

Annual Review of Biophysics

RNA Droplets

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Annu. Rev. Biophys. 2020. 49:247–65

First published as a Review in Advance on
February 10, 2020

The *Annual Review of Biophysics* is online at
biophys.annualreviews.org

<https://doi.org/10.1146/annurev-biophys-052118-115508>

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Keywords

RNA, liquid–liquid phase separation, polymer, multivalency, RNA binding protein, aberrant aggregation

Abstract

Liquid–liquid phase separation is emerging as the universal mechanism by which membraneless cellular granules form. Despite many previous studies on condensation of intrinsically disordered proteins and low complexity domains, we lack understanding about the role of RNA, which is the essential component of all ribonucleoprotein (RNP) granules. RNA, as an anionic polymer, is inherently an excellent platform for achieving multivalency and can accommodate many RNA binding proteins. Recent findings have highlighted the diverse function of RNA in tuning phase-separation propensity up or down, altering viscoelastic properties and thereby driving immiscibility between different condensates. In addition to contributing to the biophysical properties of droplets, RNA is a functionally critical constituent that defines the identity of cellular condensates and controls the temporal and spatial distribution of specific RNP granules. In this review, we summarize what we have learned so far about such roles of RNA in the context of in vitro and in vivo studies.

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1. INTRODUCTION

Membrane-bound organelles are the hallmark of eukaryotic organisms. The ability to compartmentalize different cellular components and functions and to regulate the flow of molecular traffic between organelles is essential for survival. Although these organelles have long been appreciated as the primary means of cellular organization, the discovery of membraneless organelles has highlighted a new and ubiquitous mechanism through which cells can further compartmentalize critical functions, many of which require transient assembly and timely dissolution (13, 26). Membraneless organelles form by a process called liquid-liquid phase separation (LLPS) in which proteins and associated RNA molecules condense from the surrounding dilute phase, which in cells would be the cytoplasm or nucleoplasm (7, 73, 82).

LLPS occurs when multivalent biopolymers interact transiently to coalesce into a dense condensate (Figure 1*a*). LLPS is often promoted by high protein concentration and low salt concentration where the electrostatic, hydrophobic, π -cation, cation-cation, and π - π interactions among neighboring molecules are favorable (23). Numerous studies have demonstrated that proteins harboring intrinsically disordered regions (IDRs), also known as low-complexity domains

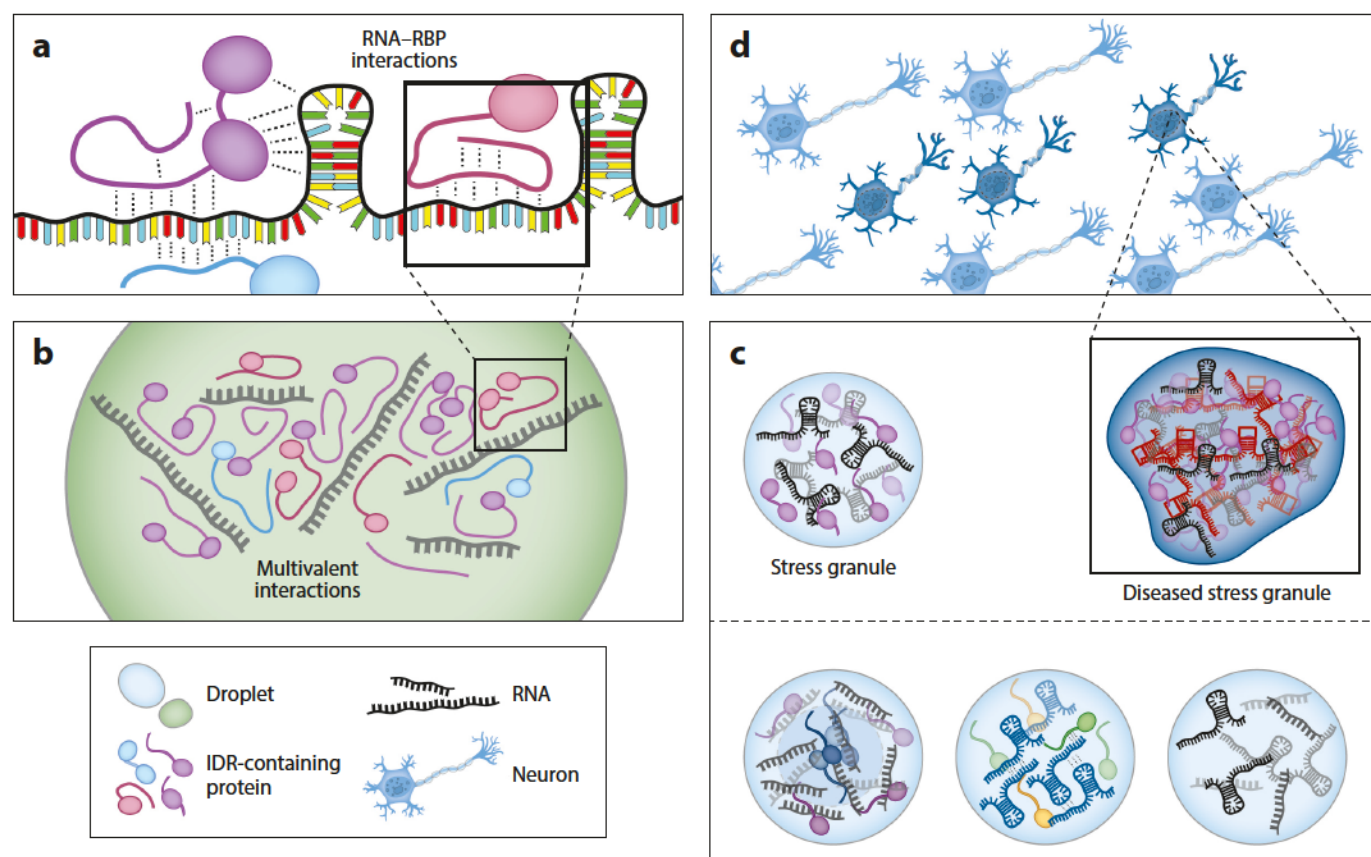


Figure 1

RNA droplets at every stage of formation and maturation. (a) RNA–RNA binding protein (RBP) contacts underlie the formation of ribonucleoprotein (RNP) complexes that contribute to droplet formation. Interactions are varied, and they can occur with any combination of structured and unstructured protein domains and RNA sequences. (b) Multivalent RNA–RBP interactions support the formation of phase-separated droplets. (c) Combination of different intrinsically disordered region (IDR) proteins and RNA allows for the formation of specific RNP granules with different cellular functions. (d) Accumulation of aberrant RNAs such as repeat expansion RNAs gives rise to aggregations via improper RNA–RNA and protein–RNA interactions that can lead to cellular toxicity and neurological disorders.

(LCDs), are necessary and sufficient to drive LLPS in vitro and in cells (16, 58). In the cellular context, the IDR-containing proteins can interact with multiple binding partners—thus achieving multivalency (Figure 1b). Self-assembly of phase-separated condensates then becomes energetically favorable (82). The physical properties of these condensates, also referred to as droplets (in vitro) or granules (in vivo), are distinct from the surrounding environment. The viscoelastic properties of different condensates are tuned by the constituent molecules (1). Functionally, condensates can accelerate enzymatic activity of the phase-separated proteins and metabolites (7, 88). Accordingly, diverse functions within the cell, including splicing, transcription, micro RNA (miRNA) processing, heterochromatin formation, DNA damage repair, and the stress response, occur in the context of phase-separated bodies (7, 17, 18, 20, 51, 74, 79, 85, 87, 99, 104). Importantly, many of these functions require RNA.

Many membraneless organelles are ribonucleoprotein (RNP) granules enriched with RNA and RNA binding proteins (RBPs). They are highly conserved across species and are found in the nucleus as well as the cytoplasm of cells (5). Despite the ubiquitous presence of the RNP granules, the constituents of different types of granules are vastly different, reflecting the selective recruitment of molecules according to their functional roles (64). One major focus in the phase

separation field has been to determine the mechanism by which particular granules are nucleated and assembled into distinct functional condensates. Understanding how these granules form will give insight into how the cell organizes itself in a spatially, temporally, and functionally regulated manner (Figure 1c).

To date, the majority of studies have primarily focused on the role of proteins in driving LLPS; they have done this by performing deletion experiments of the IDR domain from RNP-forming proteins. When these IDR proteins are removed, reduction in granule assembly or aberrant formation has been reported (14, 27, 76, 77, 78, 80, 86). Interestingly, the majority, if not all, of these proteins are RBPs with high affinity for RNA, yet the role of RNA in RNP granule formation is far less well understood. It is well documented that RNA is required for the formation of certain RNP granules, for instance, NEAT1 in paraspeckles (21, 27). RNA plays a major role in seeding or nucleating the formation of RNP granules, such as the nucleolus, P granules, and stress granules (7, 23, 96) (Figure 1c). In this article, we review the importance of RNA in RNP granule assembly, identity, function, material property, and potential transition to a disease state (Figure 1d).

2. CHARGED POLYMERS ARE THE BUILDING BLOCKS OF LIQUID-LIQUID PHASE SEPARATION

Two-phase systems arise when a polymer-rich phase segregates from the polymer-deficient phase (15). This process, termed coacervation, results in colloidal droplets that have unique viscoelastic properties compared to the dilute, polymer-deficient phase (15). Phase separation can occur in one-component systems, such as with RNA (10, 38, 95), DNA (55), peptides (101), and full-length proteins alone (73, 99) (Figure 2). By contrast, oppositely charged polymers have long been shown to promote condensation (complex coacervation), as in the interaction between DNA and the polycation spermine, which is abundant in the nucleus (3, 9, 63, 90, 95).

With recent discoveries of LLPS in the cytosol and nucleus of cells (13, 26), the biopolymeric interactions that drive cellular phase separation are now coming into focus. Proteins with LCDs

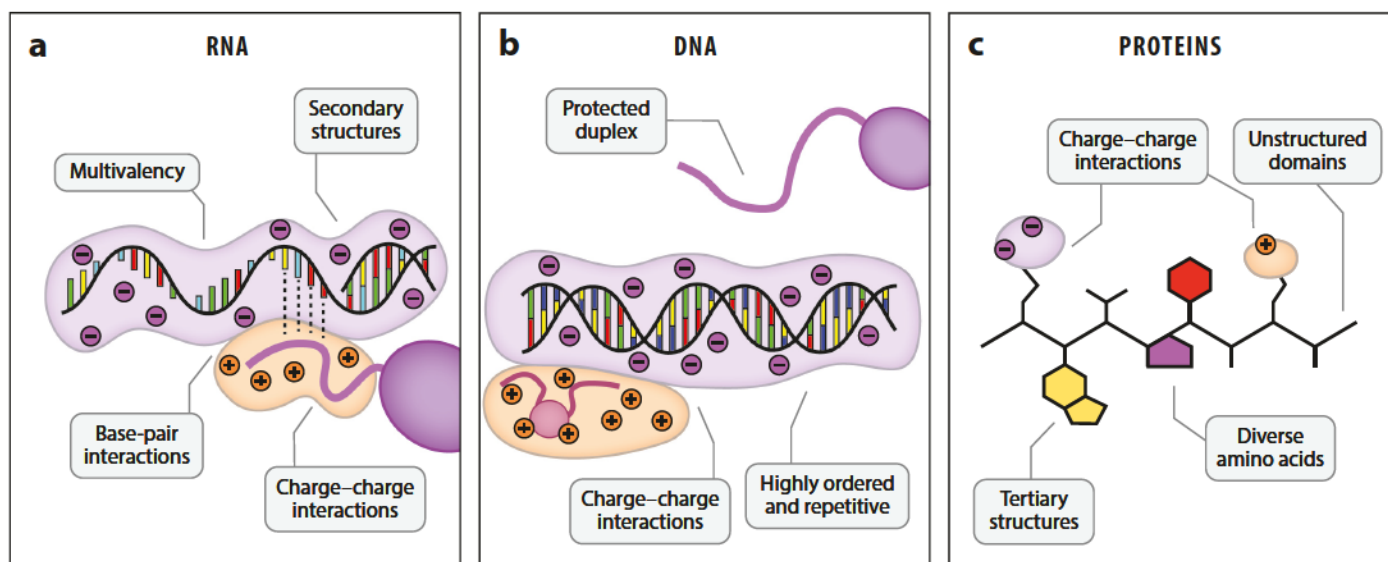


Figure 2

RNA is uniquely poised for liquid-liquid phase separation (LLPS). (a) Single-stranded RNA has a mixture of structured and unstructured regions, along with varied facets for charge-charge and other molecular interactions. (b) DNA has a protected duplex that limits interactions to the grooved or charged surface. (c) Proteins, like RNA, can be structured or unstructured and have varied opportunities to bind RNA.

and IDRs are well known to undergo phase separations through homotypic self-association (58, 73). LCDs are naturally LLPS prone, with π - π interactions between aromatic residues and non-covalent charged interactions driving phase separation (105). However, binding between polycationic protein domains (e.g., arginine- and lysine-rich motifs) and polyanionic nucleic acids can dramatically increase phase separation propensity through increased multivalency and favorable charge interactions (3). Proteins can engage in phase separation with both DNA (87) and RNA (58, 106), as well as unconventional polymers like poly(ADP-ribose) (50, 85). RNA is a uniquely potent polymer to control phase separation (Figure 2); RNA structure, sequence, and length are all parameters that can tune LLPS propensity and condensate properties, as discussed below.

2.1. RNA Innately Mimics Low-Complexity Domains

Unlike genomic DNA, the single-stranded nature of cellular RNA allows it to adopt many unique conformational structures that can promiscuously interact with many proteins (93) (Figure 2a). RNA can fold to form well-defined hairpins that are recognized by specific proteins such as MS2, tertiary structures within transfer RNAs that confer specificity to ribosome, and highly folded and enzymatically active ribozymes including Twister (8, 70, 72, 93). For example, the protein Whi3 (from the multinucleated fungus *Ashbya gossypii*) has a specific binding sequence found in the RNAs CLN3 and BNI1. These RNAs form secondary hairpin structures that also interact with Whi3 (47). In addition, the R motifs of the protein NPM1 specifically interact with ribosomal RNA (rRNA) to achieve multivalency and form the nucleolus (66). Small noncoding RNAs, including *cis*-acting riboswitches, can remain structured until base pairing with complementary DNA and RNA targets to control gene expressions in multiple cellular pathways (65, 67). Some messenger RNAs (mRNAs) are built with structured domains that reduce degradation and unstructured domains that engage in productive interactions (61).

In most unstructured cellular RNAs, both the phosphate backbone and RNA bases are exposed, enabling RNA binding partners to engage in many different specific and nonspecific interactions (57). Physiological salt conditions can stabilize charge-charge interactions between RNA and positively charged peptide sequences (6, 23, 52, 106). The physiological salt concentration is simultaneously low enough to permit protein-RNA binding but high enough to confer some selectivity to dedicated RNA binding domains (46, 56, 75). Certain RNA motifs can form additional structures, such as G-quadruplexes, which may help support LLPS (10, 28). Importantly, long RNA chains can bind multiple partners, and this multivalency is critical for LLPS (42). The ability to have numerous interactions of varying molecular specificity—and the flexibility to adopt many conformations—suggests that RNA mimics the properties of a protein's LCD.

2.2. RNA-Protein Interaction Promotes Liquid-Liquid Phase Separation

Many RBPs harbor IDRs enriched with arginine, lysine, glycine, tyrosine, and proline (16, 71). These residues drive LLPS and control the fluidity of the resulting condensate. Accordingly, deletion of IDRs greatly dampens LLPS propensity (52, 58). Although the IDRs of RBPs are sufficient for LLPS, association with RNA can significantly promote LLPS, likely by enabling multimerization of RBPs (52).

RBP interactions with RNA can be specific and nonspecific (40, 76, 81). Many RBPs have distinct and structured RNA binding domains, such as RNA recognition motifs (RRMs) and zinc fingers (ZnFs) (16, 58), which can associate with RNA in the context of RNP granules (39, 106). Meanwhile, nonspecific contacts between positively charged arginine-glycine-glycine (RGG) domains, such as those found in FUS or PGL-3, and negatively charged RNA can strengthen the

binding affinity of existing RNA binding domains and provide alternative interaction modes (23, 52, 76, 81, 105). Many classical RBPs are good candidates for phase separation given that RNA binding domains tend to be intrinsically disordered. This group of RBPs also includes the RNA helicases LAF1 and DDX-3, which have RecA-like domains that can interact with single-stranded RNA, as well as IDRs rich in RGG motifs (36, 44, 80). Above, we discuss Whi3, which also contains an RNA recognition motif, as well as a poly-Q IDR (106). The combination of protein subdomains and RNA parameters—such as structure, length, and sequence composition—can modulate phase separation.

Long RNAs can engage in multivalent protein interactions, which efficiently promotes LLPS. Most RBPs have RNA binding footprints of approximately 10–20 nucleotides (33). Therefore, the minimum size of RNA needed to enable multivalency is likely 20–40 nucleotides; smaller RNAs such as miRNAs and piwi-interacting RNAs (piRNAs) may not be able to seed phase separation independently (42, 95). Indeed, RNAs found in stress granules tend to skew longer in length (42, 81, 95). In vitro, longer RNA also more effectively phase separates into functional condensates (88). RNA concentration is also important for controlling LLPS. The high RNA-to-protein concentration ratio in the nucleus buffers phase separation of RBPs such as FUS and TAR DNA-binding protein 43 (TDP-43). By contrast, the low RNA-to-protein ratio leads to condensation of the same proteins in the cytoplasm (58). This indicates that both RNA concentration and length, which together define the total number of potential RNA binding sites, are key regulators of LLPS in cells.

2.3. RNA Can Tune the Fluidity of Ribonucleoprotein Liquid–Liquid Phase Separation

RNA properties can be altered depending on which ribonucleotides are enriched. Certain nucleotide sequences promote promiscuous RBP binding. For instance, FUS favors binding to uracil-rich sequences, though it does not have a strong sequence preference (82). By contrast, TDP-43 prefers structured RNAs (37). The poly-pyrimidine tracts (poly-uracil and poly-cytosine) tend to be more flexible due to the availability of only one aromatic ring for base stacking (3, 10, 95). These sequences may promote more dynamic RNA–protein interactions and more fluidic condensates. By contrast, poly-adenine sequences may promote stiffer interactions due to the bulky purine base, which presents two rings for stronger base stacking interactions with neighboring ribonucleotides (3, 10, 95). Poly-guanine tracts can fold into G-quadruplex structures, which are not recognized by most RBPs (10, 95). G-quadruplexes, however, may promote RNA–RNA contacts, contributing to RNA seeding (25). However, if G-quadruplexes do form within droplets, then they will likely dramatically reduce the fluidity of the resulting droplet (10).

Although many RBPs can bind and condense with unstructured or poorly structured RNAs, some RBPs require structured RNAs for binding. Secondary folds, such as hairpins, may sequester certain proteins into phase-separated droplets (47, 60, 107). For example, Whi3 binds differently shaped mRNAs to form biophysically (viscosity) and functionally distinct condensates, which become immiscible in vitro and in vivo (47, 48, 106). Disruption of the distinct RNA structure led to loss of specific Whi3–RNA interactions, resulting in miscibility between the two condensates (47). Therefore, long mRNAs encompassing both structured and unstructured regions may accommodate different types of RBPs in a multivalent manner. Tight binding with structured RNA elements may help form stable, solid-like droplet cores (see Section 3.2) (96). The multivalent nature of RNA makes it an ideal candidate for scaffolding and organizing LLPS. RNA in the form of homopolymers or highly repeated sequences, which are also pathogenic, can undergo LLPS or aggregation depending on the sequence composition and length (10, 38). Taken together,

RNA is important for controlling phase separation and altering the properties of phase-separated condensates.

3. RNA CONTRIBUTES TO PHASE SEPARATION OF PROTEINS INTO CONDENSATES

Many cellular granules contain RNA. Indeed, RNA can play a pivotal role in condensing proteins into distinct phases, acting as a scaffold to increase the frequency of protein–protein contacts. When droplets form, they exhibit different properties from the dilute phase, including altered viscosity, fluidity, size, circularity, and elasticity. Concentrating RNA and RBPs in close proximity can trigger functional cellular responses including the DNA damage response, splicing, and transcription. In this section, we discuss how RNA seeds RBP-enriched droplets, and how RNA efficiently promotes the formation of substructures within biphasic droplets.

3.1. RNA Seeds and Accelerates Condensation of RNA Binding Proteins

Many proteins can form phase-separated droplets at a high concentration threshold, referred to as the saturation concentration or C_{sat} . Therefore, a higher C_{sat} means that a higher protein concentration is needed to achieve phase separation (100). Addition of RNA can decrease C_{sat} , likely through its multivalent scaffolding of RBPs (7, 23, 52, 58, 82, 106) (Figure 3*a*). Multiple RBPs can bind to a single RNA molecule, promoting oligomeric interactions (82). For instance, the DEAD-box ATPase Dhh1 multimerizes with RNA in the presence of Pat1, and FUS is known to oligomerize with long RNAs (80, 82). In agreement with these results, disabling RNA contacts can effectively prevent LLPS of RBPs, suggesting that RNA–protein interactions are critical for RNP condensation (69, 86). The multivalency enabled by long RNAs can permit phase separation of proteins that would not ordinarily phase separate at physiological concentrations. In repeat-expansion disorders, the longer length of the encoded RNA can increase binding multivalency and lead to disease (38). Another example of the importance of multivalency is FUS, which does not usually phase separate at its concentration in the cytoplasm, 2 μM (73, 100). However, addition of RNA can drive droplet formation at this low concentration. This may be because RNA–protein contacts can stimulate subsequent protein–protein, protein–RNP, or RNP–RNP contacts, thereby concentrating RBPs in close proximity (82). RNA is also critical for the maintenance of droplet structure; RNase treatment leads to loss of the two-phase system (58). The formation and dissolution of RNA-containing droplets can occur on the order of seconds to minutes, depending on the kinetics and binding strength of the protein–RNA interaction, and RNA can readily transit between phase-separated granules in cells (68, 102). RNA-containing granules often persist in cells during all stages of the cell cycle, except during M phase, in which these granules often disassemble and reassemble during the next G1 phase (21, 24). Although RBPs can often undergo phase separations independently, RNA is important for seeding and maintaining RNP droplet structure at physiological conditions.

3.2. RNAs Alter the Physical Properties of Droplets

Phase-separated droplets exhibit physical properties that are distinct from the surrounding dilute phase, and these properties are determined by the constituent polymers of the droplet. Visual characteristics, such as droplet size and circularity, are easily influenced by protein concentration (1) (Figure 3*c*). Moreover, time can also influence the droplet properties. Aged droplets are more likely to be more solid like, especially as disordered proteins adopt conformations at the energetic

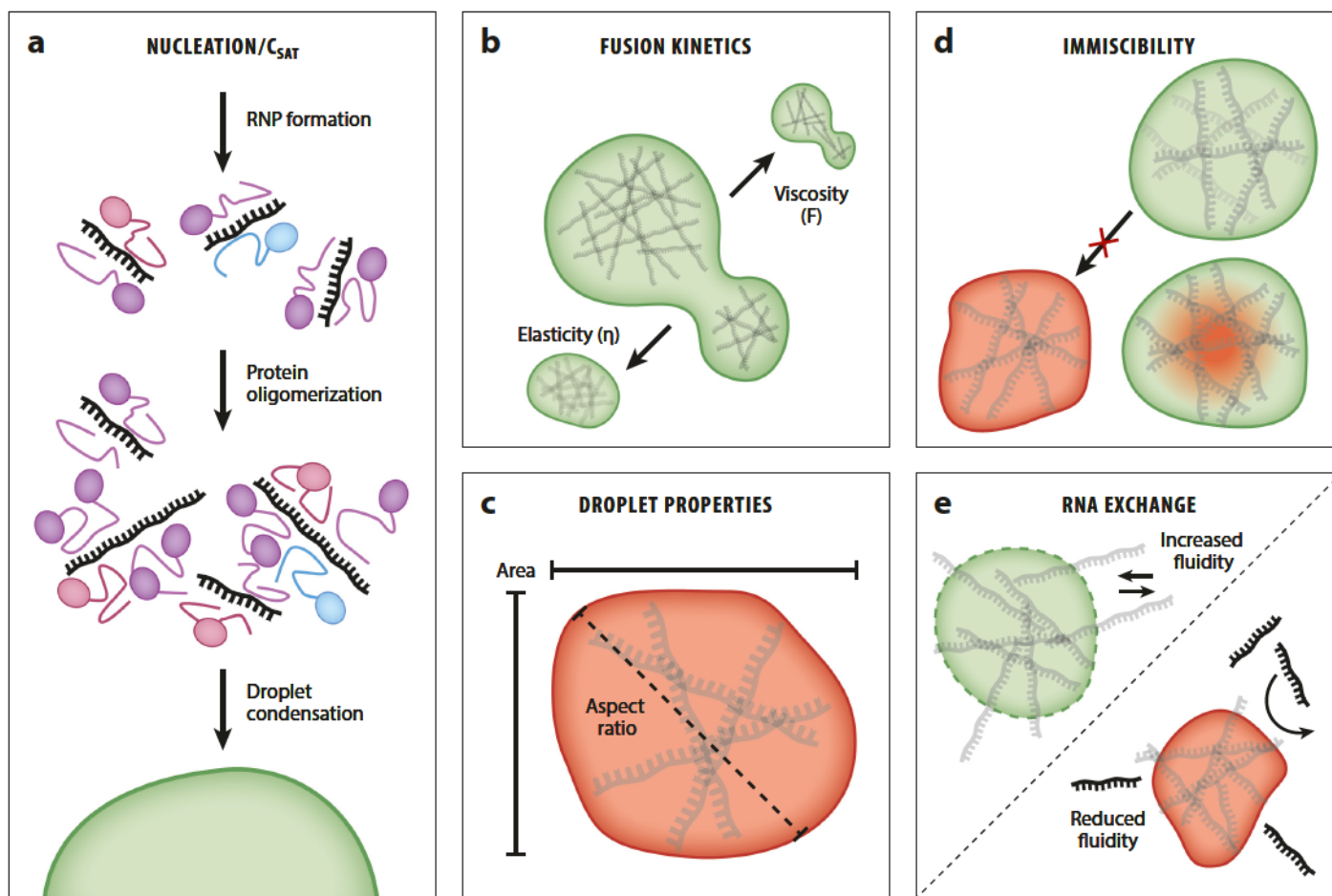


Figure 3

RNA controls the physical properties of phase-separated droplets. (a) RNA seeds droplet formation through multivalent contacts with RNA binding protein (RBP) partners. After additional RNA–RBP interactions, droplet condensation can occur. (b) The viscoelastic properties of droplets can be tuned by the RNAs incorporated into the droplet. These RNAs can affect the kinetics of the droplet fusion event (viscosity) or the return to a normal circular morphology (elasticity). (c) Droplet size and circularity (also referred to as the aspect ratio) can be controlled by RNA content. (d) Heterotypic RNA droplets can have widely divergent viscoelastic properties that prevent mixing (miscibility) or lead to formation of biphasic droplets. (e) RNA exchange rates with the surrounding environment can vary depending on the RNA contacts within the droplet and the viscoelastic properties of the droplet.

minima of their folding potential energies (73). Increased droplet size and decreased circularity may result as phase separation reaches equilibrium with the dilute phase and ages. Importantly, such in vitro aging of droplets leads to changed properties that recapitulate the aberrant state found in age-dependent neurodegenerations such as amyotrophic lateral sclerosis (ALS) and frontotemporal lobar dementia (FTD), in which liquid-like RNP droplets are converted to solid-like fibrils in motor neurons (see Section 6) (73).

Because RNA alters the C_{sat} of RBPs, RNA can also increase the observable droplet size—when more proteins can engage in LLPS, the area of the droplet increases (7, 106). Some RNAs have been observed to readily exchange between neighboring RBP granules in vivo (Figure 3e), implying that these condensates are dynamically interacting with the resident RNA (68). Similarly, addition of RNA in vitro increases droplet fluidity (6, 23, 58, 91, 106) and viscosity (58) (Figure 3b). In the case of the P granule protein LAF-1, the effect of RNA on droplet fluidity was measured by microrheology and fluorescence recovery after photobleaching (23). With both experimental methods, the addition of RNA increased the observed fluidity of LAF-1 condensates.

RNA granules also may have biphasic partitioning between a solid-like core and a fluid-like shell, and highly structured RNAs may be important for scaffolding the least fluidic subcompartments of the droplet (52). Despite these findings, how RNA affects the liquid properties of phase-separated droplets is poorly understood.

3.3. RNAs Can Help Subcompartmentalize Phase-Separated Droplets

Although most droplets appear symmetric and devoid of internal substructures, phase separations can occur within droplets, driven by differing viscosities and surface tensions (26, 84) (Figure 3d). Biphasic droplets have been observed in vivo and in vitro, and they can be modeled in simulations (26, 31, 47). The functional roles of biphasic droplets remain unknown, but they may play a role in spatial organization of nuclear compartments, as shown in *Drosophila melanogaster* embryos (45). RNAs have been shown to direct biphasic partitioning of granules. As stated above, distinct and immiscible droplets can form using different RNAs that bind to specific RBPs; Whi3 can phase separate independently with differing RNAs (48, 106). However, the functional role of biphasic RNA droplets remains unknown.

3.4. RNAs May Disaggregate Droplets by Preventing Additional Protein–Protein Contacts

Based on the aberrant LLPS observed in pathological inclusions in neurodegeneration, recent efforts have focused on how to disaggregate solid-like droplets. Potent disaggregases that disentangle specific droplets do so through strong interactions with target RBPs that eject protein molecules from aggregated droplets. For example, FUS is disaggregated by the nuclear import receptor Karyopherin- $\beta 2$ (Kap $\beta 2$), which sequesters the PY-NLS located in FUS's C terminus and thereby dissolves FUS condensates (32, 35, 77, 105). Likewise, the potentiated Hsp104 disaggregates TDP-43 and other phase separation-prone proteins (89). Although ejection of the RBP from the condensed phase was a hallmark of these and other disaggregases, high-affinity RNAs or highly concentrated RNAs may also serve as disaggregases by sequestering RBPs and thereby buffering phase separation (58). For example, if a particular structure of RNA is able to trap the RBP and preclude additional protein–protein or protein–RNA interactions, then it would limit multivalency and thus lower the phase separation potential of the RNP complex (29). This may be an effective mechanism for reducing phase separation through treatment with otherwise inert RNAs.

3.5. RNAs Are a Fundamental Component of RNA Binding Protein Droplets

As discussed above, many in vitro studies have demonstrated that RNAs have the powerful ability to concentrate RBPs and promote phase separation. They can lower C_{sat} , control intradroplet substructures, and tune the physical properties of droplets. All of these roles constitute means through which the cell can compartmentalize biological functions without the permanence of membrane-bound organelles. In cells, RNA-containing condensates can be reversibly created and destroyed, and—as with protein expression—RNA acts as another layer of droplet regulation.

4. RNA NUCLEATES RIBONUCLEOPROTEIN GRANULE FORMATION IN VIVO

Cellular RNP granules are composed of RNA and RBPs that together form a liquid-like complex to carry out a variety of processes in vivo. Both RNA and RBPs are necessary for the formation

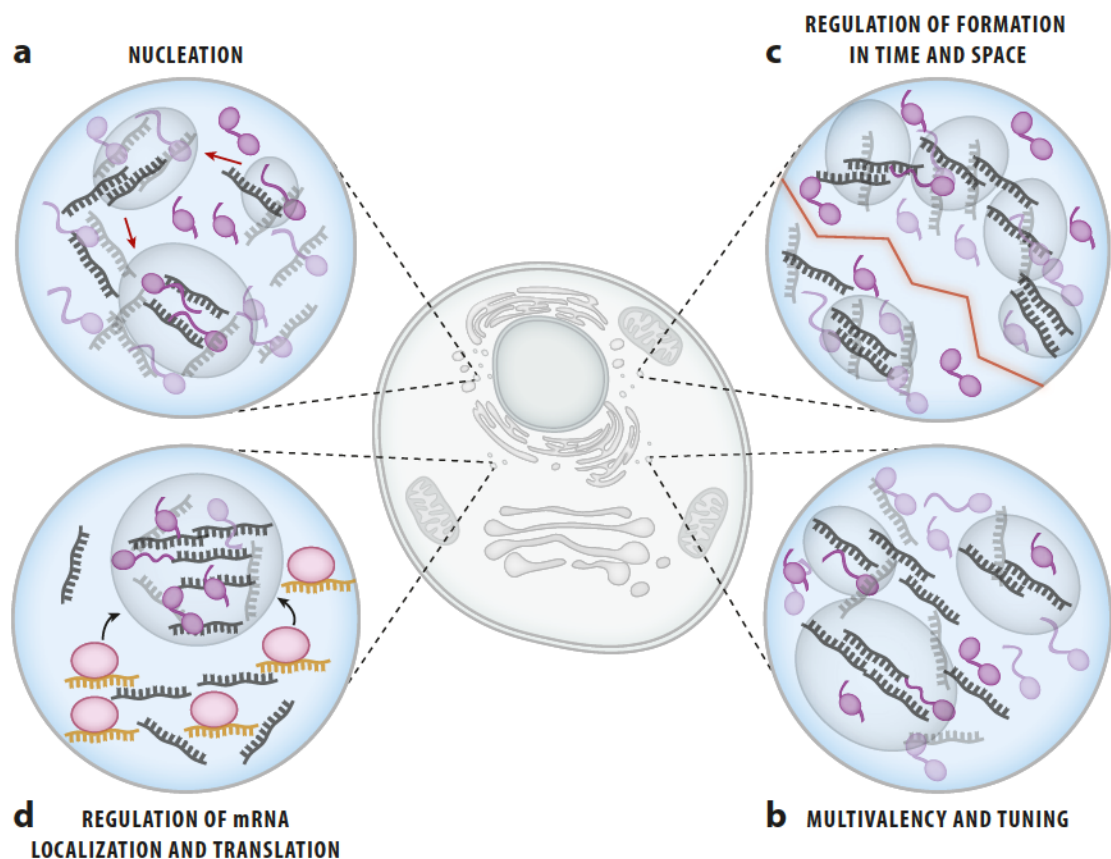


Figure 4

RNA contributes to ribonucleoprotein (RNP) granule formation and function. RNA can assist in the formation of RNP granules in many different ways. (a) RNA serves as a scaffold for RNA–RNA and protein–RNA interactions to seed the formation of RNP granules. (b) Specific RNAs can tune the elasticity or fluidity of RNP granules, forming distinct RNP granules. (c) The targeted localization of RNAs can control the spatial and temporal formation of RNP granules. (d) RNAs such as micro RNAs and piwi-interacting RNAs can assist in the recruitment of messenger RNAs to RNP granules for translational control and proper cell specification and function.

of RNP granules. The role of RNA in nucleating these bodies can vary. In many RNP granules, RNA serves as a scaffold for RNA–RNA and RNA–protein interactions to form these structures (Figure 4). These RNAs are often long, and some RNAs have expanded 3′ untranslated regions (UTRs) that allow for multivalent RNA–RNA and protein–RNA interactions (34). RNAs nucleate granules through RNA–RNA interactions and recruit RBPs to join these structures (52). Cellular granule formation can be promoted by RNA, as demonstrated in the nucleation of the nucleolus (7, 24, 66), P granules (23, 81, 86), bacterial RNP (BR) bodies of α -proteobacteria (2), processing bodies (PBs) (14, 22, 30, 54, 74, 78, 80, 83, 92), and stress granules (41–43, 96).

In the nucleolus, rRNA is necessary for nucleation (7). Pre-rRNA recruits nucleolar proteins, and reduced transcription of rRNA leads to a delay in the formation of the nucleolus with variable assembly outcomes (24). Similarly, for P granules in *Caenorhabditis elegans*, long mRNA molecules associate with PGL-3 protein with low sequence specificity and promote phase separation. This is seen at physiological conditions in vitro (81). Since MEX-5 has higher affinity for mRNA than does PGL-3, the addition of MEX-5 inhibits granule assembly of PGL-3 and RNA, thereby effectively preventing the RNA–RNA and RNA–PGL-3 interactions required to form P granules (81).

Thus, RNA can tune up or down the RNP condensation propensity in cells and may be critical for temporal and spatial regulation of cellular processes such as embryogenesis.

BR bodies are found in bacteria and form via interactions between positively charged patches of RNase E and negatively charged RNA. Subsequently, these condensates recruit proteins that function in mRNA decay pathway (2). PBs and stress granules both rely on the presence of RNA for nucleation and recruitment of proteins for assembly. In particular, PBs form by multimerization of DHH1, PAT1, and mRNA (78, 80, 92). The removal of RNA prevents PAT1 from promoting the formation of P bodies as multimerization with DHH1 is not supported without mRNA (78, 80, 92).

In certain nuclear bodies, specific RNAs are essential for formation. Assembly of paraspeckles requires NEAT1, a 4-kilobase-pair-long noncoding RNA (11, 19, 21, 27, 53, 61, 62). Accordingly, removal of NEAT1 by RNA interference completely eradicates paraspeckles, while the overexpression of NEAT1 leads to an increased abundance of paraspeckles. NEAT1 contains a self-complementary sequence that allows for intramolecular interaction, as well as intermolecular hybrids to create an interconnected lattice. In addition to nucleating paraspeckles, NEAT1 is also required for the recruitment of paraspeckle proteins PSP1 and p54 to the condensates (21, 27). Thus, certain RNP granules require specific RNAs to nucleate and recruit RBPs to further oligomerize and carry out their function.

RNA alone may be sufficient to form condensates, as demonstrated by repeat expansion RNAs that have been implicated in neurological disorders, including Huntington's and ALS. In a long chain of repeated segments, such RNAs can fold up into secondary or tertiary structures and engage in multivalent base pairing that promotes RNA focus formation *in vivo* and *in vitro*. Longer repeats promote more foci, and the RNA foci may exhibit liquid-like properties *in vivo*, as revealed by FRAP analysis (4, 25, 38). This finding demonstrates the importance of RNA–RNA interactions in RNP granule formation. Interestingly, the increased level of free mRNA in the cytoplasm induces the formation of stress granules, while an increase in protein concentration leads to their dissolution, suggesting the RNA-to-protein ratio as the key parameter in stress granule formation (12, 96). RNA is often observed to serve as the initial seeding for granule formation (Figure 4a). Therefore, RNA can impact the temporal and spatial formation of granules, regulate the recruitment of specific proteins for oligomerization, and serve as the key element that defines specific RNP granules.

4.1. RNA Provides Multivalent Interactions and Tunes Ribonucleoprotein Granule Properties *In Vivo*

In addition to providing a nucleation platform for RNP granule formation, RNAs also play a role in tuning the biophysical properties of RNP granules. The addition of total yeast RNA to a nucleolar protein FIB-1 lowered the C_{sat} for droplet formation, but it also led to accelerated coarsening of the droplets, suggesting a diverse role of RNA in phase separation dynamics (Figure 4a–b) (7). In contrast, LAF-1 droplets formed with RNA exhibited diminished viscosity and enhanced fluidity within the droplets, which points to a role of RNA as a fluidizer (23). In both cases, RNA altered the liquid-like dynamics within the droplets, reminiscent of the RNP granules formed *in vivo*. However, specific RNAs can also determine how liquid an RNP granule may be and create granules that are distinct from those formed by other RNAs. As we describe above, Whi3 form distinct granules with particular sets of mRNAs built for different cellular roles. CLN3 and BNI1 are the two mRNAs of different sequence and shape that condense with Whi3 into droplets with differing viscosity. Once formed, the CLN3 and BNI1 droplets become immiscible and physically segregated in the organism, indicating the critical function

of RNA structure, which governs assembly of distinct granules in vivo (47, 106). In summary, RNA provides a scaffold to initiate the formation of many RNP granules in vivo and tune the characteristic liquid-like properties of the cellular condensates (52, 76).

4.2. RNA Regulates Timing and Spatial Formation of Ribonucleoprotein Granules

RNA functions in regulating the temporal and spatial formation of granules in vivo (Figure 4c). In the Whi3 system, the G1 cyclin mRNAs, BNI1 and SPA2, form complexes with Whi3 in a polarized manner, thereby localizing these transcripts for the translation of polarity-setting proteins to induce symmetry-breaking events for the proper cell growth (48) (Figure 4c–d). Additionally, in *Drosophila*, approximately 200 mRNAs are enriched in the germlasm and play roles in germ cell formation, specification, survival, and migrations (94). For the development of *C. elegans*, MEG-3's access to RNA allows for the regulation of the timing and orientation of P granule formation. MEG-3 phase separates with the addition of RNA in vitro, but MEX-5 disperses MEG-3–RNA condensate by sequestering RNA needed to seed P granule formation in vivo. This mechanism allows for mRNA to act as the key regulator for P granule formation and dissolution in a space- and time-dependent manner (86). In addition, miRNAs play a role in targeting mRNAs to P bodies for directed mRNA decay (54, 74). Similarly, piRNAs that are in complex with Aub have been shown to localize to germ granules and use partial base pairing to bind mRNAs and recruit them into the germlasm (98). This suggests a mechanism that allows for localization of certain mRNAs to the posterior for translational control and thus effective germ cell fate specification and function.

5. MUTATIONS AND ABNORMAL ACCUMULATION OF RNA LEAD TO TOXICITY AND NEURODEGENERATIVE DISEASE

Disease-linked RNAs, such as G4C2 repeat expansion RNAs, can lead to an aberrant phase separation stimulated by unusual RNA structures that associate and aggregate with neighboring proteins. These pathogenic RNA granules can drive cellular toxicity and diseases including ALS and FTD (Figure 5). The addition of G4C2 RNA to cells promotes the formation of stress granules in a repeat length-dependent manner (25). The connection between repeat RNA focus formation and cellular toxicity was demonstrated by nuclear retention of pathogenic 38x and 72x G4C2 repeats, which caused apoptotic cell death (49).

In the case of myotonic dystrophies, the repeat expansion of CTG in the 3' UTR of the *dystrophia myotonica protein kinase* (*DMPK*) gene leads to a long repeat of CUG RNAs that are retained in the nucleus as a condensed focus (103). This nuclear accumulation of mutant RNA leads to the pathogenesis of both myotonic dystrophy types 1 and 2 (59, 97). Taken together, expansion of repeat RNA (both tri- and hexanucleotides) leads to the improper accumulation and aggregation of RNA in the cytoplasm and nucleus, which subsequently generates cellular toxicity and development of neurodegenerative diseases.

6. RNA CAN SEQUESTER RNA BINDING PROTEINS TO PREVENT AGGREGATION FORMATION

The ratio of RNA to protein is critical for the formation of phase-separated droplets in vitro as well as in vivo. The high RNA concentration in the nucleus buffers phase separation of FUS-like RNA binding proteins (58). TDP-43 is an RNA binding protein that forms cytoplasmic inclusions and causes neurotoxicity implicated in ALS and FTD. Adding RNAs with the TDP-43 cognate

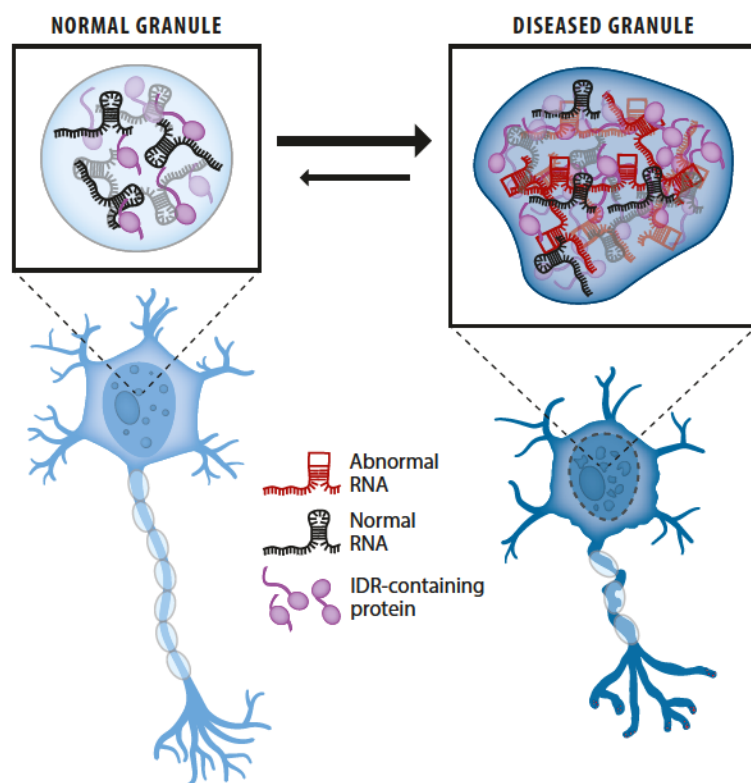


Figure 5

Role of RNA in aberrant phase separation. Expansion repeat RNAs with abnormal structures can cause accumulation and aggregation of RNA and intrinsically disordered proteins in RNP granules to promote cellular toxicity and neurological disease. Abbreviations: IDR, intrinsically disordered region; RNP, ribonucleoprotein.

sequence (Clip_34nt, rich in UG motifs) showed a loss of cytoplasmic assemblies in cells expressing optoTDP43, suggesting that RNA can rescue the formation of aberrant phase transitions and neurotoxicity. This may arise from the RNA, which buffers condensation by sequestering TDP-43 (60).

7. DISCUSSION

The role of RNA in the formation and the maintenance of RNP granules has been underappreciated. In this review, we show that RNA plays a major role in nucleating and assembling RNP droplets *in vitro* and *in vivo* via multivalent interactions (Figure 1). RNA may also serve to tune the different biophysical properties of condensates, including size, circularity, viscosity, elasticity, and surface tension. RNA can help regulate the timing and spatial formation of cellular granules by controlling the nucleation and properties of the resulting condensate. Importantly, RNAs are a functional entity of granules. RNAs can also drive liquid-like granules to an aggregated state to induce neurodegeneration. In particular, RNAs with repeat sequences can accumulate and create aberrant condensation in both the nucleus and cytoplasm that leads to plaque formation and neurodegeneration. In contrast, RNAs can also serve to fluidize RNP droplets. Physiologically, RNA-to-protein ratio is the key parameter throughout the cell, as the low and high ratios tune up and down the phase separation propensity, respectively. Therefore, RNAs are key players in cellular granule assembly and have many functions that will need to be further characterized moving forward.

7.1. The Molecular Grammar of RNA Structure and Phase Separation

The protein domains that contribute to phase separation have been extensively studied. Recently, certain amino acids have been implicated in controlling the physical properties of phase-separated condensates, revealing clues about the molecular grammar of disordered proteins (100, 105). How RNA sequence and structure modulate these properties is not understood. Above, we discuss several examples of proteins that display an RNA substrate specificity and different RNA shapes that drive condensates of varying viscosity and immiscibility (47, 106). Long and structured RNA may be associated with the stable core rather than the dynamic shell of stress granules. Meanwhile, unstructured RNAs that can engage in more promiscuous interactions may better support multivalency. In addition, there is a wide spectrum of RNA structural diversity that exists in dynamic equilibrium, but little evidence exists to demonstrate which structures may promote phase separation more effectively. Whereas a molecular grammar of amino acid composition in a subset of RNA binding proteins has been linked with droplet properties, the molecular grammar that links RNA structure to phase separation remains unsolved. Each disordered RBP has its own compendium of RNA binding specificities, but we have yet to identify how RNA folding affects phase separation of even one of these RBPs. The combinatorial effect of various structured and unstructured elements on a single RNA polymer is also unknown.

Importantly, certain RNA structures may preclude multivalency, such as GU-rich RNA sequences that inhibit TDP-43 oligomerization (29). If multivalency is inhibited, phase-separation propensity is drastically reduced (42). Therefore, a better understanding of how RNA structure promotes, inhibits, and tunes phase separation is critically important for designing RNA molecules that may act as potential disaggregases, for example. Because aberrant phase separation of certain RBPs—such as FUS and TDP-43—is linked with neurodegeneration, developing disaggregases of these proteins is important for treating patients. Nuclear import receptors are known to potently disaggregate these proteins (32), but overexpression of importins may lead to adverse effects in patients. By contrast, a designer RNA that specifically binds a certain RBP and can inhibit phase separation may be a potential therapeutic approach for neurodegenerative diseases such as ALS and FTD. Therefore, clearly linking RNA structure with phase separation is an important emerging area of study in the coming years.

7.2. RNA and Granule Function In Vivo

Many recent studies report on phase-separated granules that function in central biological processes including embryogenesis, epigenetic regulation, cell differentiation, signaling, replication, transcription, and translation. Further studies are required to fully understand RNP granules in vivo; these studies should encompass not only RBPs, but also RNAs and other polymers such as poly(ADP-ribose) that are generally found in these bodies. The understanding of RNA in the nucleation of these bodies and tuning of condensate properties demands molecular probing at the level of single RNA and single protein molecules. In addition, understanding the role of RNA–RNA interactions that can potentially seed inherently different condensates is critical, especially for disease-linked aberrant aggregates. As it did when performed on stress granules, more transcriptomic analysis of the components of RNP granules will inform us about the types of RNA that constitute the RNP granules. Finally, it will be critical to understand the normal function of RNA and RBPs in the formation and cellular activity of RNP granules to determine how normal RNP assembly can become pathological RNP granule assembly. Understanding how aberrant RNAs nucleate disparate assembly of RNA and RBPs to induce neurotoxicity will be important for our fundamental understanding of neurodegenerative diseases such as ALS, FTD, and myotonic dystrophies and may assist in developing therapies for treating these incurable diseases.

SUMMARY POINTS

1. Charge–charge and other molecular binding modes allow RNA to form multivalent interactions with proteins.
2. Phase-separated droplet properties such as viscosity, elasticity, size, area, and fluidity are tuned by constituent RNA molecules.
3. In vivo granule formation is accelerated with RNA via RNA–RBP contacts.
4. Mutations and abnormal accumulation of RNA, especially repeat-expansion RNAs, can lead to toxicity and neurodegenerative diseases such as ALS and FTD.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Our research in RNP phase separation is supported by funding from the National Institute of Neurological Disorders and Stroke, grant 1RF1NS113636, to K.R., V.V., and S.M. We also acknowledge the support from grants 1F31GM134641–01 to V.V. and 5T32GM007231–45 to K.R. and V.V.

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96. The authors report that RNA-RNA contacts may help condense stress granules in yeast.

100. The authors' mapping of FUS's LLPS grammar may serve as a roadmap for a similar effort with RNA.



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