerase I subunit 135 (RpI135) in *Drosophila*]; 7 ± 0.5 min for Nucleostemin1 (Ns1); and 8 ± 0.5 min for Pitchoune (Pit) (*Methods* has details of measurements; Fig. 1*B*). The timing of nucleolus formation is extremely reproducible and tightly constrained. At NC12, only 5 of 40 studied embryos show transient Fib and Mod and either Nopp140 or Ns1 foci; one embryo showed exclusively Mod assemblies, and two showed exclusively Fib assemblies. Visible nucleolar accumulation of all six proteins is always detectable during interphase of NC13 and stable after it is observed. The concentrations of these proteins in the nucleoplasm increase during syncytial NCs, with the maximum levels of all proteins coinciding with the first-time emergence of nucleolar assemblies at NC13 (Fig. S1) (16). Although the emergence of assemblies at high concentrations is consistent with the concentration dependence of phase separation processes, it is not sufficient to rule out the possibility of active assembly, because the latter would also lead to the formation of assemblies at higher (substrate) concentrations.

Temperature Dependence of First-Time Assembly Formation. One feature that distinguishes thermodynamic LLPSs from active assembly processes is temperature dependence. The rate of an active assembly process is expected to decrease at lower temperatures, because the collisions that drive molecular interactions are less frequent at lower temperatures and the enzymes that couple the process to a high-energy source, such as ATP, typically operate more slowly (Fig. 2*A*) (26). Thermodynamic LLPS, in contrast, is generally enhanced at lower temperatures, because the entropic cost of demixing becomes smaller than its energetic advantage at lower temperatures (the exceptions are in *Discussion*; Fig. 2*B*). Therefore, allowing the embryos of the *Drosophila* to develop at lower temperatures will delay the processes dependent on active assembly, whereas it will enable thermodynamic LLPS to occur at earlier stages of development when the concentrations of the nucleolar proteins are too low to allow accumulation at room temperature (16) (Fig. S1). We used a microfluidic device (Fig. S2) (27) that allows a *Drosophila* embryo to develop at 7 °C to 29 °C. The temperature of the embryo in the device is controlled by the temperature of the water stream passing through the channels, and it is monitored by a built-in thermometer (details are in *Methods*). When the temperature at which development occurs is lowered to 7 °C to 8 °C, all studied embryos show Fib foci at NC12 [i.e., one cycle earlier than room temperature, with one embryo also showing assemblies as early as NC11 ($n = 15$)] (Fig. 2*E* and Fig. S3). Likewise, Nopp140 always localizes to Fib foci at NC12 ($n = 3$), suggesting that the assembly of these two proteins occurs via LLPS-dependent processes. Unlike Fib and Nopp140, the accumulation of two other nuclear proteins (Mod and Ns1) is delayed at 7 °C to 8 °C and does not occur at its normal time during NC13 (Fig. 2 *C*–*E* and Fig. S3). Higher temperatures (28 °C to 29 °C) have the opposite effect on Mod and Ns1, facilitating their accumulation (NC12 vs. NC13; $n = 3$), whereas the first emergence of Fib and Nopp140 remains at NC13, unchanged from that at room temperature. The premature assemblies of Mod and Ns1 that form without the enrichment of the phase-separating components seem morphologically similar to their assemblies in the normal nucleolus at NC13. The accumulation pattern of two other tested proteins, a subunit of RNA polymerase I (Rpl135) and Pit, seems to be insensitive to temperatures in the range that we have been able to produce with our microfluidic device. It is rather striking that the assembly of different proteins proceeds through mechanisms that scale differently with temperature, despite the fact that they all accumulate to the nucleolus simultaneously at ambient temperatures at which the embryo normally develops. In addition, these mechanisms are independent of one another, because the proteins in each group can assemble in the absence of the others. The observations presented above are consistent with Nopp140 and Fib following an LLPS mechanism, whereas Ns1 and Mod behave as actively separating proteins.

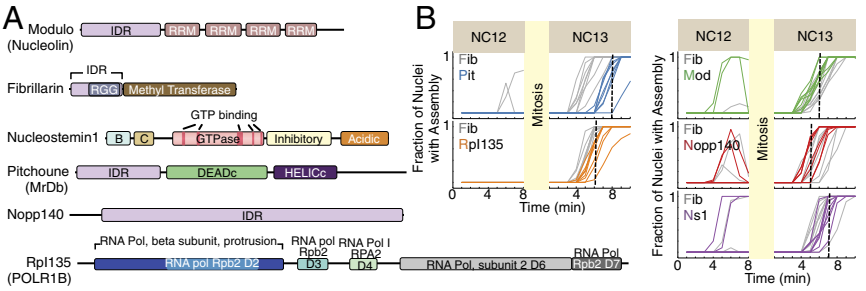

Fig. 1. First-time assembly of six nucleolar proteins at 22 °C. Structural features of six nucleolar proteins studied are depicted in *A*. The structural elements of Ns1 were previously reported (21). For other proteins, the structural features were determined using National Center for Biotechnology Information conserved domain search (22) and InterPro (23) as well as IUPRED (24) for detection of disordered regions. Fib is a methyltransferase that associates with C/D box small nucleolar RNAs to form small nucleolar ribonucleoprotein complexes; the inhibitory domain of Ns1 prevents its nucleolar localization when GTP is not bound (25). The DEADc domain in Pitchoune, characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD), contains an ATP binding region. B, basic region; C, coiled coil; D, domain; RGG, R/G-rich domain; RRM, RNA recognition motif. (*B*) The first-time emergence of assemblies for different nucleolar proteins at 22 °C at NC12–NC13 is depicted. Each line represents the fraction of nuclei in a single embryo that has detectable foci of each nucleolar protein, with the dashed lines marking the median $t_{1/2}$ for the first-time formation of each GFP-tagged protein (number of embryos in each panel = 8) (details are in *Methods*). Median $t_{1/2}$ for Fib is 6 min into interphase of NC13. Time 0 marks the beginning of interphase.

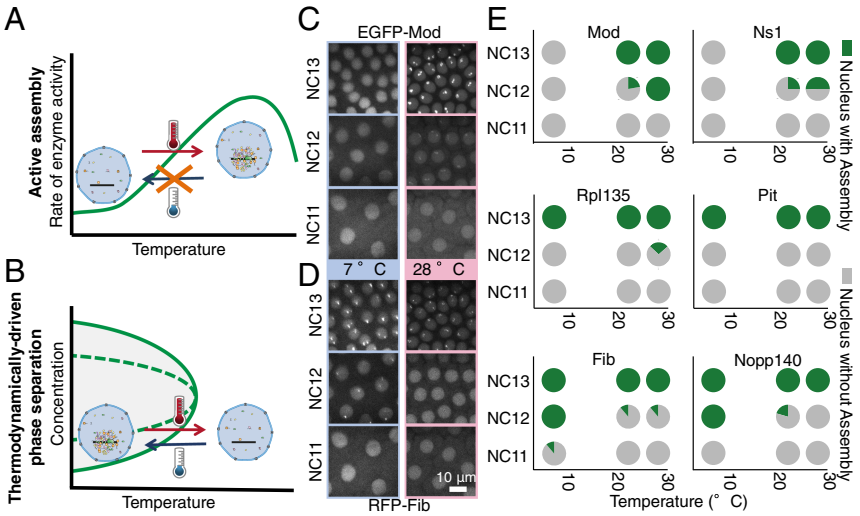

Fig. 2. Temperature uncouples the first-time assembly of different nucleolar proteins. The temperature dependence of two possible mechanisms for the formation of assemblies is illustrated qualitatively in *A* and *B*. (*A*) The rate of active processes is determined by the rate of the enzyme that couples them to an energy source. Therefore, active assembly is faster at higher temperatures and slower at lower temperatures. (*B*) This behavior is the opposite of a thermodynamic LLPS, in which more condensation occurs at lower temperatures (exceptions are in *Discussion*). (*C* and *D*) An embryo coexpressing (*C*) EGFP-Mod and (*D*) RFP-Fib is imaged at 7 °C and 28 °C to determine the first-time emergence of assemblies. Images are maximum-projected. (*E*) The percentages of nuclei with or without assemblies at each temperature for each cell cycle are depicted in a pie chart for different nucleolar proteins. Number of embryos is three or more for each condition.

Reversibility of Assembly Formation. A second distinctive feature of thermodynamically driven LLPS is its reversibility. A small increase in temperature increases the entropic cost of demixing and makes the components of the two phases more miscible, leading to a reduction in the size and concentration of the nucleolar assemblies. If the change in temperature is large enough to cross the phase boundary in Fig. 2*B*, complete mixing of the two phases should occur. This reversibility in response to changes in the temperature has been observed in vitro for several protein solutions, including bovine lens protein γII crystalline (28), hnRNPA1 (29), low-complexity domain of FUS (9), and the N terminus of Ddx4 (11). This behavior contrasts with that of an active process, which is facilitated at higher temperatures and expected to only decelerate and stall when the temperature is lowered but would not be expected to reverse (Fig. 3 *A–D*). To test this property further, we examined the reversibility of accumulation at the nucleolus in response to rapid changes in temperature. Such rapid changes should minimize the impact of the temperature shift on upstream factors, such as nuclear localization or protein synthesis. Accordingly, we allowed embryos to reach interphase of NC14 at 22 °C and then, reduced the temperature to ≈7 °C while performing confocal imaging. Next, the temperature was shifted up to ≈31 °C within a few minutes by manually changing the source of water stream from a cooling block to a heating block. The distributions of the nucleolar proteins were recorded again. To switch back to low temperature, the source of the water stream was changed back to a cooling block. This process was repeated three to six times, and changes in the nucleolar intensity of different proteins at various temperatures were measured from the resulting images. All studied embryos were coexpressing a GFP-tagged nucleolar protein with RFP-Fib for comparison. As depicted in Fig. 3 *E–J* and *G′*, Nopp140 and Fib show reversible changes in nucleolar localization in response to rapid oscillations in temperature. Higher levels accumulate at the nucleolus at lower temperatures, whereas the protein rapidly redistributes to the nucleoplasm at higher temperatures. These changes are fully reversible, because the same behavior is observed on multiple rounds of temperature oscillations. Interestingly, by shifting the temperature from 8 °C to 25 °C during early NC13, when the concentrations of these proteins are sufficiently low (16) (Fig. S1), we are able to cross the phase boundary in Fig. 2*B* and fully dissolve Nopp140 and Fib assemblies (Fig. 3*J* and Movie S1) as the concentration of the proteins increases. These results are consistent with a thermodynamically driven LLPS mechanism for the nucleolar accumulation of Nopp140 and Fib. We cannot rule out a special case with a combination of forward and reverse active processes, in which the disassembly process is more sensitive to the changes in the temperature. However, this special case seems unlikely, because it is an energetically expensive way of reaching the same behavior.

Unlike the first group of the nucleolar proteins discussed above, the formation of Mod and Ns1 assemblies is irreversible (Fig. 3E' and F'). These two proteins are recruited to the nucleolus at higher temperatures, and their accumulation is slowed down at lower temperatures. Changing the temperature does not reverse any accumulation of these two proteins that has occurred previously in the nucleolus. This observed irreversibility is consistent with an active assembly. Alternatively, the assemblies could represent an LLPS with slow kinetics. To distinguish between these two possibilities, we performed fluorescent recovery after photobleaching (Fig. S4). Both Mod and Ns1 are highly dynamic, with $t_{1/2}$ for fluorescence recovery of less than 10 s, and therefore, the assemblies of these proteins are not kinetically arrested. The high motility of these proteins at 7 °C also argues against the involvement of motor proteins in their localization. Previous studies have shown that the GTPase, Ns1, requires binding to GTP for its localization to the nucleolus (25). Another possibility is a combination of active and LLPS processes, in which an initial active reaction, such as transcription of rDNA, phosphorylation, or binding to GTP, is required for the generation of key components, but a secondary thermodynamic LLPS drives the formation of assemblies. This hypothesis is consistent with the inhibition of assembly formation at low temperatures for Mod and Ns1 (Fig. 2). However, our reversibility results do not support the secondary LLPS mechanism. During NC14, the size of assemblies of these two proteins reaches a maximum level and saturates (equilibrates), suggesting that components capable of LLPS are no longer limiting. Under these conditions, LLPS-based movement in and out of the assemblies should depend on temperature—the size and magnitude of the assemblies should be reversible and change with rapid temperature shifts. We see such reversible behavior with other nucleolar components but not with Mod and Ns1, suggesting that, even in the saturated states characteristic of NC14, those two components do not follow characteristic LLPS behavior (Fig. 3E' and F').

We also see a reversible behavior after rapid temperature shifts for the two proteins (Pit and Rpl135) that did not show obvious temperature dependence in the timing of their assembly during development. For Pit, the behavior was very similar to Nopp140 and Fib, suggesting that it may depend on LLPS (Fig. 3H'). Our previous failure to see earlier assembly of Pit at low temperatures may reflect the exclusion of Pit from nuclei before NC13 (Figs. S1 and S3). Rpl135 binds to the rDNA genes

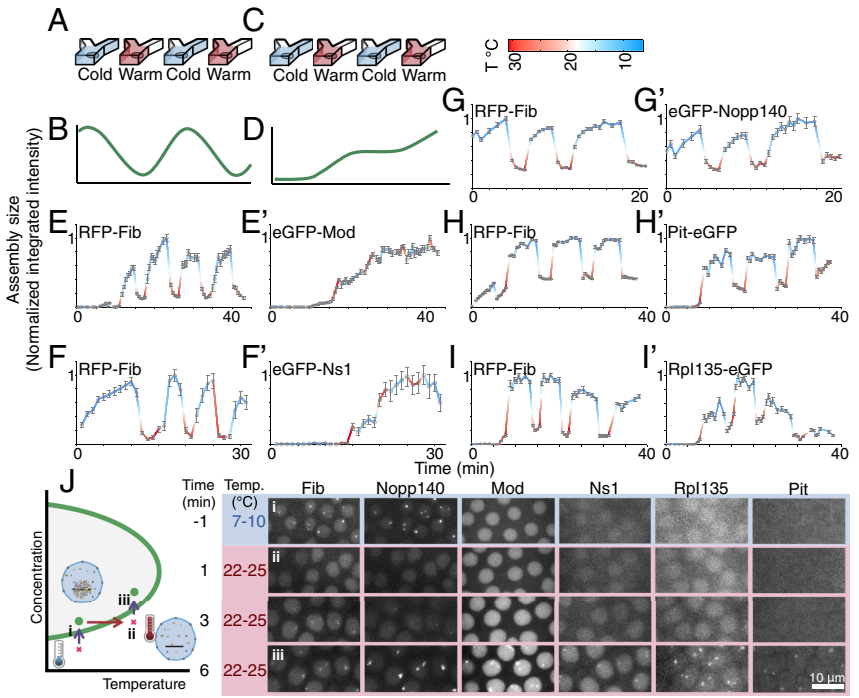

Fig. 3. An in vivo assay to test the reversibility of assembly formation by nucleolar proteins. (*A* and *C*) A microfluidic device was used to switch between cold and warm temperatures in less than 1 min during NC14. (*B* and *D*) Schematics qualitatively illustrate predicted trends in assembly size for (*B*) a thermodynamic LLPS and (*D*) an active assembly. (*B*) A thermodynamic LLPS is expected to reversibly condense and dissolve by changing the temperature, whereas (*D*) an active assembly is expected to be slow at low temperatures, fast at high temperatures, and irreversible. (*E–I* and *E′–I′*) The integrated intensity at each temperature/time for high-concentration assemblies of different nucleolar proteins is determined, and mean ± SEM for $n > 10$ nuclei is normalized to its maximum level. Time 0 is when the experiment begins. The changes in the integrated intensity of the assemblies of (*E′–I′*) EGFP-tagged nucleolar proteins compared with those of (*E–I*) RFP-Fib are shown. (*J*) During early NC13, (*i*) Fib and Nopp140 assemblies are first to form at 8 °C to 10 °C. (*ii*) As soon as Fib assemblies appear, the embryos are shifted to 22 °C to 25 °C, in which the critical concentrations for Fib and Nopp140 are higher than the nucleoplasmic concentrations of these two proteins. This shift, therefore, causes the assemblies to dissolve. (*iii*) As the concentrations of Fib and Nopp140 increase by time, the assemblies reappear. The schematic in *Left* qualitatively illustrates the state of each step (*i–iii*) in the phase diagram. Images are maximum-projected. Time 0 marks the time when the temperature shift was applied.

that are tandemly repeated hundreds of times, and this binding alone could give rise to Rpl135 foci. However, Rpl135 assemblies are present in mutant embryos lacking rDNA repeats (16) (Fig. 4*A*), arguing for the presence of alternative mechanism(s) for the recruitment of this protein. Consistent with that observation, during early NC14, successive alternations between low and high temperatures result in the reversible accumulation and redistribution of Rpl135 to the nucleolus, respectively (Fig. 3*I′*). Interestingly, unlike in the other phase-separating proteins, the nucleolar intensity of Rpl135 becomes almost insensitive to changes in temperature toward the end of NC14. This behavior suggests that the initial recruitment of Rpl135 to the nucleolus proceeds directly or indirectly through a thermodynamic LLPS but that secondary regulatory mechanisms later stabilize its nucleolar levels. Activation of transcription or association with the products of transcription could result in this observed behavior (30, 31).

Dependence of Assembly Formation on rDNA. We have previously reported that the transcription of rRNA seeds the assembly of the phase-separating proteins, Fib and Rpl135, dictating the precision of an otherwise highly variable nucleation-limited process (16). In mutant embryos that lack rDNA, Fib and Rpl135 still assemble into nucleolar-like structures but with altered kinetics and greater variability. Because the temperature shift experiments presented above suggest that Fib's incorporation and Rpl135's initial recruitment into the nucleolus may be LLPS-dependent processes, we asked whether other nucleolar proteins show similar behaviors. Similar to Fib and Rpl135, other phase-separating nucleolar proteins, Nopp140 and Pit, are capable of forming high-concentration assemblies in the mutants lacking rDNA (Fig. 4*A*). In WT embryos at 22 °C, all six studied nucleolar proteins exclusively assemble at the two rDNA repeats of X and Y chromosomes (Fig. S3). However, more variability is observed in the number and position of Fib and Nopp140 foci in the absence of rDNA (Fig. 4*A*) (16). These morphological features were also observed in the premature Fib and Nopp140 assemblies that arise in WT embryos at 8 °C (Fig. 4*B*), suggesting that assembly at low temperature may not depend on seeding by RNA. The behavior of Pit differs from that of Fib and Nopp140, in that the assemblies that it forms in rDNA mutants accumulate to the apical side of the nucleus (Fig. 4*A*). These results suggest that, in the absence of rDNA, Pit may make use of an alternative nucleation site associated with heterochromatin. In contrast to the behavior of the LLPS-dependent proteins, rDNA is necessary for the two proteins that appear to accumulate through active recruitment (Mod and Ns1), because neither form assemblies in its absence (Fig. 4*A*). This behavior is similar to what was observed for these two proteins in WT embryos at low temperatures that are not permissive for active processes, such as transcription (Fig. 2). Consistent with the role of rDNA in active recruitment of Mod and Ns1, the maximum number of assemblies for these two proteins in WT embryos at different temperatures is two (Fig. 4*B*), suggesting that they can only assemble at rDNA repeats.

Discussion

The experiments presented here constitute an in vivo assessment of the phase separation model for the formation of membraneless organelles. This assessment is based on the two main features

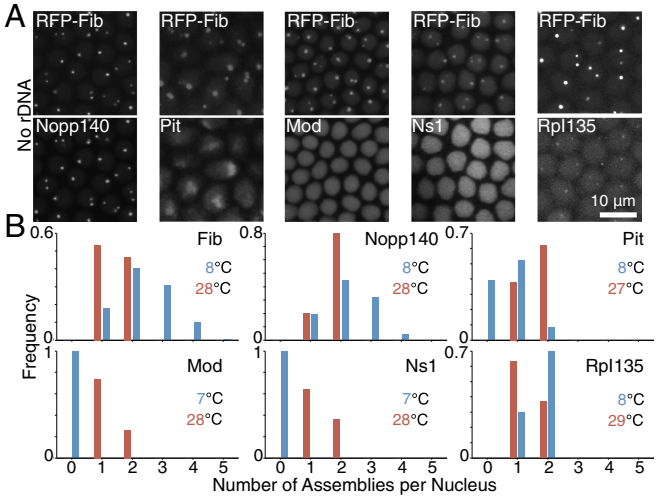

Fig. 4. Transcription of rRNA coordinates the active and thermodynamic assemblies. (*A*) Each column depicts a mutant embryo lacking rDNA repeats at 22 °C and NC14 and coexpressing RFP-Fib with another EGFP-tagged nucleolar protein (*Lower*). Fib, Nopp140, and Rpl135 colocalize to variable numbers of assemblies in a nucleation-limited process, whereas Pit forms one diffuse assembly at the apical side of the nucleus. Mod and Ns1 do not form any assemblies in the absence of rDNA. Images are maximum-projected. (*B*) Distribution of the number of assemblies per nucleus in WT embryos for each of the nucleolar proteins at NC13 is depicted at high and low temperatures. At 22 °C, assemblies form at two rDNA repeats located on X and Y chromosomes. More than two foci observed for Fib and Nopp140 at 8 °C are indicative of unseeded assembly formation, whereas other proteins can only assemble at rDNA repeats in WT embryos.

of LLPS, namely the temperature dependence and reversibility, which together can distinguish thermodynamically driven phase separations from active assemblies. Using our microfluidic-based temperature assay, we show that some nucleolar proteins are recruited to the nucleolus via a thermodynamic LLPS, whereas others are actively incorporated into the nucleolus. These findings refine our fundamental understanding of the mechanism and structural determinants for the formation of membraneless bodies in vivo in not only a developmental context but also, various diseases in which native structures are altered or pathological aggregates appear.

Several classes of LLPSs have been observed for polymer/solvent systems (Fig. S5) (32), and the assay presented in this work can distinguish between these different possibilities. Most LLPSs occur when cooling below a critical temperature because at higher temperatures, the entropic penalty of demixing increases and eventually overcomes the unfavorable energetic interactions between dissimilar entities. Such temperature dependence leads to an upper-critical solution temperature (UCST), above which the system is well-mixed at all compositions (Fig. S5*A*). Examples of UCST have been observed in vitro for the low-complexity domain of the RNA binding protein FUS (10), a disordered Nauge protein (11), and lipid bilayers (33). The phase-separating nucleolar proteins studied here (Fib, Nopp140, Pit, and Rpl135) all show this behavior, because their formation is reversibly enhanced at low temperatures. This behavior is consistent with the presence of polar residues at the disordered regions of these proteins (Fig. S6), which are predictive of LLPS with an UCST (34).

There are, however, exceptions to this general behavior, which show a lower-critical solution temperature (LCST) (Fig. S5 *B* and *C*) or no critical temperature (Fig. S5*D*). Such anomalies are observed in two types of systems. The first anomaly is mostly observed in the polymer solutions at high temperatures and pressures, at which the compressibility of the solvent affects the mixing behavior (e.g., close to the vapor–liquid critical point of the solvent) and results in phase diagrams shown in Fig. S5 *C* and *D* (32). Obviously, such conditions are not physiologically relevant. The second type of anomaly is observed when highly directional specific interactions between dissimilar molecules prevent random mixing, leading to a "closed loop" in the phase diagram (Fig. S5*B*) (32). The presence of LCST has been observed for a spindle-associated protein, BuGZ (35). In this case, decreasing the temperature can prevent the formation of assemblies, similar to an active recruitment. However, unlike active processes, an LLPS with LCST is reversible, and therefore, after the formation of assemblies, lowering the temperature results in remixing of the two phases. Such remixing is not what we observe for Mod and Ns1, for which the assembly process that happens at high temperatures is irreversible (Fig. 3E' and F'). This irreversibility is not caused by slower kinetics (Fig. S4) and is, thus, consistent with an active recruitment rather than an LLPS with LCST. In addition, these two proteins lack nonpolar residues at the disordered regions (Fig. S6) that are predictive of LLPS with LCST (34).

The large number of molecules studied here allows us to examine the structural features that govern in vivo phase separations. Previous studies suggest that the presence of intrinsically disordered regions (IDRs), repetitive modules, and RNA binding motifs in a protein enhance its phase separation propensity (3–10). The disorder tendency of the proteins studied here was assessed using IUPRED (24) (Fig. S6). Fib, Nopp140, and Pit all contain IDRs and assemble via an LLPS-dependent mechanism. In addition, Fib is a component of ribonucleoprotein complexes and therefore, has potential for multimerization through additional macromolecular constituents. Ns1 lacks IDRs and is shown to assemble actively (Fig. 3F'). An unexpected behavior, however, is observed for Mod, the *Drosophila* homologue of Nucleolin. Despite containing a disordered N terminus and four RNA binding motifs (Fig. 1 and Fig. S6), its recruitment to the nucleolus is through active assembly, suggesting that the presence of IDRs is not sufficient for an in vivo thermodynamic LLPS. Similarly, Rpl135 lacks any known structural feature that could lead to phase separation, and yet, its initial recruitment to the nucleolus is dependent on LLPS. This dependence could be direct via an unknown structural feature or indirect through binding to a phase-separating chaperon. For the three other proteins (Fib, Nopp140, and Pit), which contain IDRs and are recruited to the nucleolus through an LLPS-dependent mechanism, additional experiments will determine whether this dependence in vivo is direct or indirect. These observations underline the complexity of interactions that drive LLPS inside cells and the necessity for more in vivo studies that will allow us to refine the existing heuristics for identifying phase-separating proteins, an undertaking that could be the topic of future studies.

The observation of two independent mechanisms for the formation of a single organelle is surprising and suggests that the simultaneous accumulation observed during normal development may involve some global coordinator of the different mechanisms. Our observations confirm that the transcription of rDNA is responsible for the spatiotemporal regulation of LLPS as well as plays an essential role in recruiting nucleolar proteins, like Mod or Ns1, that depend on active assembly. Therefore, rDNA seems to function as the coordinator of the two independent mechanisms for the formation of the nucleolus.

Methods

Determination of the First-Time Emergence of Nucleolar Protein Assemblies. The nucleoli were detected using an automated algorithm described before (16). The nuclei were detected independently for the two fluorescent proteins in each embryo. The first-time emergence ($t_{1/2}$ of formation) is defined as the first time point at which at least one-half of the nuclei in the image has detectable assemblies. The average time for the first-time formation of assemblies for each protein is defined as the median $t_{1/2}$ for eight embryos studied.

Developing Embryos at Different Temperatures. The embryos coexpressing RFP-Fib with a second EGFP-tagged nucleolar protein (Nopp140, Ns1, Mod, Pit, or Rpl135) were placed in the microfluidic device before NC10. For each temperature, the flow with the desired temperature would pass through one inlet, and the second inlet was blocked. For high-temperature experiments, the embryos were exposed to continuous flow of 28 °C to 29 °C from NC10 to NC13. For room temperature experiments, no flow was applied. Because low temperatures can disrupt microtubule assembly, the embryos were exposed to 7 °C to 8 °C flow only during interphases of NC11–NC13, and the mitosis was allowed to proceed at room temperature.

Reversibility Assay. The switch between different temperatures was performed manually by alternating the inlet flow from the cooling block to the heating block. This process would be performed in about 30 s. After any change in the temperature, the objective lens and the cover glass contract or expand, shifting the embryo out of focus. The focus was, therefore, readjusted manually after each temperature shift for each image, and the temperature and time elapsed from the previous image were recorded.

SI Methods has details of materials and methods.

ACKNOWLEDGMENTS. We thank the Bloomington Drosophila Stock Center for fly stocks. We thank all members of the laboratory of E.W. and the Schupbach laboratory, especially S. Blythe for lively discussion. We also thank P. Debenedetti, S. Shvartsman, B. Machta, M. Levine, A. Haji-Akbari, and T. Schupbach for helpful comments. We thank S. Vyawahare and the Princeton Microfluidics Laboratory. This work was supported, in part, by National Institute of Child Health and Human Development Grant 5R37HD15587 (to E.W.). Additionally, E.W. is an Investigator with the Howard Hughes Medical Institute.

1. Matera AG, Izaguire-Sierra M, Praveen K, Rajendra TK (2009) Nuclear bodies: Random aggregates of sticky proteins or crucibles of macromolecular assembly?. *Dev Cell* 17(5):639–647.
2. Brangwynne CP, et al. (2009) Germline P granules are liquid droplets that localize by controlled dissolution/condensation. *Science* 324(5935):1729–1732.
3. Li P, et al. (2012) Phase transitions in the assembly of multivalent signalling proteins. *Nature* 483(7389):336–340.
4. Hyman AA, Weber CA, Jülicher F (2014) Liquid-liquid phase separation in biology. *Annu Rev Cell Dev Biol* 30:39–58.
5. Courchaine EM, Lu A, Neugebauer KM (2016) Droplet organelles? *EMBO J* 35(15):1603–1612.
6. Brangwynne CP, Mitchison TJ, Hyman AA (2011) Active liquid-like behavior of nucleoli determines their size and shape in *Xenopus laevis* oocytes. *Proc Natl Acad Sci USA* 108(11):4334–4339.
7. Berry J, Weber SC, Vaidya N, Haataja M, Brangwynne CP (2015) RNA transcription modulates phase transition-driven nuclear body assembly. *Proc Natl Acad Sci USA* 112(38):E5237–E5245.
8. Elbaum-Garfinkle S, et al. (2015) The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics. *Proc Natl Acad Sci USA* 112(23):7189–7194.
9. Murakami T, et al. (2015) ALS/FTD mutation-induced phase transition of FUS liquid droplets and reversible hydrogels into irreversible hydrogels impairs RNP granule function. *Neuron* 88(4):678–690.
10. Burke KA, Janke AM, Rhine CL, Fawzi NL (2015) Residue-by-residue view of in vitro FUF granules that bind the C-terminal domain of RNA polymerase II. *Mol Cell* 60(2):231–241.
11. Nott TJ, et al. (2015) Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles. *Mol Cell* 57(5):936–947.
12. Sear RP, Cuesta JA (2003) Instabilities in complex mixtures with a large number of components. *Phys Rev Lett* 91:245701.
13. Loschi M, Leishman CC, Berardone N, Boccaccio GL (2009) Dynein and kinesin regulate stress-granule and P-body dynamics. *J Cell Sci* 122(Pt 21):3973–3982.
14. Thomas MG, Loschi M, Desbats MA, Boccaccio GL (2011) RNA granules: The good, the bad and the ugly. *Cell Signal* 23(2):324–334.
15. Hoerndli FJ, et al. (2013) Kinesin-1 regulates synaptic strength by mediating the delivery, removal, and redistribution of AMPA receptors. *Neuron* 80(6):1421–1437.
16. Falahati H, Pelham-Webb B, Blythe S, Wieschaus E (2016) Nucleation by rRNA dictates the precision of nucleolus assembly. *Curr Biol* 26(3):277–285.
17. Dundr M, Misteli T (2010) Biogenesis of nuclear bodies. *Cold Spring Harbor Perspect Biol* 2(12):a000711.
18. Wang JT, et al. (2014) Regulation of RNA granule dynamics by phosphorylation of serine-rich, intrinsically disordered proteins in *C. elegans*. *Elife* 3:e04591.
19. Aumiller WM Jr, Keating CD (2016) Phosphorylation-mediated RNA/peptide complex coacervation as a model for intracellular liquid organelles. *Nat Chem* 8(2):129–137.
20. Blythe SA, Wieschaus EF (2015) Zygotic genome activation triggers the DNA replication checkpoint at the midblastula transition. *Cell* 160(6):1169–1181.
21. Rosby R, et al. (2009) Knockdown of the *Drosophila* GTPase nucleostemin 1 impairs large ribosomal subunit biogenesis, cell growth, and midgut precursor cell maintenance. *Mol Biol Cell* 20(20):4424–4434.
22. Marchler-Bauer A, et al. (2015) CDD: NCBI's conserved domain database. *Nucleic Acids Res* 43(Database issue):D222–D226.
23. Mitchell A, et al. (2015) The InterPro protein families database: The classification resource after 15 years. *Nucleic Acids Res* 43(Database issue):D213–D221.
24. Dosztányi Z, Csizmok V, Tompa P, Simon I (2005) IUPred: Web server for the prediction of intrinsically unstructured regions of proteins based on estimated energy content. *Bioinformatics* 21(16):3433–3434.
25. Tsai RYL, McKay RDG (2005) A multistep, GTP-driven mechanism controlling the dynamic cycling of nucleostemin. *J Cell Biol* 168(2):179–184.
26. Clark DS, Blanch HW (1997) *Biochemical Engineering* (CRC Press, Boca Raton, FL).
27. Lucchetta EM, Lee JH, Fu LA, Patel NH, Ismagilov RF (2005) Dynamics of *Drosophila* embryonic patterning network perturbed in space and time using microfluidics. *Nature* 434(7037):1134–1138.
28. Thomson JA, Schurtenberger P, Thurston GM, Benedek GB (1987) Binary liquid phase separation and critical phenomena in a protein/water solution. *Proc Natl Acad Sci USA* 84(20):7079–7083.
29. Molliex A, et al. (2015) Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. *Cell* 163(1):123–133.
30. Miller G, et al. (2001) hRRN3 is essential in the SL1-mediated recruitment of RNA Polymerase I to rRNA gene promoters. *EMBO J* 20(6):1373–1382.
31. Sanij E, et al. (2008) UBF levels determine the number of active ribosomal RNA genes in mammals. *J Cell Biol* 183(7):1259–1274.
32. Prausnitz JM, Lichtenthaler RN, de Azevedo EG (1998) *Molecular Thermodynamics of Fluid-Phase Equilibria* (Prentice-Hall PTR, Upper Saddle River, NJ).
33. Veatch SL, Keller SL (2005) Miscibility phase diagrams of giant vesicles containing sphingomyelin. *Phys Rev Lett* 94(14):148101.
34. Quiroz FG, Chilkoti A (2015) Sequence heuristics to encode phase behaviour in intrinsically disordered protein polymers. *Nat Mater* 14(11):1164–1171.
35. Jiang H, et al. (2015) Phase transition of spindle-associated protein regulate spindle apparatus assembly. *Cell* 163(1):108–122.
36. Muller HJ (1943) A stable double X chromosome. *Drosoph Inf Serv* 17:61–62.
37. Ritossa FM, Spiegelman S (1965) Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 53(4):737–745.
38. Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157(1):105–132.
39. Scheer U, Rose KM (1984) Localization of RNA polymerase I in interphase cells and mitotic chromosomes by light and electron microscopic immunocytochemistry. *Proc Natl Acad Sci USA* 81(5):1431–1435.
40. Yang Y, et al. (2000) Conserved composition of mammalian box H/ACA and box C/D small nucleolar ribonucleoprotein particles and their interaction with the common factor Nopp140. *Mol Biol Cell* 11(2):567–577.
41. Wang C, Query CC, Meier UT (2002) Immunopurified small nucleolar ribonucleoprotein particles pseudouridylate rRNA independently of their association with phosphorylated Nopp140. *Mol Cell Biol* 22(24):8457–8466.
42. Chen H-K, Pai C-Y, Huang J-Y, Yeh N-H (1999) Human Nopp140, which interacts with RNA polymerase I: Implications for rRNA gene transcription and nucleolar structural organization. *Mol Cell Biol* 19(12):8536–8546.
43. Meier UT, Blobel G (1992) Nopp140 shuttles on tracks between nucleolus and cytoplasm. *Cell* 70(1):127–138.
44. Tollervey D, Lehtonen H, Jansen R, Kern H, Hurt EC (1993) Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation, and ribosome assembly. *Cell* 72(3):443–457.
45. Ochs RL, Lischwe MA, Spohn WH, Busch H (1985) Fibrillarin: A new protein of the nucleolus identified by autoimmune sera. *Biol Cell* 54(2):123–133.
46. Ginisty H, Sicard H, Roger B, Bouvet P (1999) Structure and functions of nucleolin. *J Cell Sci* 112(Pt 6):761–772.
47. Ginisty H, Amalric F, Bouvet P (1998) Nucleolin functions in the first step of ribosomal RNA processing. *EMBO J* 17(5):1476–1486.
48. Perrin L, et al. (1998) Dynamics of the sub-nuclear distribution of Modulo and the regulation of position-effect variegation by nucleolus in *Drosophila*. *J Cell Sci* 111(Pt 18):2753–2761.
49. Perrin L, Benassayag C, Morello D, Pradel J, Montagne J (2003) Modulo is a target of Myc selectively required for growth of proliferative cells in *Drosophila*. *Mech Dev* 120(6):645–655.
50. Biggiogera M, et al. (1990) Nucleolar distribution of proteins B23 and nucleolin in mouse preimplantation embryos as visualized by immunoelectron microscopy. *Development* 110(4):1263–1270.
51. Zaffran S, et al. (1998) A Drosophila RNA helicase gene, pitchoune, is required for cell growth and proliferation and is a potential target of d-Myc. *Development* 125(18):3571–3584.
52. Romanova L, et al. (2009) Critical role of nucleostemin in pre-rRNA processing. *J Biol Chem* 284(8):4968–4977.
53. Ma H, Pederson T (2007) Depletion of the nucleolar protein nucleostemin causes G1 cell cycle arrest via the p53 pathway. *Mol Biol Cell* 18(7):2630–2635.
54. Knibiehler B, Mirre C, Rosset R (1982) Nucleolar organizer structure and activity in a nucleolus without fibrillar centres: The nucleolus in an established *Drosophila* cell line. *J Cell Sci* 57:351–364.