Translation Repressors, an RNA Helicase, and Developmental Cues Control RNP Phase Transitions during Early Development

Arnaud Hubstenberger,1 Scott L. Noble,1,2 Cristina Cameron,1 and Thomas C. Evans1,*

1Department of Cell and Developmental Biology
2Graduate Program in Molecular Biology
University of Colorado Anschutz Medical Campus, Aurora, CO 80045, USA
*Correspondence: tom.evans@ucdenver.edu
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SUMMARY

Like membranous organelles, large-scale coassembly of macromolecules can organize functions in cells. Ribonucleoproteins (RNPs) can form liquid or solid aggregates, but control and consequences of these RNP states in living, developing tissue are poorly understood. Here, we show that regulated RNP factor interactions drive transitions among diffuse, semiliquid, or solid states to modulate RNP sorting and exchange in the Caenorhabditis elegans oocyte cytoplasm. Translation repressors induce an intrinsic capacity of RNP components to coassemble into either large semiliquids or solid lattices, whereas a conserved RNA helicase prevents polymerization into nondynamic solids. Developmental cues dramatically alter both fluidity and sorting within large RNP assemblies, inducing a transition from RNP segregation in quiescent oocytes to dynamic exchange in the early embryo. Therefore, large-scale organization of gene expression extends to the cytoplasm, where regulation of supramolecular states imparts specific patterns of RNP dynamics.

INTRODUCTION

Living cells organize functions not only by membrane compartmentalization, but also by assembling supramolecular structures within aqueous environments. Small-scale molecular complexes are built by stereospecific interactions, many of which have been defined at angstrom resolution. By contrast, assembly, functions, and regulation of higher order superstructures are poorly understood.

Supramolecular assemblies are emerging as a prominent feature of gene expression pathways. Chromatin is organized into specific domains within the nucleus in a cell-type-specific manner (Bickmore and van Steensel, 2013). Upon transcription, RNAs associate with proteins to form ribonucleoprotein complexes (RNPs), and specific RNPs often coassemble into a remarkable diversity of large RNP granules or domains. In the nucleus, these structures include the nucleolus, Cajal bodies, and a variety of other nuclear RNP particles (Mao et al., 2011). Diverse RNP assemblies are also common in the cytoplasm and include P-bodies (PBs), stress granules (SGs), neuronal granules, U-bodies, germ granules, and a variety of PB/SG-related granule types that form in early development (Liu and Gall, 2007; Anderson and Kedersha, 2009; Voronina et al., 2011; Decker and Parker, 2012). Like chromatin, these RNP assemblies are regulated by developmental programs and cell state changes, suggesting important roles in controlling cell fates.

Phase transition theory has recently been used to explain large-scale organization of RNPs and other complexes (Weber and Brangwynne, 2012). Driven by reversible multivalent interactions, RNPs or other molecules can transition among diffuse, liquid, or solid states (Figure 1D). Reconstituted in vitro experiments revealed that various RNA-binding proteins can undergo liquid-liquid or liquid-solid demixing to form dynamic hydrogels (Han et al., 2012; Kato et al., 2012; Li et al., 2012). Liquid-like condensation is strongly supported for germ granules in Caenorhabditis elegans embryos and for nucleoli in Xenopus oocytes (Brangwynne et al., 2009, 2011). However, RNPs can also polymerize into solid structures. Prion-like domains are relatively common in RNP factors, and some may induce stable aggregation (Alberti et al., 2009; Si et al., 2010; Heinrich and Lindquist, 2011). Solid RNP aggregates are often associated with neurological disorders (King et al., 2012). These findings suggest that many RNPs can assemble into a variety of supramolecular states, which might be carefully controlled to promote specific functions. This hypothesis predicts that different native RNP assemblies have variable dynamics that are modulated by RNP regulators and cellular control pathways.

In this study, we find that precise regulation of RNP phase transitions leads to dramatic control of supramolecular states in vivo during early C. elegans development. Repressors of mRNA translation induce competence of specific conserved RNP components to coassemble into large viscoelastic semiliquids. The DEAD-box RNA helicase CGH-1/RCK/DDX6 controls repressor-modified RNP components in part to prevent phase transition to nondynamic solids. Developmental cues modulate these pathways to induce or repress semiliquid assembly and to control both demixing specificity and dynamics. Changes in RNP assemblies allow shifts from segregation in arrested oocytes to dynamic exchange, transformation, and localization during active early development.
RESULTS

Cytoplasmic RNPs control a progression of germ cell to early embryo development (Lasko, 2009). In *C. elegans*, several different RNP granule types assemble during this program (Figure 1A) (Schisa et al., 2001; Boag et al., 2005, 2008; Gallo et al., 2008; Jud et al., 2008; Noble et al., 2008; Voronina et al., 2011). All of these assemblies have similarities to PBs and SGs of other cells and share some components with each other. However, all differ in composition, morphology, and dynamics. Germ granules (P granules) carry germline-specific factors like PGL-1 and shift from nucleus-bound to cytoplasm during oogenesis (Figures 1A and 1B) (Updike and Strome, 2010). Distinct large RNP assemblies (here called grP-bodies or grPBs) form in arrested oocyte cytoplasm, where they recruit repressed mRNAs but exclude translationally activated mRNAs (Figures 1A and 1B) (Schisa et al., 2001; Noble et al., 2008). These grPBs contain several PB proteins and mRNA control factors including the Lsm protein CAR-1 (RAP55 in human, Trailer Hitch in fruit fly), the DEAD box RNA helicase CGH-1 (RCK/DDX6 in human, Dhh1p in yeast, Me31b in fruit fly), and mRNA-binding translational repressors such as PUF-5 and MEX-3 (Boag et al., 2008; Jud et al., 2008; Noble et al., 2008). Germ granules remain distinct from grPBs through midstages of oogenesis but merge with grPBs as oocytes differentiate (Figures 1A and 1B) (Noble et al., 2008). Remarkably, the assembly of large oocyte grPBs is inhibited by sperm signals, which cause RNPs to disperse (Schisa et al., 2001; Jud et al., 2008; Noble et al., 2008). During embryogenesis, RNP factors form PB-like assemblies distinct from grPBs (Figures 1A and 1C) (Audhya et al., 2005; Boag et al., 2005, 2008; Lall et al., 2005; Squirrell et al., 2006; Gallo et al., 2008; Noble et al., 2008). These observations suggest specific control of large-scale RNP coassembly during early development.

To investigate RNP coassembly, we examined dynamics of GFP and RFP protein fusions in gonads of living nematodes. In live animals, GFP:CAR-1 and PGL-1:RFP assembled into RNP granules in patterns indistinguishable from endogenous proteins, and fusions did not alter abundance, sizes, or transformations of RNP assemblies (Figures 1B, 1C, and 1E–1G; Supplemental Experimental Procedures available online). In gonads activated by sperm, GFP:CAR-1 labeled small (<250 nm) and diffusely distributed particles in all oocytes (Figure 1E). Thus, “active oocytes” with small RNPs include all oocytes in diakinesis of meiotic prophase (analyzed here), and not just the last oocyte undergoing meiotic maturation (excluded from analysis) (McCarter et al., 1999). In arrested oocytes of live animals lacking sperm, GFP:CAR-1 condensed into large grPBs of up
to 10 μm (Figure 1F), similar to endogenous CAR-1 in fixed gonads (Jud et al., 2008; Noble et al., 2008). However, live arrested oocytes revealed grPBs with smooth spherical surfaces, subcompartments, and protrusions, suggestive of a liquid-like state (Figure 1F). GFP:CCF-1 also localized to grPBs in live arrested oocytes, suggesting that this RNA deadenylase enzyme coassembles with CAR-1 and other grPB factors (Figure 4C). Interestingly, after loss of the RNA helicase CGH-1, GFP:CAR-1 accumulated into large sheet-like granules (Figure 1G), very similar to structures seen in fixed cgh-1(lf) gonads (Audhya et al., 2005; Boag et al., 2008; Noble et al., 2008). These results suggest that oocyte grPB RNPs may undergo regulated phase transitions from diffuse to different condensed states that can be either liquid-like or solid-like (Figure 1D).

**Small RNP Particles of Activated Oocytes Distribute Broadly by Regulated Diffusion**

RNP distribution in sperm-activated oocytes is suggestive of a decondensed “freely mixing” state, in which soluble RNP complexes diffuse to occupy available cytoplasmic space (Figures 1D and 1E). To test this, we measured fluorescence recovery after photobleaching (FRAP) of GFP:CAR-1 (Figures 2A and 2B). As predicted, recovery was total suggesting that most, if not all, GFP:CAR-1 is mobile (Figure 2B). Surprisingly however, GFP:CAR-1 FRAP rates were biphasic and ~50-fold slower than untagged GFP (Figures 2B–2D). GFP:CCF-1 FRAP rates were similar to CAR-1 supporting that these rates reflect mobility of RNP complexes (Figures 2B and 2D). However, these FRAP rates may not result from free diffusion alone. Observed RNP sizes (<250 nm) were far smaller than those predicted from simple diffusion (~3 μm), suggesting that reversible interactions or exclusion effects may suppress RNP mobility (Supplemental Experimental Procedures). Two recovery rates likely reflect heterogeneity of RNP complexes or other interacting components. FRAP occurred progressively from the periphery toward the center of bleach zones, at rates dependent on bleach area size (Figure 2E). These dynamics are consistent with a diffusion-coupled mobility, in which interactions or exclusion effects delay effective diffusion (Luby-Phelps et al., 1986; Sprague and McNally, 2005). As predicted for a diffuse state, GFP:CAR-1 intensities were mostly constant throughout the oocyte cytoplasm, with slight concentration in the cortex (Figure 2F). Therefore, small RNPs of activated oocytes retain access with the cytosol due to a diffusive noncondensed or “soluble” state. This “soluble state” could consist of individual RNP complexes in a “gas-like” distribution, small multimeric RNP coassemblies or more likely a combination.

**Oocyte Arrest Induces a Phase Transition to Viscoelastic Droplets that Segregate RNPs**

In gonads lacking sperm, GFP:CAR-1 and other RNP factors concentrate into large grPB assemblies (Figures 1F and 3A). RNP superassembly could result from a phase transition, where RNPs condense into liquid grPB droplets by a demixing process, as for germ granules (Figure 1D) (Brangwynne et al., 2009). Alternatively, grPB factors could assemble into solid polymers, similar to viral particles or ribosomes. Liquid-liquid demixing typically produces a system of coalescing droplets that lead to a scale-independent, broad size distribution (Weitz and Lin, 1986; Takayasu et al., 1988). Indeed, within each gonad, the proportion (P) of grPBs of various volumes (V) was consistent with an inverse “power-law” relationship (P ∝ V^−1) that was scale
Figure 3. RNPs Segregate into Viscoelastic, Semiliquid Droplets in Arrested Oocytes

(A) Most grPBs are quasistatic in arrested oocytes of live worms. False colored z stack projections of approximately three to five oocytes are shown at t = 0 (green), t = 2 hr (red), and with two images merged.

(B) Size distribution of grPBs support liquid-liquid demixing and coalescence. Green line is theoretical distribution for pure liquid condensation (slope of $-1.5$) (Brangwynne et al., 2011). Red line fits observed distribution, with a slope of $-1$ suggesting bias toward large grPBs.

(C) grPBs (>1 $\mu$m) tended toward a spherical aspect ratio (AR) of 1, as predicted for liquids.

(D and E) After gonad extrusion, grPBs fused and relaxed to spherical shapes like liquids, but with high viscosity. Plots of large (D) and small (E) fusing droplet AR over time yielded single exponential relaxation to spheres.
Control of RNP Phase Transitions

and rate analysis showed that recovery progressed from the periphery to the center with single-phase kinetics (Figures 3K and 3L). This behavior indicates diffusion-like mobility within grPBs, consistent with a liquid-like state. However, mobilities were 20–50 times slower than in activated oocytes, and at least an order of magnitude slower than for all other cytoplasmic RNP granules examined thus far (Figure 3N) (Kedersha et al., 2000, 2005; Andrei et al., 2005; Leung et al., 2006; Aizer et al., 2008; Chalupnikóva et al., 2008; Mollet et al., 2008; Zhang et al., 2011). We estimated viscosity from FRAP, which roughly and independently agreed with fusion relaxation kinetics (~10^2–10^3 Pa × s, Supplemental Experimental Procedures). Slowed mobility was not due to imaging conditions since arrested and sperm-activated nematodes were identically treated. Mobility was also not influenced by anesthetics used for most experiments and did not decrease with animal age (Figure S1). Taken together with the viscous and elastic nature of grPB droplets, these results show that large-scale RNP interactions greatly suppress RNP exchange, with dynamic reversibility that maintains some RNP mobility. Furthermore, FRAP, fusion kinetics, and elastic behavior indicate that the Lsm protein CAR-1/RAP55 and the deadenylase CCF-1 are components of a dynamically polymerizing/dem polymerizing matrix within these RNP assemblies.

Large grPBs with slow dynamics suggest they effectively inhibit movements of RNPs between grPBs and the cytosol. To test this, we bleached whole granules of ~2 μm (Figure 3J). Whole granule FRAP rates were approximately three times slower than same-sized areas within larger grPBs (Figures 3K and 3N). In addition, whole granule recovery was spatially uniform over time (Figure 3M). Therefore, GFP:CAR-1 exchange at the grPB-cytosol interface is rate limiting. If so, large granules with smaller surface to volume ratios should have very slow turn over. Indeed, granules >4 μm failed to recover cytosolic GFP:CAR-1 within 1 hr supporting this prediction (Figure 3J). Moreover, GFP:CAR-1 and other factors are highly concentrated in grPBs; GFP:CAR-1 was almost undetectable in cytosol, and similar localization was seen for several other grPB factors in fixed gonads, including PUF-5, CGH-1, and some repressed mRNAs (Figures 5M–5O) (Noble et al., 2008). Therefore, the macroscopic properties of grPBs influence individual RNPs: dynamic polymeric interactions within large viscoelastic droplets sharply segregate some RNP components from the cytosol. For largest grPBs (up to 10 μm), an RNP component in their center

(F) Length scales (L[μm]) of fusions against relaxation time (τ[μs]) yielded capillary velocity (slope), which is surface tension over viscosity (γ/η=0.008 μm/s) supporting a high viscosity similar to thick peanut butter (~10^3 Pa × s).
(G) After moderate pressure stress, grPB AR relaxed in seconds suggesting grPBs are elastic.
(H) Elastic recovery was far faster than fusion relaxation, supporting elasticity of transient polymeric state.
(I) Higher stresses induced stretch to long thin grPBs that failed to recover, consistent with semisolid character.
(J) Higher stresses induced stretch to long thin grPBs that failed to recover, consistent with semisolid character.
(K) GFP:CAR-1 inside grPBs has slow random mobility, and limited exchange with cytosol. (J) Time-lapse image of GFP:CAR-1 before and after photo-bleaching of 2 μm zones with white small grPBs (red arrow) or within large grPBs (light arrow). (K) GFP:CAR-1 FRAP was assayed within large grPBs (intra-grPB, blue; n = 7) normalized to whole grPB. GFP:CAR-1 exchange from cytosol to small 1–2 μm grPBs (grPB-cyo 2 μm, red, see red arrow in A) or cytosol to large (grPB-cyo 5 μm, green) grPBs was determined by whole grPB FRAP normalized to unbleached grPBs in imaged field. Single exponentials fit FRAP curves. (L) Kymograph shows spatiotemporal GFP:CAR-1 movements from grPB periphery to center indicating diffusion-coupled mobility. (M) Kymograph of whole small grPB FRAP revealed homogenous recovery suggesting cytosol to grPB mobility is rate limiting. (N) FRAP half-times (t1/2) revealed strong inhibition of RNP mobility upon oocyte arrest. GFP:CCF-1 and GFP:CAR-1 dynamics were similar, independently supporting mobility of native RNPs.
(O) In arrested oocytes, grPBs are enriched at oocyte cortex (Compare with Figure 2F).
Whisker plots (H) show minimum to maximum values (whiskers), and quartiles (boxes). Graphs show mean ± SEM (K), or 95% confidence interval (N and O). Scale bars, 5 μm (A and J); 1 μm (D, E, G, and I). See also Figure S1.
will rarely enter the cytosol. Thus, the bias toward large assemblies favors segregation in arrested oocytes.

Several observations suggest that grPBs have additional higher order organization by external cytoplasmic elements. First, grPBs were largely immobile in intact animals (Figure 3A). Second, grPBs were significantly enriched in the arrested oocyte cortex (Figure 3O). Third, grPBs had relatively regular spacing within the oocyte cortical regions, in contrast to square sheet particles of cgh-1(If) worms (Figure 4F, see below). These results suggest that grPBs are specifically tethered to cortical elements, which further supports a role in segregation of specific RNPs from other cytosolic activities.

The DEAD Box RNA Helicase CGH-1/RCK Prevents RNP Factor Polymerization to a Structured Solid State

The induction of sheet structures after CGH-1 loss suggests that this conserved DEAD box RNA helicase modulates granule fluidity or organization (Figures 1G and 4A) (Audhya et al., 2005; Boag et al., 2008; Noble et al., 2008). We therefore investigated GFP:CAR-1 dynamics after cgh-1(RNAi) or in a cgh-1(tn691) mutant (both referred to as cgh-1(If)). Sheet formation occurred both in the presence and absence of sperm (Figure 4B). Remarkably, cgh-1(If) sheets had precise geometric features: almost all sheets were nearly perfect thin squares, with mean length/width ratio of 0.99, cornicles with 90.3° angles, and depths of <300 nm (Figures 4A–4D). Sheet size varied up to ~10 µm, but all sheets retained a square geometry, suggesting regular geometrically constrained growth (Figures 4D and 4E). In addition, some GFP:CCF-1 also localized to the square sheets (Figure 4C). At least some specific mRNAs also associate with cgh-1(If) sheets in fixed gonads (Noble et al., 2008). By contrast, another grPB factor, GFP:PATR-1 (see below) became immobile sheets (Figures 4A–4D). Collectively, these results suggest that CGH-1 loss directly or indirectly induces transformations of a subset of RNP factors into regular polymeric arrays that grow by highly ordered component assembly. These results suggest that grPBs, cgh-1(If) sheets were resistant to mechanical stress, as expected for relatively strong interactions within a solid (Figure 4). Moreover, after photobleaching one-half of individual sheets, ~75% of GFP:CAR-1 failed to recover (>1 hr), whereas the remaining ~25% recovered with rapid kinetics (t1/2 = 58 s) and a spatiotemporal pattern suggesting recovery from cytosol (Figures 4G and 4H). We conclude that an immobile pool of CAR-1 resides within stable solid sheets, whereas a second pool rapidly exchanges with the solid surface. Cytosolic levels of GFP:CAR-1 were higher in cgh-1(RNAi) arrested oocytes than in controls, and GFP:CAR-1 mobility within the cytosol was similar to the fastest pool in activated oocytes (t1/2 = 40 s) (Figure 4G). Collectively, these results suggest that this RNA helicase inhibits stable polymerization of CAR-1 or a CAR-1 complex into a solid state and controls grPB composition, either as a consequence or independently of polymerization. Therefore, CAR-1 or an RNP factor complex has the intrinsic capacity to polymerize into nondynamic and ordered solid square lattices. This capacity could contribute to coassembly of dynamic grPB semiliquids in wild-type oocytes.

Translational Repressors Promote RNP Phase Transitions that Are Regulated by the CGH-1 Helicase

During oogenesis, many mRNAs are translationally repressed and selectively coassemble with their regulators in grPB droplets (Noble et al., 2008). Therefore, repressed mRNPs may influence grPB condensation. PUF-5 and PUF-6/7 bind specific 3’ untranslated mRNA elements and redundantly repress a set of mRNAs during late oogenesis (Lublin and Evans, 2007; Stumpf et al., 2008; Hubstenberger et al., 2012). We therefore asked if PUF-5/6/7 influences grPB dynamics. Surprisingly, loss of PUF-5 alone strongly inhibited condensation and activated mobility of GFP:CAR-1 (Figures 5A–5F and 5J–5L). We previously found that PUF-5 depletion alone also caused derepression of several mRNAs, but most oocytes formed and arrested (due to PUF-6/7 redundancy) indicating that grPB disruption is not due to defective oocyte arrest (Hubstenberger et al., 2012). Loss of grPB assembly was specific to late oogenesis, where PUF-5 is expressed and functions and was not seen in distal gonads, indicating that grPB dynamics depend on PUF-5 protein and not put-5 mRNA per se (data not shown, see Figure 5S). In fixed arrested oocytes, depletion of PUF-5 and PUF-6/7 disrupted coassembly of gfp-1 mRNA and CGH-1 in grPBs, showing that PUF-5/6/7 are required for coassembly of multiple grPB components (Figures 5M–5R). GFP:CAR-1 FRAP rates in put-5(RNAi) arrested oocytes were 100 times faster than control RNAi (Figures 5J and 5L). Even in activated oocytes, PUF-5 loss induced a significant mobility increase of GFP:CAR-1, suggesting that PUF-5 influences CAR-1 RNPs in the diffuse state (Figures 5K and 5L). Therefore, PUF-5 is required for suppression of RNP dynamics, and for grPB coassembly. To test if other mRNA repressors controlled grPB dynamics, we also knocked down PUF-3/11 proteins. PUF-3/11 proteins bind different 3’ UTR elements to repress a distinct subset of mRNAs during late oogenesis (Stumpf et al., 2008; Hubstenberger et al., 2012). Loss of PUF-3/11 significantly reduced grPB size and increased the fraction of diffuse cytosolic GFP:CAR-1 (Figures 5G–5I). Together, these results show that grPB assembly, growth, and dynamics depend on mRNA repressors, consistent with a model where repressed mRNP states or repression of specific mRNAs are essential for condensation.

The apparently opposing functions of PUF proteins and CGH-1/RCK in controlling CAR-1 assembly and mobility prompted us to ask how these activities may interact. Remarkably, whereas cgh-1(tn691) mutants produced only large square sheets, loss of PUF-5 in cgh-1(tn691) animals prevented square sheet formation and instead produced very small GFP:CAR-1 puncta (Figures 5S and 5T). Similarly, PUF-3/11 depletion in cgh-1(tn691) worms transformed square sheets to small, rounded assemblies (Figures 5S and 5T). Furthermore, sheet transformations were specific to oogenesis and later oocytes, where both PUFs function, as sheets still formed in the distal gonad (Figure 5S). Collectively, these findings suggest that these PUFs are required for RNP competency to coassemble into either semiliquid droplets or solid polymers; the CGH-1 RNA helicase could act on repressor-altered RNPs in part to prevent a nondynamic solid state. If so, other RNA-binding repressors may have similar interactions with CGH-1. In support of this prediction, we found that loss of the KH domain repressor...
GLD-1 also suppressed square sheet formation in earlier stages of cgh-1(lf) gonads, where GLD-1 functions (Figure 5U) (Jones and Schedl, 1995). Therefore, divergent translation repressors induce the capacity of RNP factors to assemble into either liquid or solid supramolecular phases, the properties of which depend directly or indirectly on the CGH-1 RNA helicase.
Figure 5. Translation Repressors Induce RNP Factor Competence for Semiliquid or Solid Assembly, which Is Regulated by the CGH-1/RCK Helicase

(A–I) Depletion of PUF translation repressors inhibited grPB condensation. Control RNAi (mock(RNAi)) did not alter GFP:CAR-1 (A) and PGL-1:RFP (B) assembly in large grPBs of arrested oocytes (A–C). Following PUF-5 RNAi, GFP:CAR-1 and PGL-1:RFP were diffuse or in small particles (D–F). Following PUF-3 depletion, GFP:CAR-1 particles (G) became small, whereas PGL:RFP (H) remained in distinct small aggregates.

(J–L) PUF-5 depletion strongly increased RNP mobility rates. (J and K) FRAP of 2 μm zones are shown for mock(RNAi) (blue) or puf-5(RNAi) (red) in arrested (J) or active (K) oocytes (mock(RNAi) FRAP in (J) was for intra-grPB as in Figure 3K). (L) FRAP half-times (t[1/2]) revealed strong activation of RNP mobility following PUF-5 depletion in arrested oocytes, and significant mobility increase in active oocytes in each of two half-times, with the fast pool showing a strong increase (n = 6–24).

(M–R) Fixed arrested gonads were costained for glp-1 mRNA and CGH-1 after mock(RNAi) or puf-5/6/7(RNAi).

(S and T) Depletion of PUF-5 or PUF-3/11 in cgh-1(tn691) animals block solid square sheet induction. Whole gonad sections (S) show disruption of sheets was specific to late oogenesis. Oocyte sections (T) show small rounded puncta in puf-5(RNAi);cgh-1(tn691) and puf-3/11(RNAi);cgh-1(tn691) gonads.

(U) Depletion of the GLD-1 repressor impairs square sheet formation in distal gonad of cgh-1(tn691) animals (epifluorescence image).

Error bars are SEM (J and K) or 95% confidence intervals (L). Scale bars, 5 μm (A–I and M–U).
Embryogenesis Modulates RNP Droplet Dynamics and Demixing Specificity

During development, RNP factors rearrange into different granule types (Figures 1A–1C). Control of RNP coassembly dynamics may underlie these transformations. To test this idea, we analyzed various grPB factors and the germ granule factor PGL-1 in live animals. As oogonia formed, PGL-1 particles detached from nuclei into the cytosol and eventually merged with grPBs in arrested oocytes (Figures 1B and 6D–6I). Strikingly however, PGL-1 within grPBs remained in distinct puncta that did not overlap with a large surrounding CAR-1 domain (compare D to A and G). Spherical subdomains were depleted of RNP components (blue arrowheads). GFP:CAR-1 exchange between granules and cytosol is ~100 times faster for embryonic germ granules than for similar size grPBs (~2 μm).

GFP:CAR-1 and SYTO-60 surrounded numerous spherical regions devoid of grPB factors (Figures 1F and 6A–6I). Therefore, specific RNP components remain segregated within several grPB subdomains in live animals, revealing clear molecular specificity in substructure that was previously suggested by imaging of fixed gonads (Jud et al., 2008; Noble et al., 2008; Patterson et al., 2011). RNP condensation into “disordered” grPB assemblies is nonetheless driven by molecularly specific interactions, which can prevent mixing of different condensates even within a common coassembly.

After sperm-driven activation and fertilization, grPBs disassemble and RNP factors reorganize into coassemblies in the embryo (Figures 1A and 1C) (Boag et al., 2008; Gallo et al., 2008). Embryonic germ granules continually localize to posterior cytoplasm at each cell division and appear to be more liquid than oocyte grPBs (Figure 1C) (Brangwynne et al., 2009; Updike and Strome, 2010). We therefore directly tested whether dynamics of
a single component (CAR-1) that is shared by both assemblies changes after the oocyte to embryo transition. Strikingly, GFP:CAR-1 exchange between embryonic germ granules and cytosol was two orders of magnitude faster than grPB-cytosol exchange in arrested oocytes (Figure 6P). GFP:CAR-1 FRAP rates in embryonic germ granules were similar to previously reported PGL-1 diffusion rates (Brangwynne et al., 2009). Thus, FRAP rates and droplet fusion kinetics independently demonstrate that embryonic germ granules are far more fluid (~100-fold) than grPBs (Figures 3D–3F, 3J–3N, and 6P) (Brangwynne et al., 2009). In addition, the arrested oocyte to embryo transition induced large shifts in CAR-1 distribution: GFP:CAR-1 was ~10-fold concentrated in embryonic germ granules over cytosol, compared to >50-fold concentration in oocyte grPBs (Figure 6O). Moreover, embryogenesis triggered alterations in demixing specificity and granule organization. Although GFP:CAR-1 and PGL-1:RFP were segregated within grPBs in arrested oocytes, they tightly overlapped within germ granule domains in embryonic germ blastomeres (compare Figure 6M with 6N). Therefore, phase transitions of CAR-1 complexes are modulated in two ways upon transition from arrested oocyte to early embryo. First, CAR-1 dynamics within and from RNP assemblies are activated, leading to far more extensive droplet-cytosol exchange. Second, RNP modifications allow mixing of components that are segregated in oocyte grPBs.

**DISCUSSION**

This study reveals that RNP phase transitions are controlled with surprising precision in early development, leading to starkly different supramolecular states that alter RNP organization and dynamics. In the cytoplasm, a subset of RNP components can transition among diffuse, semiliquid, or solid polymer forms. RNP assemblies transform from viscoelastic semiliquids with subdomains to far more dynamic liquid droplets during early development. Therefore, reversible interactions among thousands of RNP complexes impart regulated patterns of RNP dynamics, and large-scale organization of gene expression pathways in the cytoplasm.

Alterations in RNP states mirror shifts in RNP regulation that are critical to early development. In arrested oocytes, high viscosity of RNP droplets strongly inhibits access of repressed mRNPs to the cytosol compartment where translation is known to be active (Noble et al., 2008). Because tightly regulated mRNAs are repressed in both active and arrested oocytes, grPB condensation is not essential for repression. However the sharp segregation of RNP factors likely plays a reinforcing, redundant, or modulatory role. Inducers (PUFs and CAR-1) and modulators (CGH-1) of assembly promote mRNA repression and stability, consistent with links among these processes (Lublin and Evans, 2007; Boag et al., 2008; Noble et al., 2008; Hubstenberger et al., 2012). In activated oocytes, RNP droplet dissolution and mobilization may be important to promote rapid changes in RNP organization after fertilization. In the embryo, far more liquid granules favor continuous RNP localization and remodeling that are tied to new patterns of mRNA activity important to cell specification.

Collectively, these results suggest that mRNP modification pathways control an underlying capacity of specific RNP components to polymerize, thereby driving or suppressing phase transition to semiliquid granules, and preventing assembly of nondynamic solids (Figure 7A). In one model, RNA-binding proteins induce repressed mRNP states (or RNP factor states) that are then competent for coassembly, and the CGH-1/RCK RNA helicase could modulate assembly-competent RNPs in part to prevent transition to solid polymers and maintain semiliquid
subdomains. Alternatively, RNA-binding repressors could act indirectly by repressing mRNAs for negative regulators of assembly. Because several different repressors promote coassembly, a simpler view is that they transform various RNPs to an assembly competent state. RNA helicases like CGH-1 remodel RNP structures, which could directly stimulate superstructure dynamics or indirectly maintain RNP components essential for dynamics (Fairman et al., 2004; Ernoult-Lange et al., 2012; Hilliker, 2012). Regardless, these findings support the idea that RNP remodeling is a prerequisite for RNP supramolecular coassembly and determines biophysical properties of these structures in vivo.

Regulated RNP phase transitions appear to be prevalent in the nucleus and cytoplasm of diverse eukaryotes and many cell types, from oocytes to brain neurons. Several common themes are emerging. Although cytoplasmic RNP particles vary in their properties, many specifically recruit repressed mRNPs and several shared protein types, including CAR-1 and CGH-1 homologs (Anderson and Kedersha, 2009; Decker and Parker, 2012). Thus, related mechanisms of RNP transformation may be a common requirement for supramolecular condensation and control. To induce coassembly, some RNP components must have multivalent interaction potential, which is known to drive liquid–liquid demixing in vitro (Han et al., 2012; Kato et al., 2012; Li et al., 2012). These could be RNAs with their multiple sites for binding factors. Multivalent factors could also be proteins; PGL proteins may promote germ granule condensation through multivalent interactions, and many RNP proteins have low complexity or prion-like sequences that can induce sol–gel or liquid–liquid demixing phenomena (Gilks et al., 2004; Alberti et al., 2009; Hanazawa et al., 2011; Updike et al., 2011; Han et al., 2012; Kato et al., 2012; King et al., 2012). So far, the few RNP assemblies examined in vivo have liquid or semiliquid properties, suggesting that liquid-like states may be typical (this study) (Brangwynne et al., 2009, 2011). However, our studies suggest such states are modulated, which can dramatically slow or stimulate RNP dynamics, and also inhibit or promote mixing of subdomains. ATP-dependent RNA helicases may commonly regulate such RNP dynamics. In addition to CGH-1/DDX6, Vasa-type RNA helicases promote germ granule integrity in several systems, and nucleolus viscosity increases after ATP depletion (Hay et al., 1988; Kawasaki et al., 1998; Kuznicki et al., 2000; Brangwynne et al., 2011; Updike et al., 2011). Together, these findings suggest that many RNP complexes can inherently coassemble into large superstructures by phase transition. Repressors, helicases, and other factors likely modulate these states through control of reversible interaction kinetics. Defects in these pathways could lie at the heart of solid RNP aggregates common to several neurological disorders (King et al., 2012).

**Live Imaging**

Live adult nematodes were anaesthetized in M9 buffer with 2% Tricaine methanesulfonate (Sigma), 0.2% tetramisole hydrochloride (Sigma), mounted on 2% agarose pads with coverslips, and imaged on a Leica TCS SP5 II confocal microscope (using HCX PL APO CS 40x oil objective, 1.25 aperture). Unless stated otherwise, single confocal sections (1 Airy unit pinhole) are shown. For image analysis, ImageJ, and the plugin collection MBF ImageJ for microscopy by Tony Collins, was used (Schneider et al., 2012). The HILO look-up table was used to ensure below saturation fluorescence and full background subtraction. Nematodes with activated (+ sperm) or arrested (− sperm) oogenesis were generated in parallel using gravid hermaphrodites (N2) or virgin fog-2(q71) females, respectively. Unmated fog-2(q71) females only lack sperm and were indistinguishable from sperm-depleted N2 hermaphrodites (Schedl and Kimble, 1988; Jud et al., 2007, 2008; Noble et al., 2008).

To stain RNA in live worms, 100 µl of 0.25 mM SYTO 60 (Invitrogen), 118 mM NaCl, and 48 mM KCl were added on bacterial lawns and fed to nematodes for 15 hr at 20°C. Worms were incubated 15 hr at 20°C, washed, and then mounted on slides in M9 buffer with 30 mM NaN3 or with Tricaine/tetramisole (as above); both conditions revealed tight SYTO 60 colocalization with GFP-CAR-1 (Figures 6A–6C), but Tricaine/tetramisole had higher cytoplasmic staining (data not shown). SYTO 60 labeled nucleoli but not chromosomal nuclear compartments, supporting specificity for RNA (data not shown).

**Fluorescence Recovery after Photobleaching Analysis**

For fluorescence recovery after photobleaching (FRAP), circular zones 2 µm in diameter were photobleached and confocal time series were recorded at minimal laser intensities, using 2 Airy unit pinhole size. Only differentiated oocytes still in meiotic prophase (diakinesis) were imaged (~2 to 3 active oocytes, ~2 to 5 arrested oocytes). Embryos were imaged at 4- to 8-cell stages. For all FRAP series, imaging-induced bleaching was normalized by dividing fluorescence intensities of bleach zones by unbleached fluorescent areas or by fluorescence of entire image field, which gave similar FRAP rates. Normalized FRAP curves were quantified according to FRAP(t) = [I(t) − I(t0)]/[I(t0) − I(t−)], where FRAP(t) is relative fluorescence recovered at time t, I(t) is normalized fluorescence at time t, I(t0) is normalized intensity at time 0, and I(t−) is normalized intensity prior to photobleaching. To determine spatiotemporal FRAP patterns, kymographs were generated by measuring fluorescence across a zone of interest over time, using ImageJ “reslice.” To mitigate movements during imaging, time series were aligned to an image at t=0, using ImageJ, Stacks-Shuffling-MultiStackReg plugin.

**RNP Granule Size Distribution, Shape, and Gonad Organization Analyses**

To analyze RNP granule size distributions, maximum intensity projections of confocal z stacks were generated and late oocyte regions were manually defined. To delimit granules from cytosol and each other, ImageJ “smooth,” “threshold,” “watershed” functions were combined. Automatic particle analysis of granules >1 µm was then shifted to 25°C for 12 hr. cgh-1(tn691ts), animals were grown at 15°C for 36 hr and then shifted to 25°C for 161–173, October 28, 2013 ©2013 Elsevier Inc. 171

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**EXPERIMENTAL PROCEDURES**

**RNAi and Genetic Manipulations**

RNAi was done by the feeding method using nonspecific sequence as a control (mock RNAi), as described (Timmons et al., 2001; Hubstenberger et al., 2012). L4 larvae were grown on test or control RNAi plates at 20°C for 48 hr. To induce square sheet formation in cgh-1(bn691ts), animals were grown at 15°C up to 36 hr post-L4 stage and then shifted to 25°C for 12–20 hr. For RNAi of cgh-1(bn691ts), L4 larvae were grown on RNAi plates at 15°C for 36 hr and then shifted to 25°C for 12 hr.
recorded with 2 Airy unit pinholes, and ARs of pairs of fusing granules were analyzed over time. To impose mechanical stress by pressure over time, dissected worms and extruded gonads were placed in 10 μl dissection buffer between coverslip and slide. Distorted GFP-CAR-1 particle aspect ratios were determined. At early times, elastic responses were detected in oocytes leaking through gonad openings. Higher pressures induced at later times caused grPB extensions passed a tipping point that remained distended.

**In Situ Detection of RNA and Proteins**

Dissected and fixed gonads were simultaneously stained for glp-1 mRNA and CGH-1 protein, by combined fluorescent in situ hybridization (FISH) and immunofluorescence, as described (Noble et al., 2008). Antisense and sense RNA probes derived from glp-1 cDNA were made as described (Barbee and Evans, 2006; Noble et al., 2008); only antisense probes stained grPBs and small RNPs ((Noble et al., 2008; data not shown). Similar results were seen with RNA probes to pos-1 mRNA (data not shown).

**Statistical Analyses and Calculations**

All statistics analyses were conducted using Prism 5 (GraphPad Software). FRAP recovery curves were fitted using the least square method to determine fit to single or 2 phase exponentials as follows, FRAP = P × (1−e−t/τ1), or FRAP = P × [1−f(solution)(1−e−t/τ2)] × [1−s(solution) × 0.01 × e−t/(t/τ3)], respectively, where t is time, P the plateau reached at infinite time, and τ the time constant. Half-times were calculated as t(1/2) = τ × ln(2). To compare best-fit values, we used the extra sum-of-squares F test. Diffusion rates and size estimates were calculated from FRAP and the Stokes-Einstein relationship (calculations in Supplemental Experimental Procedures) (Soomsnes, 1983). Viscosities were estimated by two independent approaches: (1) grPB fusion kinetics and (2) FRAP-based diffusion rates within grPBs, as previously described (calculations in Supplemental Experimental Procedures) (Brangwynne et al., 2009, 2011).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.09.024.

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