RNA granules: functional compartments or incidental condensates?

Andrea Putnam,[1] Laura Thomas, and Geraldine Seydoux

Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, Johns Hopkins University, Baltimore, Maryland 21205, USA

RNA granules are mesoscale assemblies that form in the absence of limiting membranes. RNA granules contain factors for RNA biogenesis and turnover and are often assumed to represent specialized compartments for RNA biochemistry. Recent evidence suggests that RNA granules assemble by phase separation of subsoluble ribonucleoprotein (RNP) complexes that partially demix from the cytoplasm or nucleoplasm. We explore the possibility that some RNA granules are nonessential condensation by-products that arise when RNP complexes exceed their solubility limit as a consequence of cellular activity, stress, or aging. We describe the use of evolutionary and mutational analyses and single-molecule techniques to distinguish functional RNA granules from "incidental condensates."

RNA granules are intracellular RNA–protein assemblies not enclosed by membranes that range in size from ∼100 nm to several micrometers. RNA granules have been observed in animal, fungus, plant, and prokaryotic cells and fall into three general classes: ubiquitous, cell type-specific, and stress-induced. Over 20 types of RNA granules have been described so far, each with a unique composition, comprising in some cases dozens of proteins and thousands of RNAs (Emenecker et al. 2020; Gao et al. 2021; Lacroix and Audas 2022; Hirose et al. 2023; Rostam et al. 2023).

Proteins enriched in RNA granules function in many aspects of RNA metabolism, from transcription and processing in the nucleus to translation and RNA turnover in the cytoplasm. By extension, RNA granules are often assumed to represent functional compartments that house RNA-focused activities that require the granule environment (Hirose et al. 2023). For example, nucleoli assemble around nascent ribosomal RNAs and concentrate ribosomal proteins and ribosome assembly factors, implicating the nucleolus as the main cellular compartment for ribosome biogenesis (Lafontaine et al. 2021). Similarly, many other RNA granules have been assigned putative functions based on composition, including P-bodies as sites of mRNA storage or decay and nuclear speckles as sites of mRNA splicing (Standart and Weil 2018; Faber et al. 2022; Vidya and Duchaine 2022).

Recent findings have linked RNA granule assembly to phase separation of RNA–protein (RNP) complexes (Shin and Brangwynne 2017). Phase separation is a thermodynamic process that causes interacting molecules to "demix" from the cytoplasm or nucleoplasm into dense condensates (Hyman et al. 2014). Unlike compartments delimited by membranes, which require energy to assemble and maintain, condensates form spontaneously under conditions such as high concentration, when components exceed their solubility limit. In this review, we explore the possibility that some RNA granules are condensation by-products that form when subsoluble RNP complexes saturate the cytoplasm or nucleoplasm. We introduce the term "incidental condensates" to refer to condensates that are tolerated by cells but do not add functionality beyond that provided by the soluble pool of saturating RNP complexes. We begin by describing how the biophysical properties of phase-separated condensates provide a strong theoretical framework to describe the dynamics and composition of RNA granules and, at the same time, raise questions as to their potential role as cellular compartments. Next, we review experimental evidence in support of and against functions commonly assigned to RNA granules. Finally, we consider best practices for distinguishing functional RNA granules from incidental condensates. Table 1 summarizes the main themes addressed in this review.

Properties of condensates

What is a condensate?

Banani et al. (2017) first introduced the term "biomolecular condensate" to refer to any mesoscale assembly that

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[1]**Present address: Department of Biomolecular Chemistry, University of Wisconsin-Madison, Madison, WI 53706, USA.**
Corresponding author: gseydoux@jhmi.edu
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Table 1. *Recurrent themes in the biomolecular condensate literature*

Theme	Expanded theme
Membraneless assemblies arise by phase separation.	Condensates arise by phase separation, but not all membraneless assemblies are condensates.
Condensates are liquids.	Condensates are viscoelastic liquids that exhibit a range of dynamics that evolve over time.
Protein–RNA complexes assemble in condensates.	Protein–RNA complexes concentrate in condensates but may be more abundant in the dilute phase.
IDRs mediate low-affinity interactions that drive phase separation.	IDRs participate in a range of interactions, including high-affinity binding in soluble complexes.
Condensates organize cellular biochemistry.	Condensates may be functional or "incidental" condensation by-products of subsoluble complexes.

Listed are common themes in the biomolecular condensate field (left column) expanded to integrate primary literature highlighted in this review (right column). (IDRs) Intrinsically disordered regions.

concentrates biomolecules without the help of a limiting membrane, irrespective of mechanism. In this review, we use the term "condensate" in a narrower sense to refer to the product of condensation or, more precisely, phase separation (Shin and Brangwynne 2017). Phase separation is an emergent property of diffusive molecules that interact with each other in solution. Above a critical concentration (c_{sat}), the sum of favorable intermolecular interactions offsets the entropic cost of demixing, causing interacting molecules to redistribute ("phase separate") into a dense phase (condensate) and a dilute phase (Hyman et al. 2014). Unlike ordered assemblies, where molecules assume specific configurations (e.g., actin in microfilaments), molecules in condensates adopt multiple conformations and binding stoichiometries, exchanging binding partners within the condensate and exchanging with the dilute phase. The time scales of these dynamics can vary greatly, causing the condensates to appear "liquid-like" or "solid-like," although most biological condensates are likely neither simple liquids nor solids but rather viscoelastic fluids (Jawerth et al. 2020; Mittag and Pappu 2022). Condensates do not have a prescribed size and theoretically can grow infinitely if provided unlimited components. Although molecules in the condensate flux in and out, condensates have a sharp inside/outside boundary or interface (Fig. 1A). Molecules inside the condensates experience a chemical and diffusive environment distinct from that experienced by molecules in the surrounding "dilute" phase (cytoplasm or nucleoplasm) (Israelachvili 2011).

It is important to emphasize that not all assemblies visible by microscopy are necessarily phase-separated condensates (McSwiggen et al. 2019b). Reversible binding to a multivalent scaffold, such as DNA or RNA molecules, can colocalize diffusive proteins into dynamic assemblies that superficially resemble condensates (Fig. 1B). Amyloid protein aggregation—and possibly other forms of polymerization where proteins form ordered fibrils that extend in three dimensions—could also in principle generate supramolecular assemblies that resemble condensates (Fig. 1C; Kato et al. 2022). Unlike condensates, however, these types of assemblies do not have a distinct "surface" (interface) separating molecules in the assembly from the surrounding medium and thus do not create true "compartments" (Fig. 1).

In principle, condensates can be distinguished from other assemblies by their unique growth, fusion, and wetting properties, specified by their interfacial and internal dynamics. For example, condensates exhibit switch-like growth and degrowth in response to changes in concentration, fuse upon contact, and wet surfaces such as membranes (Fig. 1D–F), as illustrated by Brangwynne et al. (2009), who demonstrated that the P granules of *Caenorhabditis elegans* are liquid-like condensates. However, these types of analyses are difficult to perform on assemblies that have slow dynamics and/or are diffraction-limited in size. Other reviews have addressed the challenges associated with determining whether RNA granules correspond to condensates, other assembly types, or a mixture of the two (Erdel and Rippe 2018; Alberti et al. 2019; McSwiggen et al. 2019b; Forman-Kay et al. 2022). In the following sections, we describe how the biophysical properties of condensates explain certain aspects of RNA granule dynamics in cells and, at the same time, complicate function determination.

Phase separation is a spontaneous, concentration-dependent process

Phase separation is driven by associative ("binding") interactions and segregative ("repulsive") interactions that cause molecules to sort into distinct phases above c_{sat}. The landmark study of Li et al. (2012) showed that sequence-specific protein-binding domains can drive phase separation when present in multiple copies (multivalency) capable of generating large networks of interacting molecules. Long, flexible RNA molecules are ideal multivalent scaffolds for RNA-binding proteins and essential components of several types of RNA granules (Decker et al. 2022). Condensation is also enhanced by sequence-nonspecific interactions, involving protein–protein, protein–RNA, and possibly also RNA–RNA interactions (Box 1).

Because phase separation is a concentration-dependent equilibrium process, no energy input is required to initiate

Box 1. What types of interactions drive the formation of RNA granules?

Genetic studies have shown that sequence-specific protein-binding domains contribute to RNA granule assembly in cells. For example, oligomerization domains in Edc3, G3BP, and PGL-1 are required to assemble P-bodies, stress granules, and P granules, respectively, likely because these domains mediate the formation of subsoluble RNP complexes (Ling et al. 2008; Hanazawa et al. 2011; Kedersha et al. 2016; Guillén-Boixet et al. 2020; Sanders et al. 2020; Yang et al. 2020). In addition to globular domains, RNA-binding proteins often also contain intrinsically disordered regions (IDRs) that can phase-separate in isolation in vitro. Best studied is the FUS IDR, which is stabilized in condensates by a variety of binding interactions involving most amino acids along the length of the IDR, which remains disordered in the condensates (Murthy et al. 2019; Martin and Holehouse 2020; Peran and Mittag 2020). Whether IDR–IDR interactions drive the phase separation of native RNA granules in cells, however, is less clear. IDRs are rarely sufficient to drive phase separation in cells (unless overexpressed) but can augment the condensation of oligomerizing globular domains by linking condensation to environmental inputs such as RNA availability, pH, oxidation state, and temperature (Riback et al. 2017; Franzmann et al. 2018; Kato et al. 2019; Guillén-Boixet et al. 2020; Iserman et al. 2020; Sanders et al. 2020; Yang et al. 2020; Putnam and Seydoux 2023). For example, the IDR of the P granule protein MEG-3 is not essential for condensation but binds RNA in a sequence-nonspecific manner and is required to recruit low-translation mRNAs to P granules (Lee et al. 2020; Schmidt et al. 2021)

RNA can promote or disrupt protein condensates. In vitro, RNA can lower the c_{sat} of RNA-binding proteins by functioning as a multivalent scaffold or raise c_{sat} by competing with protein–protein interactions (Zhang et al. 2015; Saha et al. 2016; Maharana et al. 2018; Lee et al. 2020; Rhine et al. 2020). Both effects have been observed in cells. Some nuclear proteins, such as nucleolar RNA-binding proteins, condense around point sources of nascent RNAs, likely because binding to colocalized RNAs raises their concentration locally above c_{sat}, allowing them to phase-separate (Berry et al. 2015; Lawrimore et al. 2021). Other RNA-binding proteins, such as FUS, require RNA binding and high RNA concentrations to remain soluble in nuclei (Maharana et al. 2018). In the cytoplasm, the assembly of RNA granules is often stimulated by abundant low-translation transcripts. For example, P-bodies assemble around low-translation deadenylated mRNAs, and P granules and stress granules assemble around low-translation, polyadenylated mRNAs (Tharun and Parker 2001; Hubstenberger et al. 2017; Khong et al. 2017; Lee et al. 2020).

RNA molecules can phase-separate in vitro even in the absence of proteins through non-sequence-specific π–π, hydrogen-bonding, and electrostatic interactions (Nakano et al. 2007; Aumiller et al. 2016; Van Treeck et al. 2018; Onuchic et al. 2019; Bevilacqua et al. 2022; Forman-Kay et al. 2022). These observations have led to the proposal that RNA–RNA interactions contribute to condensation in cells, especially under stress conditions that block translation initiation and release thousands of "naked" mRNAs in the cytoplasm (Van Treeck et al. 2018). mRNAs sort into homotypic clusters inside RNA granules in *Drosophila* embryos (Niepielko et al. 2018; Trcek et al. 2020), and RNA structure influences the material properties of RNA–protein condensates in vitro and in reconstituted systems in tissue culture (Maharana et al. 2018; Ma et al. 2021; Roden and Gladfelter 2021; Decker et al. 2022). Remarkably, some RNAs appear immobile in condensates even when bound by dynamic proteins (Moon et al. 2019; Cabral et al. 2022), indicating that RNAs can assemble static scaffolds inside RNA granules. In summary, RNA molecules have a high propensity for condensation, especially when not engaged in translation, and can play a dominant role in specifying condensate organization and dynamics.

condensation. Changes in the concentration, valency, or binding affinity of protein and/or RNA molecules are sufficient to induce condensation (or dissolution). Consistent with these theoretical predictions, RNA granule assembly in cells has been correlated with changes in the concentration or valency of proteins and RNAs. For example, the polarized condensation and dissolution of P granules coincides temporarily with the formation of concentration gradients across the cytoplasm of the *C. elegans* zygote (Fig. 2; Brangwynne et al. 2009; Wang et al. 2014; Saha et al. 2016; Smith et al. 2016; Folkmann et al. 2021). The assembly of stress granules and P-bodies is linked to translation inhibition and RNA deadenylation, respectively, which increase the pool of RNA molecules available for binding by stress granule and P-body proteins (Chen and Shyu 2013; Bounedjah et al. 2014). Some nuclear condensates, such as nucleoli, condense around point sources of nascent RNAs (Berry et al. 2015; Lawrimore et al. 2021). Condensation is also predicted to be affected by factors that impact the solvation capacity of the cytoplasm or nucleoplasm. Lowering ribosome numbers in yeast and HEK293 cells decreased the condensation of an artificial condensate, which could be rescued partially by osmotic shock, likely due to changes in molecular crowding (Delarue et al. 2018). Although no energy is required to initiate condensation, cells use ATP-consuming mechanisms to counter condensation and enhance the solubility of proteins and RNAs (Box 2).

The 'dark' side of condensation: the dilute phase

A common assumption is that molecules in RNA granules are highly concentrated in the granules and only active in the granule environment. This assumption has led to the widespread hypothesis that RNA granules represent functional compartments, akin to organelles, that house specialized functions not possible in the more dilute environment of the cytoplasm or nucleoplasm (Banani et al. 2017; Fare et al. 2021). However, the realization that many RNA granules likely are condensates that arise by phase separation challenges this view. As described above, phase separation involves partitioning of

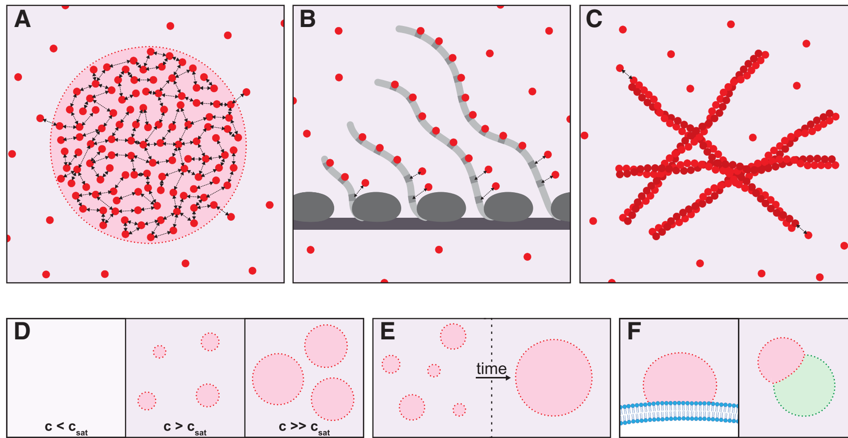

Figure 1. Condensates and other assemblies. (*A*) Condensates arise when diffusive multivalent molecules (red circles) interact reversibly (double arrows) to form a dynamic network. Condensates are defined by an interface (red dotted line), with associated surface tension separating the condensed phase (red) from the dilute phase (purple). The surface tension arises from the energy differential between molecules at the interface (which are pulled into the condensate by their neighbors) and molecules in the interior. The molecules inside the condensate experience a chemical and diffusive environment distinct from the dilute phase. (*B*) A multivalent scaffold (such as nascent RNA molecules) can concentrate proteins (red) that bind to the scaffold (gray). Such an assembly may resemble a condensate by microscopy but does not possess an interface and therefore is not phase-separated. However, this type of assembly could evolve into a condensate if the proteins, in addition to binding to the scaffold, also interact with one another and binding to the scaffold causes the proteins to exceed c_{sat} locally. (*C*) Proteins containing low-complexity, prion-like domains can interact via β-sheet stacking to form extended fibers in multiple dimensions. This type of assembly does not constitute a phase-separated condensate but could arise within a condensate that concentrates proteins with prion-like domains. (*D*–*F*) Properties of condensates. (*D*) Condensates form above c_{sat} (saturation concentration), the maximum concentration allowed in the dilute phase. Above c_{sat}, further increases in concentration cause the condensates to grow larger without any changes to the concentration in the dilute phase, which remains at c_{sat}. However, this theoretical prediction is difficult to apply in vivo, where multiple components contribute to c_{sat}, leading to complex concentration-dependent behaviors (Riback et al. 2020). (*E*) Surface tension drives condensates to minimize surface area, causing them to coarsen over time to create fewer, larger condensates with lower surface:volume ratios. The time scale of coarsening will depend on the material properties of the condensates (less dynamic condensates will coarsen more slowly). Also, agents that adsorb to the interface can reduce surface tension and coarsening. (*F*) Condensates wet surfaces, including membranes (blue) and other condensate types (green) that provide favorable interaction interfaces (Gouveia et al. 2022). For example, P granules wet nuclear membranes, and P-bodies wet stress granules.

molecules between two phases: a dilute phase and a condensed phase. The fraction of molecules in each phase will depend on the partition coefficient and on concentration. At concentrations right above c_{sat}, the highest concentration permitted in the dilute phase, only a small fraction of molecules will populate the condensates. If molecules in the dilute phase are also active (i.e., in RNP complexes), redistribution of activity from the dilute phase to the condensates will be minimal.

Several lines of evidence indicate that phase-separating proteins also assemble complexes in the dilute phase. First, classical biochemistry experiments have defined many protein and protein–RNA complexes that assemble in solution (Musacchio 2022). Binding domains defined by those experiments drive phase separation when multimerized in vitro (e.g., Li et al. 2012) and are required to assemble RNA granules in vivo (Ling et al. 2008; Hanazawa et al. 2011; Kedersha et al. 2016; Guillén-Boixet et al. 2020; Sanders et al. 2020; Yang et al. 2020). These observations suggest that phase separation in cells is intimately linked to the networking potential of multivalent macromolecular complexes, as recently articulated by Mittag and Pappu (2022). Consistent with this view, proteomic analyses in cell lysates have revealed that the connectivity of stress granule proteins does not change following stress granule assembly, suggesting that the RNP complexes that populate stress granules also exist as soluble species in the cytoplasm (Markmiller et al. 2018; Youn et al. 2018). Similarly, certain yeast mutants that lack P-bodies still assemble P-body protein complexes that can be detected by nanoparticle tracking (Rao and Parker 2017). Interestingly, even simple model condensate proteins that self-interact using distributed IDR–IDR interactions (e.g., FUS) form heterogeneous oligomers or "clusters" in solution (Murthy et al. 2019; Zhao et al. 2021; Kar et al. 2022; Seim et al. 2022). The clusters range from a handful to hundreds of molecules, grow larger with increasing concentrations, and are thought to lower c_{sat} by increasing valency and decreasing solubility as a function of size (Kar et al. 2022). The polyQ condensing protein Whi3 of *Ashbya gossypii* also forms soluble oligomers in vitro, although in that context the soluble oligomers appear to compete with condensation (Seim et al. 2022). The emerging view is that RNA granules arise from condensation of RNP complexes that also form in the dilute phase.

How many RNP complexes are left in the dilute phase when condensates form in cells? Quantitative studies addressing this question for native RNA granules are rare (Lyon et al. 2021). A systematic survey in yeast revealed that most P-body proteins are present in higher proportions in the cytoplasm. When P-body proteins are labeled with GFP, P-bodies appear brighter than the cytoplasm—but the total fluorescence intensity in the cytoplasm is actually higher than that in P-bodies—due to modest enrichment in P-bodies and the small fraction of total cell volume occupied by P-bodies (Xing et al. 2020). Similarly, most mRNAs recruited to stress granules in mammalian

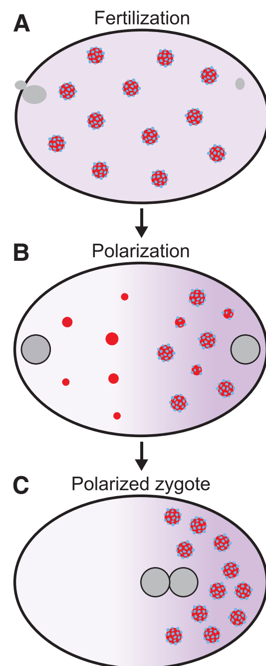

Figure 2. P granules are condensates that undergo localized dissolution and condensation in the *C. elegans* zygote. P granules consist of a central liquid core (containing dozens of proteins; red) covered by solid-like clusters (blue) adsorbed to the interface of the liquid core. All P granule components exchange between the granules and cytoplasm. The solid clusters recruit the kinase DYRK3, which accelerates P granule/cytoplasm exchange. The solid clusters also lower surface tension to stabilize P granules against coarsening ("Pickering effect"). (*A*) In unpolarized zygotes, P granules distribute throughout the uniformly saturated cytoplasm. (*B*) During polarization, P granules dissolve in the anterior cytoplasm and grow in the posterior cytoplasm in response to two spatial inputs: (1) A subset of P granule components enrich in the posterior, forming a saturation gradient across the cytoplasm, and (2) interfacial clusters are depleted from anterior granules and enriched on posterior granules by an unknown mechanism, preferentially stabilizing posterior granules. (*C*) In polarized zygotes, P granules are only found in the supersaturated environment of the posterior cytoplasm.

cells or P granules in *C. elegans* are more abundant in the cytoplasm (Khong et al. 2017; Moon et al. 2019; Lee et al. 2020; Glauninger et al. 2022). Although the dilute phase may appear "dark," it may be the primary compartment for mRNA regulation.

Incidental condensates

Based on the considerations above, we propose a new null hypothesis for RNA granule assembly in cells that does not impose functionality: RNA granules arise when RNP complexes exceed their solubility limit in the cytoplasm (or nucleoplasm) and a fraction demixes into phase-separated condensates. If the condensates enrich active RNP complexes or change their activity, the condensates will represent functional compartments that house (or suppress) the activity associated with the RNPs localized therein. On the other hand, if the condensates do not create (or localize) new activity, the condensates will have no functional consequences. We refer to such nonfunctional condensates as "incidental" to denote the fact that their assembly is a secondary consequence of developmental, physiological, or stress-induced changes in the concentration, affinity, or valency of RNP complexes, leading to oversaturation of the cytoplasm or nucleoplasm (Fig. 3). In the next section, we examine proposed functions for RNA granules in light of this new null hypothesis.

Possible functions for RNA granules

RNA granules typically have been assigned functions based on composition and/or biochemical experiments using condensates reconstituted in vitro (Lyon et al. 2021). Six general themes have emerged, which we consider in turn, evaluating supporting evidence and alternative interpretations. Exemplary RNA granules and their proposed functions are listed in Table 2.

Translational repression

An often-cited role for RNA granules is translational repression. RNA granules have been proposed to block the translation of mRNAs in granules by excluding ribosomes and/or enriching factors that compete with the translational machinery for access to transcripts (Parker et al. 2022). Observations in several granule models, however, argue that assembly into RNA granules is a consequence rather than a cause of translation repression. First, targeting of mRNAs to P-bodies by miRNAs, or to stress granules in stressed cells, correlates with translational repression, but translational repression does not require P-bodies or stress granules (Eulalio et al. 2007; Ohn et al. 2008; Kedersha et al. 2016). Second, RNAs that enrich in the polar granules of *Drosophila* oocytes are translationally repressed prior to entering the granules. Third, mRNAs that enrich in P granules maintain low ribosome occupancy and translational repression even in embryos that lack P granules (Lee et al. 2020).

The weight of the evidence today favors a model in which loss of elongating ribosomes enhances RNA condensation into stress granules, P-bodies, and P granules by generating "naked" RNA molecules that can interact with each other and with RNA-binding proteins. Indeed, treatments that decrease translation initiation and lead to ribosome runoff increase recruitment of RNAs into RNA granules. In contrast, active or stalled elongating ribosomes limit the assembly of stress granules, P-bodies, and P granules and the accumulation of translated mRNAs in stress granules and P granules (Mazroui et al. 2002; Brengues et al. 2005; Teixeira et al. 2005; Khong and Parker 2018; Lee et al. 2020). Elongating ribosomes, however, are not an absolute barrier to accumulation in stress granules, as translating mRNAs have been observed on the surface and inside stress granules (Mateju et al. 2020). These findings do not exclude the possibility that phase separation of translational

Box 2. Cells expend energy to minimize condensation and maintain condensates in a dynamic state

Theoretical considerations indicate that multivalent polymers in initially liquid condensates will "harden" over time by maximizing interactions with their neighbors, leading to "kinetic trapping," where molecules are unable to exit or enter the condensate (Ranganathan and Shakhnovich 2020; Chatterjee et al. 2022; Jiang et al. 2023). Experiments in vitro have shown that proteins in RNA granules form condensates that either (1) remain liquid for hours and evolve glass-like properties over days (Jawerth et al. 2020), (2) start out liquid and arrest dynamics within minutes (Bose et al. 2022), or (3) are immediately solid within the experimental time frame (Putnam et al. 2019; Jawerth et al. 2020; Bose et al. 2022). Cryo-EM analyses have confirmed that initially liquid condensates aged in vitro maintain an amorphous appearance with no internal structure, consistent with an arrested liquid (Jawerth et al. 2020; Bose et al. 2022).

Condensate hardening is potentially problematic because slow dynamics extend the time scale at which condensates will respond to environmental perturbations. When placed in a dilute environment below c_{sat}, condensates will dissolve (lose molecules) at a rate proportional to the rate at which molecules in the condensates liberate themselves from their neighbors to enter the dilute phase. Similarly, when placed in a concentrated environment above c_{sat}, condensates will grow on a time scale proportional to the rate at which molecules at the interface rearrange to accommodate new neighbors incoming from the dilute phase. These theoretical considerations generally mean that reducing binding interactions between condensate components will increase c_{sat}, increase the rate of disassembly in undersaturated conditions ($c < c_{sat}$), and increase the rate of growth in oversaturated conditions ($c > c_{sat}$).

Several lines of evidence suggest that cells expend energy to enhance the solubility of RNAs and proteins and maintain condensates in a responsive, dynamic state. The cytoplasm of *Escherichia coli* behaves like a glass-forming liquid that requires constant energetic input from metabolism to remain fluid, becoming "vitrified" under low energy conditions (Parry et al. 2014). Similarly, the liquid-like dynamics of nucleoli require ATP (Brangwynne et al. 2011). Several ATP-dependent mechanisms have been identified that minimize condensation in cells. ATP-consuming protein chaperones clear heat-induced condensates (Yoo et al. 2022). DEAD-box (DDX) proteins are a large family of ATPases proposed to tune condensation by binding RNA (Hilliker et al. 2011; Elbaum-Garfinkle et al. 2015; Mugler et al. 2016; Hondele et al. 2019; Marnik et al. 2019). DDX proteins bind RNA when ATP-bound and release RNA upon ATP hydrolysis (Putnam and Jankowsky 2013). DDX proteins that contain intrinsically disordered regions promote condensation when bound to RNA and trigger dissolution upon ATP hydrolysis (Mugler et al. 2016; Hondele et al. 2019). The DDX translation initiation factor eIF4A dissolves RNA condensates in vitro in an ATP-dependent manner and limits stress granules assembly in cells (Tauber et al. 2020). Protein modifications can also modulate solubility. Nucleoporins stockpiled in oocytes depend on phosphorylation and sugar modifications to remain soluble and limit the formation of potentially toxic condensates (Thomas et al. 2022). Phosphorylation by the DYRK kinase MBK-2 is essential to accelerate P granule dynamics and ensure that their polarized dissolution and condensation occur sufficiently fast to keep up with embryonic cell divisions (Folkmann et al. 2021). The prion-like domain of FUS exhibits strong selection for phosphorylation sites predicted to prevent hardening (Dasmeh and Wagner 2021). The emerging view is that many cellular components are naturally close to saturation and cells have evolved energy-consuming mechanisms to limit condensation. A corollary is that treatments that interfere with energy production will lead to condensation.

repressors enhances their activity, as suggested for FMRP (Kim et al. 2019b; Tsang et al. 2019), but this hypothesis remains to be tested directly in vivo.

RNA storage and suppression of RNA entanglement

Another proposed function for RNA granules is long-term storage of translationally repressed mRNAs that eventually recycle back into the cytoplasm when favorable conditions return. When yeast cells are shifted from stress conditions to conditions favoring growth, transcripts in P-bodies are returned to translation (Brengues et al. 2005; Bhattacharyya et al. 2006). Similarly, RNAs and proteins in stress granules are released into the cytoplasm and "recycled" following release from stress (Wallace et al. 2015; Wilbertz et al. 2019; Das et al. 2022). In *C. elegans*, mRNAs in P granules disperse back into the cytoplasm upon translational activation in the germline founder cell (Lee et al. 2020; Parker et al. 2020; Cassani and Seydoux 2022). RNA granules are also prominent in oocytes and early embryos, which stockpile maternal mRNAs to be used for embryonic development (So et al. 2021). Embryonic RNA granules in zebrafish accumulate mRNAs during oogenesis that become activated for translation in developing embryos (Sato et al. 2022). Ripin and Parker (2022) have proposed that packaging of RNAs into RNA granules could limit the tendency of "naked" RNAs to become hopelessly tangled by cohousing RNA with RNA-binding proteins that compete with RNA–RNA interactions. Consistent with this possibility, stress granules contain proteins that can prevent RNA entanglement and melt RNA-only condensates in vitro (Sauer et al. 2019; Guillén-Boixet et al. 2020; Tauber et al. 2020; He et al. 2021).

However, direct evidence for RNA entanglement in cells remains lacking. After recovery from stress, RNAs in the cytoplasm are translated and decayed at the same rate as RNAs in stress granules and P-bodies (Wilbertz et al. 2019). Similarly, in mutants defective in P granule assembly, translational activation in the germline founder cell proceeds with the same timing as in wild type (Lee et al. 2020; Cassani and Seydoux 2022). These observations suggest that storage in RNA granules is not essential to maintain the functionality of translationally repressed

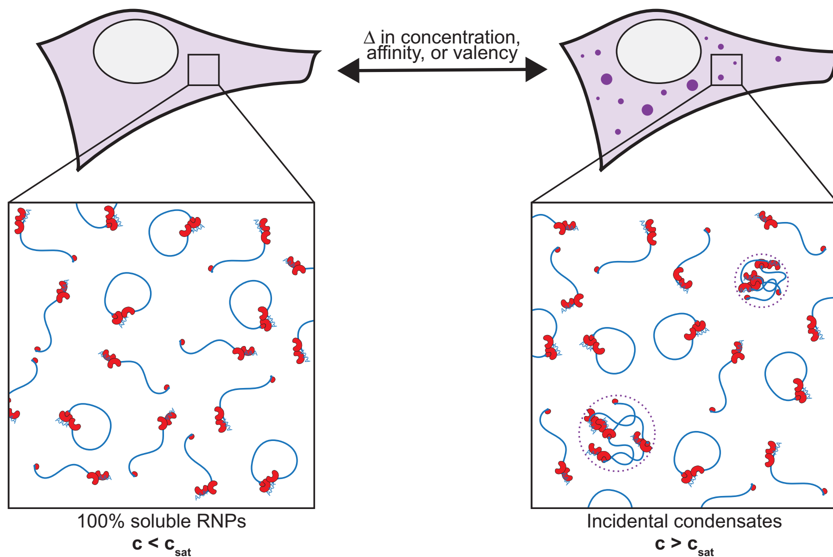

Figure 3. Near saturation conditions, changes in concentration, valency, or affinity of RNP complexes (or in the solvation capacity of the cytoplasm or nucleoplasm) are sufficient to induce condensation or dissolution of RNP complexes. Incidental condensates appear concentrated when visualized by fluorescence microscopy but contain only a fraction of RNP complexes, many of which remain in the dilute phase. Incidental condensates are tolerated by cells but add no functionality beyond that provided by RNP complexes in the dilute phase. Although nonessential, incidental condensates can be useful markers of cellular activity supported by saturating complexes, as well as markers of stress and aging (see the text).

mRNAs, but more precise measurements may be needed to reveal a possible quality control role for RNA granules.

RNA transport, enrichment, and localized 'translation factories'

One of the best-documented roles for RNA granules is to localize RNAs to specific cellular areas by active transport or passive enrichment. RNA granules in neurons are an example of active transport: Neuronal granules travel down axonal processes, transporting mRNAs to synapses and other sites distal from the cell body (Das et al. 2019). One proposed mechanism involves "hitchhiking" using a specific tether that links the granules to lysosomes that travel on microtubules (Liao et al. 2019). Other RNA granules localize mRNAs by assembling at specific cellular locations and passively trapping mRNAs. In *Drosophila*, mRNAs coding for germ cell determinants are captured as single molecules by germ granules that assemble at the posterior-most end of oocytes, marking the site where germ cells will form in the future embryo (Niepielko et al. 2018). Similarly, in *C. elegans* embryos, repeated cycles of polarized assembly and disassembly of P granules in synchrony with cell division enrich maternal mRNAs in the germline founder cell (Schmidt et al. 2021). In the multinucleate hyphae of *A. gossypii*, the phase-separating RNA-binding protein Whi3 is required to both regulate translation and localize transcripts to specific cellular areas (Lee et al. 2015; Zhang et al. 2015), although whether the latter involves transport or local entrapment is not known.

mRNAs in granules are often translationally repressed during transport and activated postlocalization. In *Drosophila*, mRNAs in germ granules are translated at specific developmental stages, possibly by polysomes on the surface of the granules (Mahowald 1968; Rangan et al. 2009). In *C. elegans*, P granule mRNAs are released into the cytoplasm coincident with translation, although in this system, granule localization is not a prerequisite for translational activation (Lee et al. 2020; Schmidt et al. 2021; Cassani and Seydoux 2022). Other RNA granules have been proposed to function as localized "translation factories" that coordinate the translation of mRNAs coding for proteins that function in the same process. For example, granules that enrich glycolytic enzymes also contain mRNAs coding for those same enzymes (Morales-Polanco et al. 2021). Similarly, growing yeast enrich mRNAs coding for translation factors at bud tips—sites of rapid growth (Pizzinga et al. 2019). mRNAs coding for axonemal dynein concentrate in a granule at the growing end of cilia in *Drosophila* sperm (Fingerhut and Yamashita 2020). In some cases, the RNAs become localized in granules indirectly through cotranslational protein targeting, as suggested for RNAs that colocalize with centrosomes, the cell cortex, and nucleoporin condensates (Sepulveda et al. 2018; Hampoelz et al. 2019; Parker et al. 2020). In *Drosophila* oocytes, nucleoporin condensates have been proposed to function as preassembly sites for nuclear pore complexes stored in membrane structures called annulate lamellae (Hampoelz et al. 2019). A study in *C. elegans*, however, suggests that nucleoporin condensates are dispensable for viability and correspond to minor condensation by-products of a large stockpile of highly cohesive FG-Nups maintained near c_{sat} in oocytes for use later during embryogenesis (Thomas et al. 2022). Whether RNAs localize to condensates to promote, or as a consequence of, binding between encoded proteins during translation remains to be determined.

Compartmentalization of biochemical reactions

By enriching and excluding specific components, RNA granules could also theoretically function to compartmentalize biochemical reactions in cells, separating reaction substrates from products for example. Early pioneering studies revealed that condensates assembled with the intrinsically disordered region of the helicase Ddx4 exclude duplex nucleic acids, enrich single strands,

Table 2. *Proposed functions for RNA granules*

Cytoplasmic RNA granules	Proposed function	Evidence	Counterevidence
P-bodies	Translational repression	P-bodies contain translationally repressed mRNAs (Hubstenberger et al. 2017).	Mutants defective in P-body assembly are still competent for translational repression (Chu and Rana 2006; Decker et al. 2007; Eulalio et al. 2007).
	RNA decay	P-bodies concentrate RNA decay factors (Sheth and Parker 2003; Cougot et al. 2004). RNAs targeted to P-bodies degrade with unique kinetics (Wang et al. 2018). In vitro: Condensation enhances deadenylation of target RNAs in Ago2-containing condensates (Sheu-Gruttadauria and MacRae 2018). In vitro: Condensation enhances Dcp1/Dcp2 autoinhibition, which is relieved by Edc3 (Schütz et al. 2017; Tibble et al. 2021).	Mutants defective in P-body assembly are still competent for RNA decay (Stoecklin et al. 2006; Decker et al. 2007; Eulalio et al. 2007). Many P-body proteins are also abundant in the cytoplasm (Xing et al. 2020). RNA decay intermediates do not enrich in P-bodies (Horvathova et al. 2017). RNAs in the cytoplasm are translated and decayed at the same rate as RNAs in P-bodies (Wilbertz et al. 2019).
Stress granules	Translational repression	Stress granules enrich translationally repressed mRNAs and stalled translation initiation factors (Kedersha and Anderson 2002; Mazroui et al. 2002; Wilczynska et al. 2005; Yang et al. 2006). Stress granule assembly correlates with repression of translation initiation (Anderson and Kedersha 2008).	Only a fraction of translationally repressed mRNAs are in stress granules (Mollet et al. 2008; Khong et al. 2017). Stress granules are neither sufficient nor required for translational repression (Ohn et al. 2008; Kedersha et al. 2016). RNAs in the cytoplasm are translated and decayed at the same rate as RNAs in stress granules (Wilbertz et al. 2019). Translation can occur in stress granules (Mateju et al. 2020). *Drosophila*: The stress granule scaffold G3BP promotes translation and RNA stability (Laver et al. 2020).
	RNA chaperone	The DEAD-box ATPase eIF4A reduces RNA condensation in vitro and limits stress granule assembly in cells (Tauber et al. 2020). In vitro: G3BP prevents RNA entanglement (Guillén-Boixet et al. 2020). In vitro: RNA can condense and form aggregates (Van Treeck et al. 2018; Boeynaems et al. 2019; Ripin and Parker 2022).	Irreversible RNA tangles have not yet been observed in vivo (Glauninger et al. 2022).
	Viral defense	Antiviral immunity factors block translation and induce stress granule assembly (Reineke and Lloyd 2015; Reineke et al. 2015; Burgess and Mohr 2018). Loss of stress granules (depletion of G3BP or TIA-1) restores translation and viral replication of MERS-CoV (Nakagawa et al. 2018). Several viruses target the stress	Stress granule proteins, including G3BP, promote translation of interferon-stimulated mRNAs (Bidet et al. 2014). G3BP facilitates alphavirus replication (Götte et al. 2019).

Continued

Table 2. *Continued*

Cytoplasmic RNA granules	Proposed function	Evidence	Counterevidence
		granule scaffold G3BP and other stress granule proteins to counteract stress granule assembly (Eiermann et al. 2020; Mateju and Chao 2022).	
Neuronal transport granules	Translational repression	mRNAs are translationally repressed while in transit in the granules (Das et al. 2021). In vitro: Condensates of the neural granule protein FMRP can inhibit translation (Tsang et al. 2019).	*Drosophila*: FMRP enhances translation in oocytes (Greenblatt and Spradling 2018).
	RNA transport	Stress-induced RNA granules attach to moving lysosomes for transport along microtubules (Liao et al. 2019). *Drosophila*: Mutations in the FMRP RNA-binding motif disrupt condensate formation and RNA localization (Starke et al. 2022).	Some RNAs are transported in single copy (Turner-Bridger et al. 2018).
Germ granules	Localize mRNAs to germ plasm to specify germ cell fate	Germ granules assemble in germ plasm (a specialized cytoplasm that segregates RNA-binding proteins to the germline) and recruit mRNAs required for germ cell development (So et al. 2021; Thomas et al. 2023). *Drosophila*: Mutants that do not assemble germ plasm and germ granules do not localize mRNAs and are sterile (Ephrussi and Lehmann 1992; Dobrynin et al. 2022; Thomas et al. 2023). Zebrafish: The germ granule (Balbiani body) scaffold Bucky ball is required to localize germ plasm mRNAs in oocytes, and its overexpression induces ectopic germ cells in embryos (Bontems et al. 2009).	*C. elegans:* Mutants that assemble germ plasm but lack germ (P) granules do not enrich mRNAs in granules but still stabilize mRNAs in germ plasm and are mostly fertile (Lee et al. 2020).
	Translational repression/ derepression of mRNAs in oocytes/embryos	*Drosophila*: Polysomes appear on the surface of germ granules in embryonic germline precursors (Mahowald 1968). *Drosophila*: In mutants that do not assemble germ plasm/germ granules, germ granule-enriched mRNAs remain translationally repressed and are not translated (Trcek and Lehmann 2019). Zebrafish: Repositioning of *nanos* RNA at the periphery of germ granules correlates with translational derepression (Westerich et al. 2022).	*Drosophila:* Enriched RNAs are translationally repressed before entering germ granules (Trcek and Lehmann 2019). *C. elegans*: mRNA enrichment in granules correlates with but is not required for translational repression/derepression (Lee et al. 2020; Cassani and Seydoux 2022).
	Compartmentalization of sRNA pathways in germ cells (*C. elegans*)	sRNA biogenesis proteins enrich in compositionally distinct perinuclear condensates (Aravin et al. 2009; Phillips et al. 2012; Wan et al. 2018; Dodson and Kennedy 2020; Manage et al. 2020;	sRNA pathway proteins are also present outside of granules in cytoplasm and do not assemble into granules in somatic cells where sRNAs are also active

Continued

Table 2. *Continued*

Cytoplasmic RNA granules	Proposed function	Evidence	Counterevidence
		Phillips and Updike 2022). Silenced mRNAs and RNA fragments tagged for siRNA production accumulate in perinuclear germ granules (Shukla et al. 2020; Ouyang et al. 2022).	(Aoki et al. 2007; Phillips et al. 2012; Uebel et al. 2018).
Whi3 bodies	RNA localization (*A. gossypii*)	The Whi3 polyQ domain is required for condensation in vitro and in vivo and localization of Whi3-bound mRNAs in vivo (Lee et al. 2015; Zhang et al. 2015). Whi3 condensates containing mRNAs targeted to different cellular regions have different material properties and do not mix in vitro. RNA mutations that enable RNA colocalization in condensates in vitro enable RNA colocalization in cells (Langdon et al. 2018).	In vitro: Whi3 forms soluble oligomers (Seim et al. 2022). Not all mRNAs targeted by Whi3 colocalize with Whi3 granules (Lee et al. 2015).
TIS granules	Promote 3′ UTR-dependent protein–protein interactions	In vitro: TIS11 and disordered RNAs phase-separate in irregular mesh-like condensates associated with ER (TIGER domain) (Ma et al. 2021). TIS11 enriches AU-rich mRNAs in the TIGER domain and is required to promote binding between nascent membrane proteins and 3′ UTR-bound cofactors that enhance trafficking to cell surface (Ma and Mayr 2018).	TIS granule components are also present in the dilute phase (Ma and Mayr 2018). TIS11 family proteins shuttle between nucleus and cytoplasm and have been implicated in mRNA degradation (Smith and Costa 2022).
NORAD condensates	Maintain genome stability	Pumilio-binding sites are required for the noncoding RNA *NORAD* to promote genome stability. The single RBMX-binding site in *NORAD* is not essential (Elguindy et al. 2019). Multivalent *NORAD*–Pumilio and Pumilio–Pumilio interactions drive condensation in vitro and in cytoplasm in vivo (Elguindy and Mendell 2021). *NORAD* condensates deplete the cytoplasmic concentration of Pumilio by 50%, leading to derepression of Pumilio targets required for genome stability (Elguindy and Mendell 2021).	*NORAD* binds RBMX to assemble a nuclear RNP that contains suppressors of genomic instability, including topoisomerase (Munschauer et al. 2018).
Nucleoporin foci	Intermediates in nuclear pore assembly	Cytoplasmic foci enrich nuclear pore proteins (Cordes et al. 1996; Hampoelz et al. 2019; Thomas et al. 2022). *Drosophila*: Transport of nuclear pore condensates is required for the formation of annulate lamellae (potential nuclear pore precursors) in oocytes (Hampoelz et al. 2019).	*C. elegans*: Condensates contain <10% of nucleoporins and are not required for viability or nuclear pore assembly (Thomas et al. 2022).

Continued

Table 2. *Continued*

Nuclear RNA granules	Proposed function	Evidence	Counterevidence
Nucleolus	Ribosome biogenesis: Nucleolar layers facilitate assembly-line biogenesis of ribosomes	Nucleolar layers enrich different ribosome assembly factors involved in distinct steps in ribosome assembly (Németh and Grummt 2018). In vitro: Purified nucleolar proteins that localize to distinct nucleolar layers form layered droplets (Feric et al. 2016). Cryo-electron tomographic analyses support a gradient of assembly intermediates in the GC layer (Erdmann et al. 2021). rRNA exits the nucleolus by advective flow (Riback et al. 2022).	Nucleolar morphology varies between species (Thiry and Lafontaine 2005), including two-layer coaxial organization around chromatin in yeast (Lin et al. 2022). In human nucleoli, rRNAs remain near transcribing rDNA repeats (Mangan and McStay 2021). Disruption of nucleolar layers is compatible with viability and fertility in *C. elegans* (Spaulding et al. 2022).
Transcription-related condensates	Enhance transcription	In vitro: The C-terminal domain of RNA polymerase II and IDR-containing transcription factors undergo phase separation (Guo et al. 2022). Clusters of RNA Pol II and transcription factors are visualized around active transcription sites (Cho et al. 2018; McSwiggen et al. 2019a; Rippe and Papantonis 2022). Propensity for phase separation correlates with increased transcriptional output (Boija et al. 2018; Wei et al. 2020).	Condensation does not correlate with transcriptional output measured in real time (Trojanowski et al. 2022). Low concentrations of an IDR promote transcription, but high concentrations that promote condensation suppress transcription (Chong et al. 2022). Not all transcription foci are condensates (Cho et al. 2018; Patange et al. 2021).
Nuclear speckles	Gene expression hubs	Nuclear speckles correlate with sites of enhanced gene expression (Quinodoz et al. 2018; Kim et al. 2019a; Zhang et al. 2021; Leidescher et al. 2022). Splicing-defective transcripts are retained in nuclear speckles (Johnson et al. 2000). Blocking splicing leads to accumulation of spliceosomes in nuclear speckles (Girard et al. 2012).	Speckles accumulate inactive splicing factors that must leave the speckles to serve active genes (Misteli et al. 1997). Speckles are not major sites of transcription and do not contain active RNA polymerase II (Xie et al. 2006). *C. elegans*: Speckle markers are diffuse in the nucleoplasm (Pham et al. 2021).
	Sequestration of excess splicing factors	Disassembly of nuclear speckles leads to accelerated splicing (Hochberg-Laufer et al. 2019).	
Paraspeckles	Sequestration of RNAs and proteins	The paraspeckle RNA scaffold *NEAT1* is required for export of RNAs with Alu repeats (Chen and Carmichael 2009). Knockdown of *NEAT1* releases paraspeckle resident proteins and activates gene expression (Hirose et al. 2014).	Paraspeckle composition diverges across evolution. Paraspeckles also assemble in *Drosophila* and *C. elegans* (Lacroix and Audas 2022), but the RNA scaffold *NEAT1* is mammalian-specific. Mouse knockout of *NEAT1* is viable but deficient in corpus luteum function required for successful pregnancy (Nakagawa et al. 2014).
	pri-miRNA processing	Knockdown of *NEAT1* disrupts pri-miRNA processing (Jiang et al. 2017).	
Cajal bodies	Assembly of small nuclear ribonucleoproteins (snRNPs) essential for splicing	The Cajal body scaffold coilin multimerizes and interacts with Nopp140 to assemble Cajal bodies on snRNA genes (Courchaine et al. 2022).	*Drosophila*: Coilin-null mutants do not assemble Cajal bodies yet have normal levels of Cajal body-specific snRNAs and are viable (Deryusheva and Gall 2009).

Continued

Table 2. *Continued*

Nuclear RNA granules	Proposed function	Evidence	Counterevidence
		Zebrafish: Depletion of coilin leads to decreases in snRNPs and splicing, as well as developmental arrest, which can be rescued by injection of mature human snRNPs (Strzelecka et al. 2010).	

Examples of eukaryotic RNA granules and their proposed functions with supportive and counterevidence. Only a subset of known RNA granules is listed. For more comprehensive lists of RNA granules, including prokaryotic RNA granules, consult Hirose et al. (2022), Lacroix and Audas (2022), and Rostam et al. (2023).

and even melt duplex structures, acting as "biomolecular filters" (Nott et al. 2015, 2016). Based on in vitro reconstitutions and observations in *Xenopus* oocytes that assemble nucleoli around extrachromosomal rDNA, the nucleolus has been modeled as a multilayered condensate where differential binding to nascent versus folded ribosomal RNA promotes vectorial transport of assembly intermediates through the nucleolar layers (Feric et al. 2016; Mitrea et al. 2016; Yao et al. 2019; Riback et al. 2020; Lafontaine et al. 2021). Consistent with vectorial flow, cryo-electron tomography studies suggest that ribosomal intermediates distribute in a gradient in the outer layer of the nucleolus, with fully mature complexes enriching at the periphery (Erdmann et al. 2021). However, interpretation of the functional significance of nucleolar layers is complicated by the observations that ribosome assembly factors cycle between active and latent forms, nucleolar morphology varies considerably between cells and species, and other functions have been attributed to nucleoli besides ribosome production (Hernandez-Verdun et al. 2010; Tartakoff et al. 2022; Hori et al. 2023). Tagging of a subset of rDNA repeats in human cells revealed that rRNAs remain around their site of origin while in nucleoli, suggesting that ribosome biogenesis occurs entirely in subnucleolar territories anchored around the transcriptionally active rDNA repeats (Mangan and McStay 2021). Consistent with this view, a recent study reported that disruption of nucleolar layers is compatible with viability and fertility under normal growth conditions in *C. elegans* (Spaulding et al. 2022). One hypothesis posits that the protein-rich nucleolar layers arise as a consequence of ribosome biogenesis proximal to rDNA repeats and function primarily as storage sites for latent ribosome biogenesis factors and partially unfolded proteins during heat stress (Alberti and Carra 2019; Frottin et al. 2019; Tartakoff et al. 2022).

The model of the nucleolus as a compartmentalized condensate that supports RNA transcription and processing has been extended to mRNA-coding genes (Hnisz et al. 2017). The C-terminal domain of RNA polymerase II, as well as several IDR-containing transcription factors, undergoes phase separation in vitro. Clusters of RNA polymerase II and transcription factors have been observed around active transcription sites in nuclei, although whether these represent phase-separated condensates or smaller assemblies ("hubs") is under debate (McSwiggen et al. 2019a; Darzacq and Tjian 2022; Palacio and Taatjes 2022; Rippe and Papantonis 2022). Interestingly, for several natural and engineered transcription factors, propensity for phase separation correlates with transcriptional output, and condensates nucleated by the IDR of Mediator are sufficient to activate transcription of a reporter gene (Wei et al. 2020; Trojanowski et al. 2022; Lyons et al. 2023). However, one study examining accumulation of nascent RNAs in real time found no correlation between transcriptional output and the presence of a condensate at the locus (Trojanowski et al. 2022), and another found that overexpression of an IDR driving condensation inhibits gene expression (Chong et al. 2022). Rather than promoting condensation, IDRs could facilitate the assembly of soluble complexes linking transcription factors to RNA polymerase II (Ferrie et al. 2022; Rippe and Papantonis 2022). IDRs can facilitate complex assembly by dynamic binding to ordered domains or to other IDRs, in some cases with remarkably high (picomolar) affinity (Pontius 1993; Borgia et al. 2018; Hong et al. 2020). Because the same domains that drive condensation could also drive the formation of smaller, non-phase-separated assemblies, assigning a function to condensates is challenging. One possibility is that both condensates and non-phase-separated assemblies facilitate transcription but at different loci (Palacio and Taatjes 2022).

RNA-rich condensates that form in the cytoplasm have also been proposed to compartmentalize RNA-based processes. For example, in the *C. elegans* adult germline, components of the small RNA biosynthetic machinery sort into at least four distinct perinuclear germ granules, each with a unique composition (Sundby et al. 2021). However, this complex organization is unlikely to be essential for small RNA biogenesis, since small RNAs are also active in somatic cells, which do not assemble condensates (Phillips et al. 2012). Compartmentalization of small RNA biogenesis in germ cells has been proposed to prevent dangerous feed-forward silencing loops that could silence genes for generations, but direct evidence for this hypothesis is not yet available (Ouyang and Seydoux 2022). Alternatively, multiphase condensation could simply be a consequence of the different types of

RNPs involved in small RNA biogenesis and their relatively high concentration in the adult germline, a tissue with above-average rates of transcription. Consistent with this view, treatments that lower transcription reduce germ granule condensation (Sheth et al. 2010).

RNA granules have also been suggested to promote the assembly of protein complexes on nascent proteins during translation. TIS granules are ER-associated condensates that enrich transcripts coding for trafficked proteins. The condensate-forming TIS11B protein recruits mRNAs to TIS granules and facilitates their binding to SET, a cofactor that promotes protein trafficking to the plasma membrane (Ma and Mayr 2018). How TIS granules enhance such interactions is not yet known, but transient entrapment of newly translated proteins in the dense environment of the TIS11B condensates has been proposed as a possible mechanism (Chen and Mayr 2022).

Enhanced RNA biochemistry

An exciting possibility is that condensation of RNA in protein-rich condensates creates a solvent environment uniquely suited for RNA biochemistry. Studies using artificial dextran-rich condensates found them to be potent RNA concentrators and enhancers of ribozyme activity (Strulson et al. 2012; Poudyal et al. 2019). Similarly, condensates of the Argonaute Ago2 and the GW182 protein TNRC6B sequester Argonaute-bound RNAs and accelerate their deadenylation in vitro (Sheu-Gruttadauria and MacRae 2018). Reconstitution of minimal P-bodies using purified components has revealed that condensation can modulate RNA-decapping activity (Schütz et al. 2017; Tibble et al. 2021). Condensation stabilizes the decapping complex Dcp1/Dcp2 in an autoinhibited conformation while preserving the complex's ability to respond to stimulation by the decapping activator Edc3. The lower basal activity of Dcp1/Dcp2 in condensates causes Edc3 to raise decapping activity by 90-fold in condensates, compared with a mere threefold in solution (Tibble et al. 2021). Together, these studies demonstrate that, in principle, condensates can support RNP enzymology and enhance reactions by increasing concentrations and modulating protein and RNA conformation. Direct evidence that native RNA granules provide a unique solvent environment for RNA biochemistry in cells, however, is still lacking.

Titration of soluble RNPs

Several lines of evidence suggest that some RNA granules affect RNA biochemistry indirectly by removing RNA regulators from the soluble pool. Nuclear speckles and paraspeckles accumulate splicing and other nuclear proteins, which when released in the nucleoplasm lead to dysregulated gene expression (Hirose et al. 2014; Hochberg-Laufer et al. 2019). Condensation of poly(A)-binding protein is enhanced by heat stress and antagonized by RNA, driving poly(A)-binding protein into RNA-free gel-like condensates that enhance stress resistance (Riback et al. 2017). In human cells, the noncoding RNA *NORAD* is induced by DNA damage and condenses the normally soluble translational repressor Pumilio. High *NORAD* copy number and valency (18 Pumilio-binding sites) drive highly efficient condensation of Pumilio, depleting the soluble pool by half, which in turn activates the translation of Pumilio mRNA targets (Elguindy and Mendell 2021). These findings demonstrate that efficient condensation can tune RNA biochemistry by reducing the concentration of RNA regulators in the nucleoplasm or cytoplasm.

Best practices for determining RNA granule function

Several challenges complicate the assessment of RNA granule function in cells. First, because most RNA granules likely assemble by phase separation of RNP complexes also present in the dilute phase, it is not straightforward to parse out whether the activity under study comes from complexes in the dilute phase, the condensates, or both. Second, "part lists" alone cannot be used to predict function, since some proteins may become inactive or change function in the condensate environment. Third, condensates may facilitate activities that only become essential under specific conditions, such as protein and/or RNA chaperoning under stress, and these functions may require new assays to fully assess. Last, because condensation is a spontaneous process sensitive to fluctuations in concentration and binding affinities, the possibility that some condensates are not functional and incidental to the assembly of subsoluble complexes cannot be discounted. In vitro reconstitutions will continue to serve as powerful tools to explore the effect of condensation on enzymatic activity and protein and RNA folding (Lyon et al. 2021). In the next sections, we consider in vivo experiments that can be used to complement in vitro reconstitutions to probe the function of native RNA granules.

Quantitative analyses of protein and RNA enrichment in condensates

A first step toward functional characterization of an RNA granule is to determine the percentage of molecules inside versus outside of the granules. These experiments should be performed using probes that detect endogenous components, without transgenes that could lead to overexpression, and ideally in living animals to minimize nonphysiological stresses associated with cell culture. Care should also be taken to avoid fixation or microscopy conditions that enhance or suppress condensation (Uebel and Phillips 2019; Elaswad et al. 2022a; Irgen-Gioro et al. 2022). The relatively small volume of RNA granules compared with the rest of the cell may mean that, even when prominent by microscopy, RNA granules may only account for a small proportion of molecules, as demonstrated for stress granules, P-bodies, and nucleoporin condensates (Khong et al. 2017; Xing et al. 2020; Thomas et al. 2022). Such a finding may help rule out models that require a significant proportion of RNPs to localize to the condensates (such as inhibitory/titration models) but does not necessarily rule out other functions. For example, in

situ hybridization experiments revealed that P granules capture at most only ~30% of molecules for a specific translationally repressed transcript, ruling out granule localization as causal for translational repression (Lee et al. 2020). Comparisons of embryos with and without P granules, however, confirmed that recruitment into granules correlates with enrichment of those transcripts in the germline founder cell, supporting a role for P granules in RNA localization (Schmidt et al. 2021).

Titration experiments

Because condensation is ultrasensitive to concentration, one approach to determine whether condensates are essential is to titrate the concentration of a critical condensate scaffold. The titration range should flank c_{sat} to generate cells with and without condensates and should be small enough to not significantly affect the concentration of soluble complexes. This approach has been used to evaluate the functional relevance of transcriptional condensates (Chong et al. 2022; Trojanowski et al. 2022). For example, by monitoring transcriptional output in real time in cells expressing variable levels of a condensing transcription factor, the investigators concluded that the presence of condensate on the transcribing locus had no, or a slightly negative, effect on transcriptional output (Trojanowski et al. 2022). However, one drawback to this approach is that, in the case of multiscaffold condensates, titration of only one scaffold could skew condensate composition, leading to nonfunctional condensates (Riback et al. 2020).

Genetic and evolutionary analyses

Another approach is to use genetic mutants to correlate activity in cells and in reconstituted systems. Mutations that affect condensation without affecting enzymatic activity or RNA binding can be used to disentangle effects due to loss or disruption of condensates versus loss of RNP complex activity (Riback et al. 2017; Iserman et al. 2020; Tibble et al. 2021). In such analyses, it is important to verify that the mutations do not affect RNP complex assembly in the dilute phase. Sequences between binding sites ("spacer" sequences) can be good mutagenic targets if they contribute to the overall solubility of RNP complexes, but their effects may be weak due to redundancy (Kar et al. 2022; Mittag and Pappu 2022). Evolutionary analyses can be used to reveal conserved sequence features selected to tune condensation to environmental inputs (Pritišanac et al. 2020). For example, sequence analyses comparing 351 poly(A)-binding protein orthologs revealed a strong selection signature for hydrophobic amino acids in a proline-rich low-complexity domain. These observations guided the construction of an allelic series with predicted reduced hydrophobicity, which was then used to demonstrate a correlation between propensity for phase separation in vitro and stress resistance in vivo (Riback et al. 2017). Similarly, examination of orthologs of the *Saccharomyces cerevisiae* Ded1 RNA helicase revealed a region required for temperature-induced condensation. Mutations that lowered the temperature threshold required for Ded1 condensation were detrimental to yeast fitness and revealed that Ded1 condensation is adapted to species-specific thermal niches (Iserman et al. 2020). Interestingly, in both cases, condensation was correlated with loss of activity, allowing for a selective shift in the types of mRNAs translated in the cytoplasm upon heat-induced stress. These examples also illustrate the importance of assaying function under a range of conditions that explore the full fitness landscape, as some condensates may only contribute to organismal fitness under stress or other specific conditions.

In vivo imaging

Mutational analyses can be complemented with imaging to directly locate activity in live cells. In cases where the RNA granules are predicted to promote RNA biochemistry, it may be necessary to develop probes to measure enzymatic activity in situ. As mentioned above, studies examining transcriptional output in real time have shown that the presence of a condensate at a transcriptionally active locus does not always correlate with increased transcriptional activity (Trojanowski et al. 2022). Single-molecule studies have also debunked assumptions about stress granules as compartments incompatible with translation by showing that RNAs inside the granules are accessible to the translation machinery (Mateju et al. 2020). Single-molecule analyses also revealed that mRNA degradation events occur throughout the cytoplasm and do not enrich in P-bodies (Horvathova et al. 2017). Recent advances in superresolution microscopy techniques that permit precise counting of molecules colocalized in cells may also provide insights into whether condensates or smaller clusters (or both) underlie biological processes (Bond et al. 2022; Castells-Garcia et al. 2022).

Quantitative observations in cells can also be used to refine in vitro reconstitutions. Molecular parameters measured in vivo (concentrations, diffusion rates/internal viscosity, and surface tension) can be used to adjust conditions for reconstitutions in vitro to better match the in vivo setting. Parameters measured in vivo and in vitro in turn can inform theory to test and refine quantitative models (e.g., Folkmann et al. 2021). Ultimately, combinations of in vivo, in vitro, and in silico experiments will be needed to develop a quantitative understanding of RNA granule function and dynamics.

Outlook—incidental condensates as markers of cellular activity, stress, and aging

Phase separation of multivalent RNP complexes into condensates is an attractive model for the compartmentalization of RNA-focused activities in cells. For some native RNA granules, observations in cells are consistent with the physical properties of condensates, providing a sound theoretical framework for modeling RNA granule dynamics. Because the inside of phase-separated condensates is

chemically and diffusionally distinct from the surrounding phase, condensates hold great promise as compartments with unique biochemistry. At the same time, the exquisite sensitivity of phase separation to small changes in solubility and concentration raises the possibility that some condensates are "incidental," tolerated by cells as by-products of cellular activity but providing no new function.

Although not functional, incidental condensates could still be useful to experimentalists as reporters of cellular activities involving subsoluble RNP complexes. For example, the sudden appearance of P-bodies signals the onset of maternal mRNA turnover in embryos (Gallo et al. 2008). Analysis of the composition and dynamics of incidental condensates could report on the types of molecular complexes and their dynamics in the cytoplasm or nucleoplasm during cellular and developmental transitions. Mutations that disrupt incidental condensates could be used to guide the identification of binding sites in proteins and/or RNAs that mediate RNP complex assembly in vivo. Incidental condensates could also serve as useful markers of stress responses and aging, as exemplified by stress granules that arise under stress conditions that block translation initiation (Kedersha and Anderson 2002; Kedersha et al. 2005) and cytoplasmic nucleoporin condensates, whose assembly is enhanced by heat shock and organismal aging (Patterson et al. 2011; Thomas et al. 2022). Because cells use energy-consuming mechanisms to counter condensation (Box 2), incidental condensates may also prove useful markers to identify quiescent cells with lower ATP production. For example, arrested oocytes accumulate many condensate types (Jud et al. 2007; Elaswad et al. 2022b), which we speculate arise as a consequence of the suppression of metabolic activity that accompanies dormancy (Rodríguez-Nuevo et al. 2022). Incidental condensates are tolerated by healthy cells but could in theory become toxic if allowed to evolve into irreversible sinks that deplete soluble proteins and RNAs, as suggested by several studies linking accelerated condensation to disease (Boeynaems et al. 2018; Nedelsky and Taylor 2022). Understanding how cells minimize incidental condensates may suggest strategies to reverse pathological condensates and aggregates. Finally, we anticipate that in vivo experiments, combined with in vitro reconstitutions conducted under physiological conditions, will help distinguish incidental condensates from functional RNA granules where evolution has harnessed phase separation to drive biological function.

Competing interest statement

The authors declare no competing interests.

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