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It's not just a phase: function and characteristics of RNA-binding proteins in phase separation

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Abstract

Biomolecular condensates that form via phase separation are increasingly regarded as coordinators of cellular reactions that regulate a wide variety of biological phenomena. Mounting evidence suggests that multiple steps of the RNA lifecycle are organized within RNA-binding protein (RBP)-rich condensates. In this review, we discuss recent insights into the influence of phase separation on RNA biology, which has implications for basic cell biology, pathogenesis of human diseases, and the development of novel therapies.

Introduction

Cells need to orchestrate an enormous number of chemical reactions that allow life to persist. During the last decade, macromolecular condensation that often occurs via phase separation has emerged as a mechanism for organizing these chemical reactions (Box 1). Membrane-less compartments that house biomolecules can be visualized as cellular speckles or foci by fluorescent microscopy and will be broadly referred to here as "condensates". Proteomic studies have revealed that these condensates are often rich in RNA-binding proteins (RBPs)[1,2]. RBPs are critical regulators of all steps of the mRNA lifecycle, which includes transcription, pre-mRNA processing, localization, translation and decay, and therefore they have a large impact on gene expression patterns. This, in turn, can affect cellular fate determination, tissue identity, and organism development[3–5].

Biomolecular condensates range in size from 20 nm (interchromatin granules) to up to 1–6 μm (P granules) in diameter[6,7]. One potential function of these condensates is to organize RNA synthesis, processing, metabolism, expression, and silencing, in different cellular regions, mediated at least in part by the RBPs contained within them[1,8–11] (Fig. 1).

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Competing interests

The authors declare no competing interests.

The coordination of RBP function through phase separation presents a new framework for understanding how gene expression is regulated in the cell. In the following sections, we will discuss the contribution of RBPs and RNA to condensate formation, and evidence for a functional interplay between RNA processing and phase separation.

RBP sequence features contribute to their phase separation capacity

A central feature of phase-separating biomolecules is their ability to orchestrate multiple weak noncovalent interactions[12–14]. RBPs achieve this multivalency through intrinsically disordered regions (IDRs), RNA-binding domains (RBDs), and dynamic post-translational modifications (PTMs) (Fig. 2).

Intrinsically disordered regions (IDRs)

'IDR' is a broad term that refers to a stretch of amino acids with low sequence complexity, low hydrophobic amino acid content, and lack of a well-defined 3D structure[15]. IDRs exist as an ensemble of conformations that facilitate multivalent interactions, which bolster phase separation capacity (Fig. 2a). Compared to the entire proteome, RBPs are enriched in IDRs and low complexity sequences[16,17]. IDRs in RBPs are repetitive and have an unusually high prevalence of glycine, arginine, lysine, and tyrosine residues, which are commonly found in domains that interface with RNA[17]. One specific type of IDR often present in RBPs are prion-like domains (PrLDs), which share sequence similarities with prion proteins[18]. Computationally, PrLDs have been identified based on the enrichment of glutamine and/or aspargine (Q/N) residues[19]. Cation-π interactions between tyrosine residues in PrLDs and arginine residues are the primary drivers of PrLD-mediated phase separation in several RBPs[14,20]. Glycine content increases condensate fluidity while glutamines and serines decrease dynamics within condensates[14]. PrLD containing proteins can form liquid-like condensates but also have the propensity to form solid or gel-like condensates, that may irreversibly aggregate[21,22] (Fig. 2b).

RNA-binding domains (RBDs)

RBPs interact with RNA through multiple types of RBDs, including RNA-recognition motifs (RRM), K-homology domains (KH), arginine-glycine-glycine (RGG) motifs, and zinc finger domains[23,24]. Interestingly, proteins that contain both RBDs and PrLDs are predicted to have a particularly high phase separation propensity, suggesting that interactions between these domains can cooperate to increase the ability of an RBP to condense[14,25]. Indeed, while IDRs are often sufficient to trigger phase separation, well-structured RRMs can modulate the effect, especially in the presence of RNA[22]. In contrast to RRMs, low complexity RGG motifs are poorly structured sequences often found within IDRs[26]. Because RGG motifs are typically repetitive, they can greatly expand multivalent interactions among protein and RNA substrates[26] (Fig. 2c).

Post-translational modifications (PTMs)

PTMs change the physical or chemical properties of amino acids, altering their hydrophobicity, bulkiness, or charge, and thereby strengthen or impair the multivalent interactions that underlie phase separation[27,28] (Fig. 2d). Arginine methylation and

phosphorylation of serine, tyrosine, and threonine residues are the best-studied PTMs that tune RBP phase separation. In general, arginine methylation impairs phase separation by reducing cation-π interactions between arginine and aromatic amino acids[14,20,29–31]. Accordingly, hypomethylation can induce the formation of pathological hydrogels and inhibits the dynamic properties of condensates[20]. Phosphorylation, on the other hand, can either enhance or block phase separation of RBPs. The introduction of a negatively charged phosphate group may promote electrostatic interactions that drive phase separation[32,33]. Alternatively, phosphorylation can weaken intermolecular interactions and may introduce electrostatic repulsion[34,35]. Therefore, the effects of phosphorylation are highly context specific and the modification of two different sites on a single protein may have opposite effects. Other PTMs including PARylation, ubiquitination, lysine acetylation, SUMOylation, and O-linked GlncNAc have been linked to phase separation in other proteins and may also impact RBPs[36–39]. Given that a single PTM can strongly suppress or promote phase separation, it will be interesting to determine how different PTMs cooperate to regulate RBP phase separation.

Alternative splicing affects the ability of RBPs to phase separate

Alternative splicing of RBPs can alter the presence of domains capable of multivalent interactions, thereby affecting the phase-separation propensity of the protein[29,40–43] (Fig. 3). For example, within the family of heterogeneous nuclear ribonucleoproteins A and D (HNRNPA and HNRNPD), five out of six members have mammalian-specific alternative exons that harbor IDRs[40]. Inclusion of these exons increases the number of glycine-tyrosine motifs, which enhances phase separation and promotes multimeric assembly of the proteins on pre-mRNA[40] (Fig. 3a).

Splice isoforms can also produce condensates with unique dynamics. Each of the three splice variants of HNRNPDL exhibits a different propensity of phase separation[41]. The longest HNRNPDL isoform contains an arginine-rich and tyrosine-rich IDR and has the highest tendency to phase separate in human cells, whereas the isoform that lacks these two IDRs does not phase separate under any condition[41]. Interestingly, the HNRNPDL isoform that only lacks the N-terminal IDR has the tendency to form amyloid aggregates (Fig. 3b), possibly due to the loss of electrostatic repulsion provided by the excluded IDR[41].

Furthermore, isoform expression can change the morphology of condensates. The shortest splice variant of the RBP FMR1 autosomal homolog-1 (FXR1) forms large, spherical condensates in U2OS cells, while the isoform containing a larger IDR is found in small, irregularly shaped condensates[42] (Fig. 3c). This may be explained by the fact that a longer IDR presents more sites for PTMs, which can discourage condensate fusion[42]. Indeed, the loss of low complexity sequences in RBPs through alternative splicing does not always lead to a reduction in phase separation. A short splice isoform of TAR DNA binding protein 43 (TDP-43) that lacks the C-terminal low complexity sequence is highly insoluble and has the ability to translocate full length TDP-43 into the cytoplasm where these two protein isoforms form aggregates[43] (Fig. 3d). Overall, these observations suggest that phase separation can be regulated by controlling expression of RBP isoforms, which might adjust the physical properties of a condensate and protect from pathological aggregation.

RNA sequence, structure, and concentration tune the properties of phase separated condensates

RNA itself can also nucleate the formation of condensates[44–47], possibly by acting as a scaffold that allows higher order assembly of RNAs and proteins. RNAs can recruit multiple copies of a single RBP or different RBPs[48–51] (Fig. 4a). For example, the long noncoding RNA *Xist* (X-inactive specific transcript) contains an E-repeat element that triggers condensate formation by multivalent interactions between four RBPs: polypyrimidine tract binding protein 1 (PTBP1), matrin-3, TDP-43, and CUGBP Elav-like family member-1[50]. The formation of this condensate, termed the Xi compartment, might contribute to *Xist*-dependent X-chromosome inactivation[50].

RNA is particularly important in dictating condensate composition[44–46] (Fig. 4a–b). RNA secondary structure can control the physical properties of a condensate and, even in the absence of protein, promote gel-like phase separation[52] (Fig. 4c). RNAs can alter protein conformation to induce phase separation in response to environmental stimuli. During cellular stress, mRNAs bind to the stress granule assembly factor G3BP1 and induce a conformational change which frees the RBD of this RBP to interact with RNA and promote protein clusters, eventually leading to condensate formation[53–55] (Fig. 4d).

RNA concentration also buffers phase separation[56,57]. A lack of RNA can lead to pathological liquid-to-solid phase transitions and RBP aggregation; however, high RNA concentrations can inhibit liquid-liquid phase separation[56,58] (Fig. 4e). For example, *in vitro*, the RBP FUS undergoes phase separation in the absence of RNA and the addition of 50 ng/μL of total RNA increases the amount of FUS incorporated into the condensates[56]. Increasing the RNA concentration inhibits the phase separation of FUS, as evidenced by decreased condensate size and reduced FUS levels in the condensates[56]. Nuclear RNA concentrations are estimated to be over ten times higher than the threshold required to dissolve these condensates *in vitro*[56]. These results may explain why RBPs that are soluble in the nucleus are often found as aggregates in the cytoplasm, where RNA concentrations are relatively lower[56].

The mechanisms by which RNA can promote and inhibit phase separation is an area of active investigation. One hypothesis is that RNA promotes RBP solubility either by competitive or allosteric inhibition of interactions between low complexity domains[57]. Another possibility is that at low levels, RNA facilitates phase separation by expanding electrostatic interactions between positively charged protein side chains and the negatively charged phosphate backbone of the RNA[59]. In this model, high levels of negatively charged RNAs would destabilize condensates due to increased electrostatic repulsion[59].

Distinct cellular condensates containing RBPs

Condensates allow creation of diverse, transient microenvironments without the costly process of generating a lipid membrane. Under this framework, sequestering or concentrating related RBPs and RNAs in condensates can modulate interactions between

these molecules. In fact, different steps of RNA biogenesis, processing, and decay appear to occur in unique condensates[1,8–11].

Nuclear condensates

Functional specialization of compartments is thought to expedite interactions between RBPs and their target RNAs. For example, histone pre-mRNA processing factors and histone mRNAs are concentrated within histone locus bodies that are distinct from nuclear speckles where other mRNAs are spliced[8–10,60] (Fig. 1). Less is known about the function of other nuclear condensates, including the perinucleolar compartment, nuclear stress bodies, and cleavage bodies. The perinucleolar compartment (PNC) is located on the edge of the nucleolus and harbors RNA splicing factors, especially PTBP1, and newly synthesized RNA polymerase III (pol III) transcripts. This led to the hypothesis that the PNC functions in pol III RNA processing[51,61]. Recent work has demonstrated that sequestration of PTBP1 in the PNC by the long noncoding RNA *PNCTR* can control splicing patterns of RNA polymerase II transcripts[51]. Like the PNC, nuclear stress bodies have long been thought to regulate splicing, but mechanistic details have been unclear until recently. Emerging evidence now indicates that nuclear stress bodies contain both RBPs as well as kinases, and can serve as a platform for the phosphorylation of splicing factors, ultimately blocking intron retention in over 400 transcripts[62]. Cleavage bodies are known to contain 3’ processing factors that aid in post-transcriptional pre-mRNA processing. Their presence increases during S phase, suggesting that these condensates regulate gene expression during the cell cycle[63,64]. Still, no studies have conclusively demonstrated that processing actually occurs in these condensates, and the identity of regulated RNAs is unknown. New technologies that allow a more thorough characterization of the composition and physical properties of these condensates will help in determining their cellular function.

Cytoplasmic condensates

Several cytoplasmic condensates regulate mRNA translation and degradation[1,11,65]. TIS granules form a mesh-like structure with the endoplasmic reticulum and are mainly composed of the RBP ZFP36 ring finger protein like-1 (ZFP36L or TIS11B)[11]. Although the mechanism is unknown, the assembly of TIS granules promotes the translation of AU-rich mRNAs[11]. In contrast, P-bodies sequester untranslated mRNAs and prevent access of the translational machinery, resulting in gene repression[1,66]. After temporary storage, some mRNAs are degraded while others are eventually released to be translated[1,65]. In response to cellular demands, P-bodies disassemble, possibly to provide a reservoir of mature mRNAs[1]. Release of their contents bypasses transcription to facilitate quick responses to stimuli.

Another function of cytoplasmic condensates may be to provide protection during cellular stress[67–71]. In response to environmental insults, such as heat, pH fluctuations, hyperosmotic stress, or UV radiation, RBPs translocate from the nucleus to the cytoplasm and interact with resident cytoplasmic proteins to promote the formation of stress granules, which contain stalled translation initiation complexes[2,54,72]. Stress granules are composed of over 100 different proteins and approximately two thousand mRNAs[73], and the mechanism by which they assemble is not fully understood. Nevertheless, G3BP1 has emerged as an essential protein for initiating stress granule formation. G3BP1 coordinates multivalent interactions

between RNAs and proteins to propel the system past the saturation threshold, resulting in phase separation[53–55]. Stress granule assembly may also be enhanced by interactions between stalled mRNAs[74]. Studies of individual stress granule components have revealed that some RBPs, such as the poly(A) binding protein cytoplasmic 1 (PABPC1) and the eukaryotic peptide chain release factor GTP-binding subunit (SUP35), are “stress sensors” that adapt their function to specific cellular environments[68,70]. SUP35 induces translational termination under normal conditions but forms liquid-like condensates in response to low pH[70]. Sequestration of SUP35 in these condensates inhibits protein production and protects the SUP35 catalytic domain from denaturation[70]. Upon the cessation of stress, SUP35 condensates disassemble and quickly shift the cellular metabolic program towards growth[70]. In this manner, the temporary storage of RBPs in condensates can allow rapid cellular recovery.

Sequestration of RBPs within stress granules can also alter gene expression programs to favor cell survival[71]. For example, the yeast DEAD-box RNA helicase (DED1P) moves into stress granules in response to heat. Because DED1P is thought to initiate scanning of housekeeping genes that contain complex 5’UTR structures, DED1P sequestration in stress granules silences housekeeping gene expression[71]. Functionally, this process is thought to promote the production of stress response proteins containing simple 5’ UTRs[71]. DED1P condensation is readily reversible when the stressor is removed, illustrating a switch-like nature of regulation by stress granule formation[71].

Given that condensates can regulate the processing, expression, and decay of RNAs, one open question is how RNAs translocate between condensates and whether a network of condensates exists to guide RNAs through various steps of their lifecycle. In one model, RNAs shuttle between polysomes, P-bodies, and stress granules to regulate gene expression in response to cellular demands, but the molecular details of this process are still unclear[75].

Recent evidence connecting phase separation and RBP activity

Although the functional relevance for RBP-mediated phase separation is still uncertain, there are indications that the phase separation propensity of RBPs correlates with their activity[35,76–78]. For example, mutations of the conserved glycine residues in the low complexity domain of TDP-43 increase both its phase separation and splicing activity[78]. Inversely, disruption of TDP-43 condensates through an N-terminal phosphomimetic substitution reduces its splicing function[35]. However, systematic mutagenesis of the TDP-43 IDR revealed several mutations that prevent TDP-43 phase separation but appear to have no effect on its splicing activity[79]. Therefore, additional studies are required to determine the role of TDP-43 phase separation and whether condensation per se enhances splicing. There is also evidence that phase separation of deadenylation factors is associated with acceleration of RNA decay[76,77]. Phosphorylation of the FMRP translational regulator-1 (FMR1) and the cell cycle associated protein-1 (CAPRIN1) drives phase separation which results in accelerated RNA deadenylation and repression of translation *in vitro*[77]. In cellular lysates, eukaryotic translation initiation factor 4E was excluded from these condensates, suggesting that translational inhibition may occur due to the physical separation of translation initiation factors from the target RNA[77].

Functional importance of condensate formation is further supported by recent studies demonstrating that removal or mutation of protein domains that promote phase separation in RBPs alters mRNA processing[35,40,80–82]. Both the RNA-binding fox-1 homolog-1 and HNRNPA/HNRNPD family members require formation of IDR-mediated higher order assemblies to promote exon inclusion in target RNAs[40,80]. Similarly, the PrLD of flowering control locus A (FCA) in *Arabidopsis thaliana* facilitates the formation of nuclear phase-separated bodies containing 3'-end processing factors which promote the use of proximal polyadenylation sites on target RNAs[82].

Pathological roles of RBP-mediated phase separation and therapeutic strategies

Dysregulation of condensates can cause gain or loss of RBP function, which can contribute to disease pathology. Indeed, aberrant RBP phase separation has been observed in the context of cancer and neurodegenerative diseases, as discussed below.

Cancer

Because phase separation influences gene expression and cell survival, it is unsurprising that condensates can be co-opted by cancer cells. For example, overexpression of the splicing regulator A-kinase anchoring protein-8 (AKAP8, also known as AKAP95) enhances the formation of condensates that are necessary for AKAP8-mediated intron retention in cyclin A2 (CCNA2), blocking nonsense-mediated decay of this transcript[81]. Ultimately, this results in upregulation of the CCNA2 oncogene which promotes cancer cell proliferation[81]. Oncogenic gene expression patterns can also be achieved by localizing spliceosome factors to condensates to promote aberrant isoform expression[60].

Oncogenic chromosomal translocations can give rise to a variety of RBP fusion proteins with altered phase separation properties. In Ewing's sarcoma, gene translocation of EWS RNA-binding protein-1 (*EWSR1*) and Fli-1 proto-oncogene (*FLI1*) gives rise to a EWSR1-FLI1 fusion protein. EWSR1-FLI1 recruits chromatin remodelers to tumor-specific microsatellites[83]. Interestingly, the domain responsible for EWSR1-FLI1 phase separation is also required for the regulation of cancer gene expression programs[83]. Recent work has illustrated that condensates formed by the FUS/EWS/TAF15 (FET) fusion protein recruit RNA-pol II to DNA which initiates aberrant gene expression[84]. This suggests the physical properties of oncogenic fusion proteins can drive cancer genome reprogramming, but whether phase separation is a common trait of fusion proteins remains to be determined.

Cancer cells can also hijack the formation of stress granules. This aids survival in harsh environments and allows the cells to outcompete the native cell population[85]. In metastatic tumors, the Y-box binding protein-1 is upregulated and stimulates translation of G3BP1 by binding to its 5'UTR[86]. G3BP1 expression initiates stress granule assembly and is correlated with poor survival in human sarcoma[54,86]. Whether stress granules enhance tumor metastasis directly is unknown, but one hypothesis is that they repress expression of genes that inhibit metastasis by sequestering their mRNAs.

These studies are only beginning to indicate links between phase separation-mediated processes and cellular proliferation and metastasis. An interesting topic of future investigation will be the contribution of phase separation to cancer hallmarks, including immune system modulation and maintenance of the tumor microenvironment.

Neurodegenerative diseases

Certain RBPs are commonly mutated in ALS and frontotemporal lobar degeneration[87,88]. These diseases are characterized by aggregation of proteins in the nucleus or cytosol. While FUS, TDP-43, HNRNPA1, and HNRNPA2 all undergo liquid-liquid phase separation under physiological conditions, ALS-associated mutations in these RBPs promote the formation of dense, pathological aggregates[22,89–91]. In ALS, mutations in the PrLDs of HNRNPA1 and HNRNPA2 strengthen their steric zipper motif, which increases polymerization and fibrillization[89]. Atomic structures of TDP-43 low complexity domain segments suggest that mutations associated with familial ALS may favor irreversible aggregation and promote the formation of amyloid fibrils[92]. Therefore, mutations that alter RBP structure may promote aberrant phase transitions, but pathological aggregates are also often found in patients without mutations in these RBPs[93]. In these cases, improper protein folding, or aberrant PTM deposition may govern the presence of aggregates[94–98].

How RBP-associated condensates transition to dense aggregates and how this contributes to neurodegenerative disease pathology is still uncertain[99]. Neuronal death may result from the failure to clear toxic aggregates, possibly due to a decline of protein degradation pathways[99]. Another hypothesis is that the incorporation of RBPs into aggregates blocks their ability to access their RNA targets and leads to dysregulated gene expression. Since a single RBP regulates thousands of transcripts, RBP loss-of-function can have profound consequences for gene expression; however, the identity of dysregulated genes that drive pathology in these neurodegenerative diseases is unknown, and represents an area of intense study.

Therapeutic strategies

Given the relevance of phase separation and protein aggregation to human health, strategies to prevent aberrant aggregation might offer therapeutic options. RNA oligonucleotides with high affinity for the TDP-43 RRM can block phase transitions and reduce neuronal cell death in tissue culture[57], suggesting that pathological aggregate formation may be targeted by RNA-based therapies. There has also been interest in identifying small molecules that can inhibit pathological aggregation[100–102]. One caveat is that several of these molecules inhibit aggregation by blocking liquid-liquid phase separation, which might impact physiological processes mediated by phase separation. Recently, a class of small molecules have been identified that induce TDP-43 liquid-liquid phase separation at low concentrations and promote the disassembly of condensates at a high concentration[102]. It remains to be determined if these compounds behave similarly *in vivo* and how they interact with other RBP condensates.

It has also been demonstrated that certain small molecules preferentially interact with specific condensates due to their physiochemical properties[103]. This kind of partitioning can impact concentration and pharmacodynamic properties of the drug[103]. This knowledge

can be harnessed to engineer compounds that are incorporated into particular condensates in order to increase target engagement.

Controversies and open questions

From the time that intracellular phase separation was first described, its application to explain diverse cellular phenomena, including signal transduction, transcription, RNA processing, and intracellular trafficking, has rapidly expanded. The sudden rise in popularity of phase separation has created challenges. What precisely constitutes phase separation in a biological setting is vaguely defined, exacerbated by a lack of clear standards for rigorous experimental characterization of phase-separation processes[104]. Analysis of phase separation has traditionally relied on qualitative observations of the physical properties of droplets (for example their roundness and tendency to fuse)[105]. Such qualities have recently been observed in systems that do not form via phase separation, underscoring the need to discriminate between alternative mechanisms[106]. These findings have evoked skepticism about the biological reality of phase separation in certain settings.

A further challenge is that condensate function is difficult to determine. Condensates are transient, and their extremely complex composition makes them difficult to reconstitute. Optogenetic platforms that allow inducible assembly and disassembly of condensates are now being used to probe their biological function[107,108]. High resolution microscopy techniques provide another approach for elucidating condensate function by tracking their protein and RNA constituents.

It is also important to consider whether the experimental methods used to manipulate phase separation also influence RBP function independent of condensate formation. Current studies often rely on mutagenesis of multivalent domains to alter phase separation and subsequently assess function. An important caveat to this approach is that loss of multivalency itself may alter RBP function. Recent work has demonstrated that transcription factor activation is enhanced by multivalency, but not via formation of phase-separated condensates[109]. Further studies dissecting the contributions of multivalency versus phase separation to RBP function are required to address this question.

Crucially, many RNAs and RBPs have only been studied *in vitro*, and their ability to phase separate in a physiological context is unknown. Experimental findings of *in vitro* studies are greatly influenced by the concentration of proteins and RNAs as well as buffer conditions. Another problem with *in vitro* studies is that they typically rely on bacterial expression systems A lack of physiological PTMs in the bacterially-expressed proteins may change their ability to phase separate. Furthermore, since high enough concentrations can cause virtually any protein to aggregate, it is important to consider whether the conditions of an assay are physiologically relevant. An attractive strategy to circumvent these issues may be to use cellular extracts, coupled with phase separation and functional assays[104]. These points underscore an urgent need for studies addressing the biological purpose of phase separation within the constraints of the cellular and organismal environment. Understanding the properties of condensates *in vivo* will be essential for harnessing their power as a therapeutic target.

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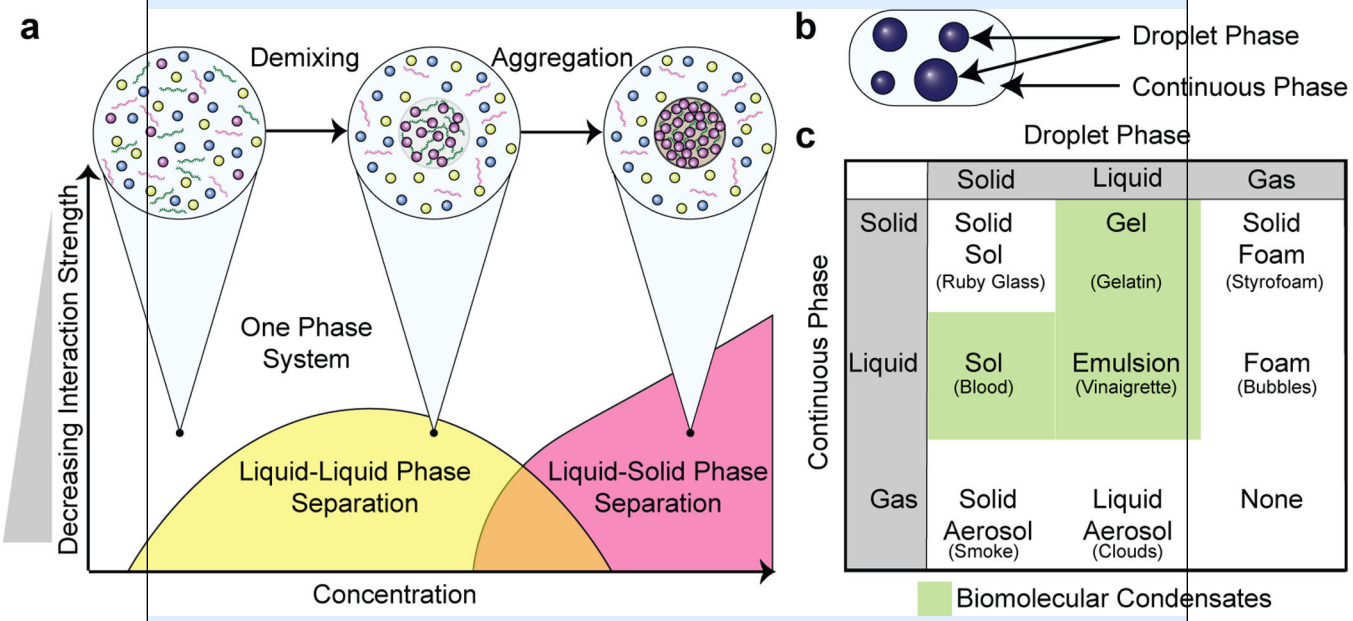
Box 1.

Phase separation: from colloidal chemistry to biological importance

Simply put, a phase is a system that is chemically homogenous and has distinguishing physical properties. Often, the term “phase” is used to refer to states of matter (i.e. solids, liquids, and gases); however, two substances in the same state of matter can exist in distinct phases. In biological systems, phase separation can occur when interactions between specific proteins and/or RNAs are energetically favored over other interactions. Such interactions must overcome the loss of entropy due to demixing[110]. The tendency for a system to phase separate is dependent on a multitude of chemical factors including temperature, ion concentration, pH, and macromolecular concentration (**a**). Mixtures containing a droplet phase and a continuous phase, separated by a phase boundary, are known as colloids (**b**). Before colloids were recognized in the cellular environment, the properties of such multi-phase systems were rigorously analyzed by the physicists Josiah Willard Gibbs and Johannes Diderik van der Waals[111]. William Hardy and Edmund Wilson were among the first to postulate that colloids could serve as a convenient way to compartmentalize the cell[112,113]. Still, phase separation was largely ignored by cell biologists until the late 20th century when new microscopy techniques permitted the high-resolution visualization of droplets within the nucleus and cytoplasm of the cell. These have been broadly termed “biomolecular condensates” which encompass a variety of physical interactions, with the most biologically relevant being liquid-solid and liquid-liquid phase separation (**c**).



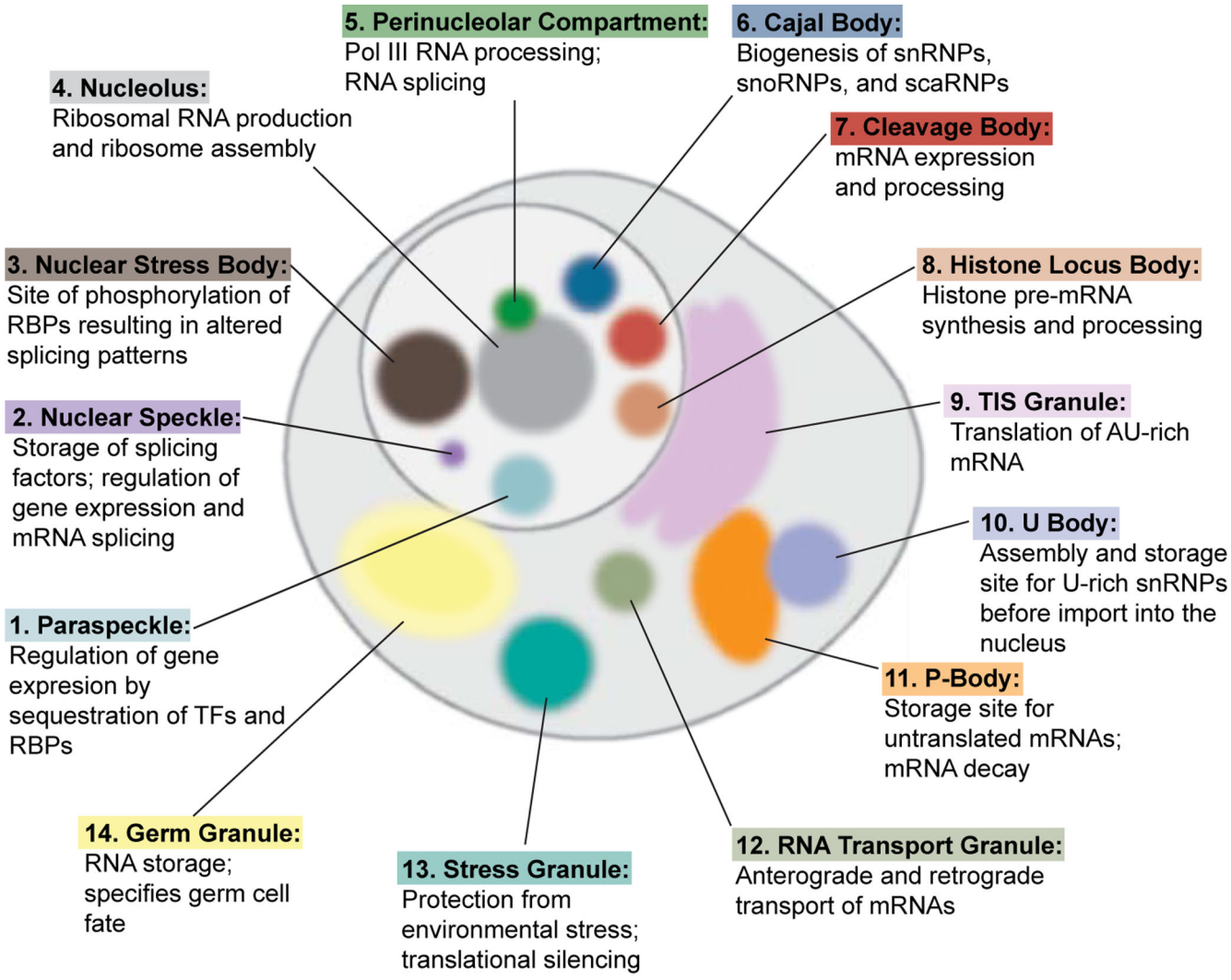


Figure 1. Distinct condensates impact various aspects of the RNA lifecycle.

Depiction of cellular condensates and descriptions of their proposed functions.

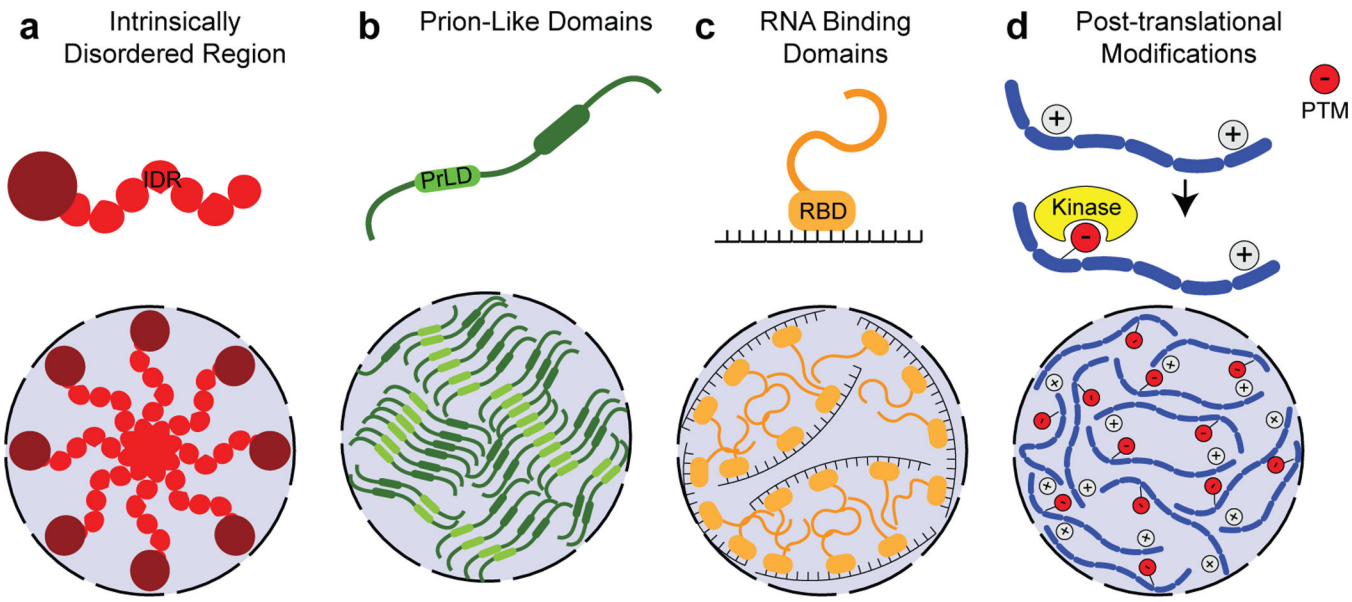


Figure 2. Protein domains and post-translational modifications coordinate multivalent interactions to drive RBP phase separation.

a, Intrinsically disordered regions (IDRs) adopt multiple conformations and often associate with other regions of low complexity. **b**, Prion-like domains (PrLDs) can form liquid-like droplets that may transition to aggregated or fibril-like condensates. **c**, RNA-binding domains (RBDs) allow RBPs to bind RNA motifs. Repeated RBD motifs coordinate RNA:RNA, protein:protein, and RNA:protein interactions to promote phase separation. **d**, Post-translational modifications (PTMs) can alter the charge of local protein stretches or induce conformational changes that strengthen interactions between proteins.

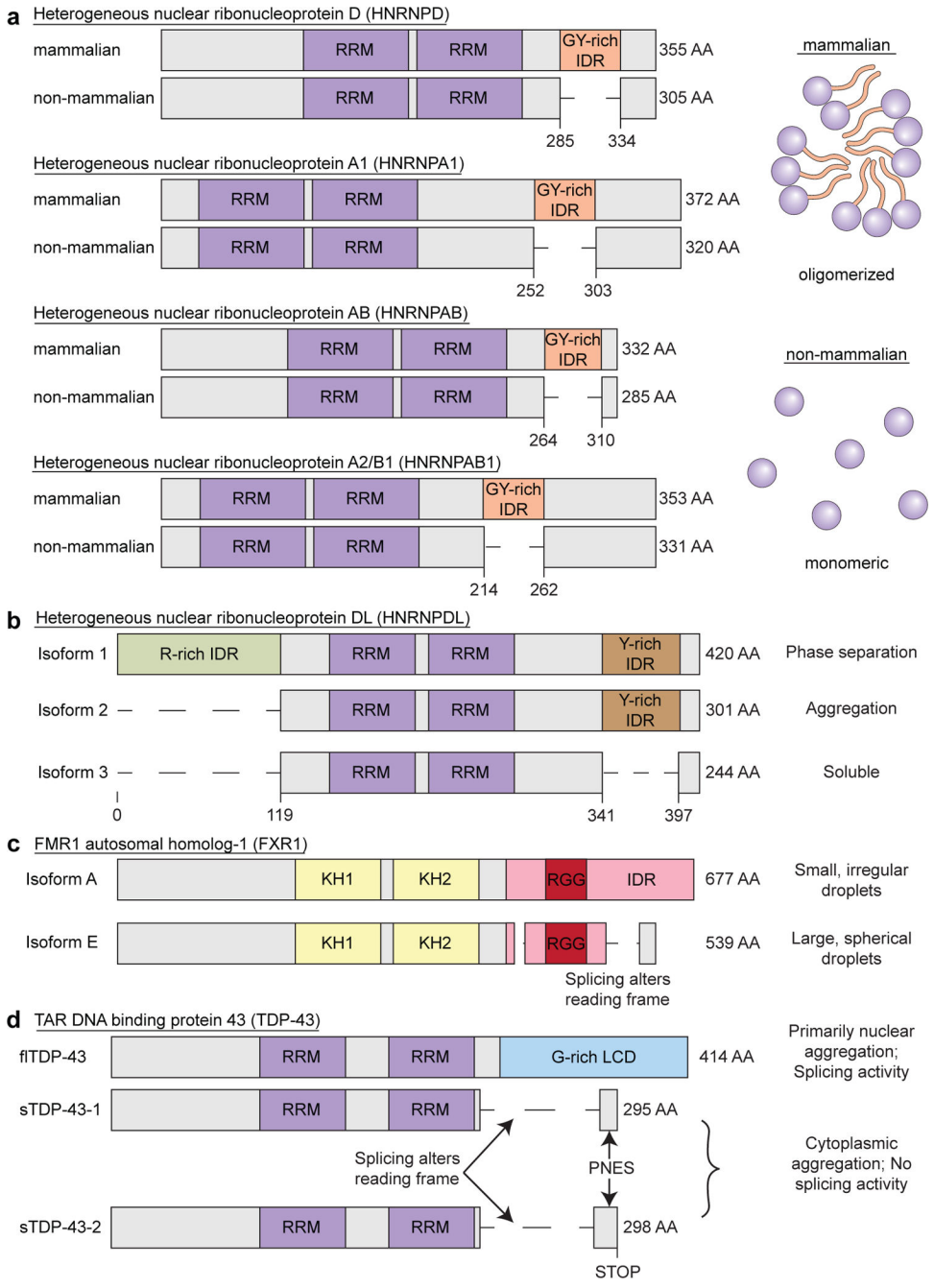


Figure 3. Alternative splicing changes the propensity of RBPs to phase separate and the physical characteristics of their condensates.

a, Mammalian isoforms of the HNRNPA and HNRNPD families include a glycine-tyrosine-rich intrinsically disordered region (GY-rich IDR, orange) that increases their ability to form high order assemblies. The IDR is skipped in the non-mammalian isoforms which do not form self-assemblies. **b**, Different isoforms of HNRNPDL display altered degrees of phase separation, likely due to the inclusion or skipping of two IDRs. **c**, FXR1 condensate morphology can be altered by alternative splicing. **d**, Alternative splicing shifts the reading frame of the short TDP-43 isoforms which generates a putative nuclear

export signal (PNES). The PNES alters the localization of granules from being primarily nuclear to cytoplasmic. AA, amino acids; RRM, RNA recognition motif (purple); R-rich IDR, arginine-rich IDR (green); Y-rich IDR, tyrosine-rich IDR (brown); KH, K-homology domain (yellow); RGG, arginine-glycine-glycine containing motif (red); IDR, intrinsically disordered region (pink); G-rich LCD, glycine rich low complexity domain (blue).

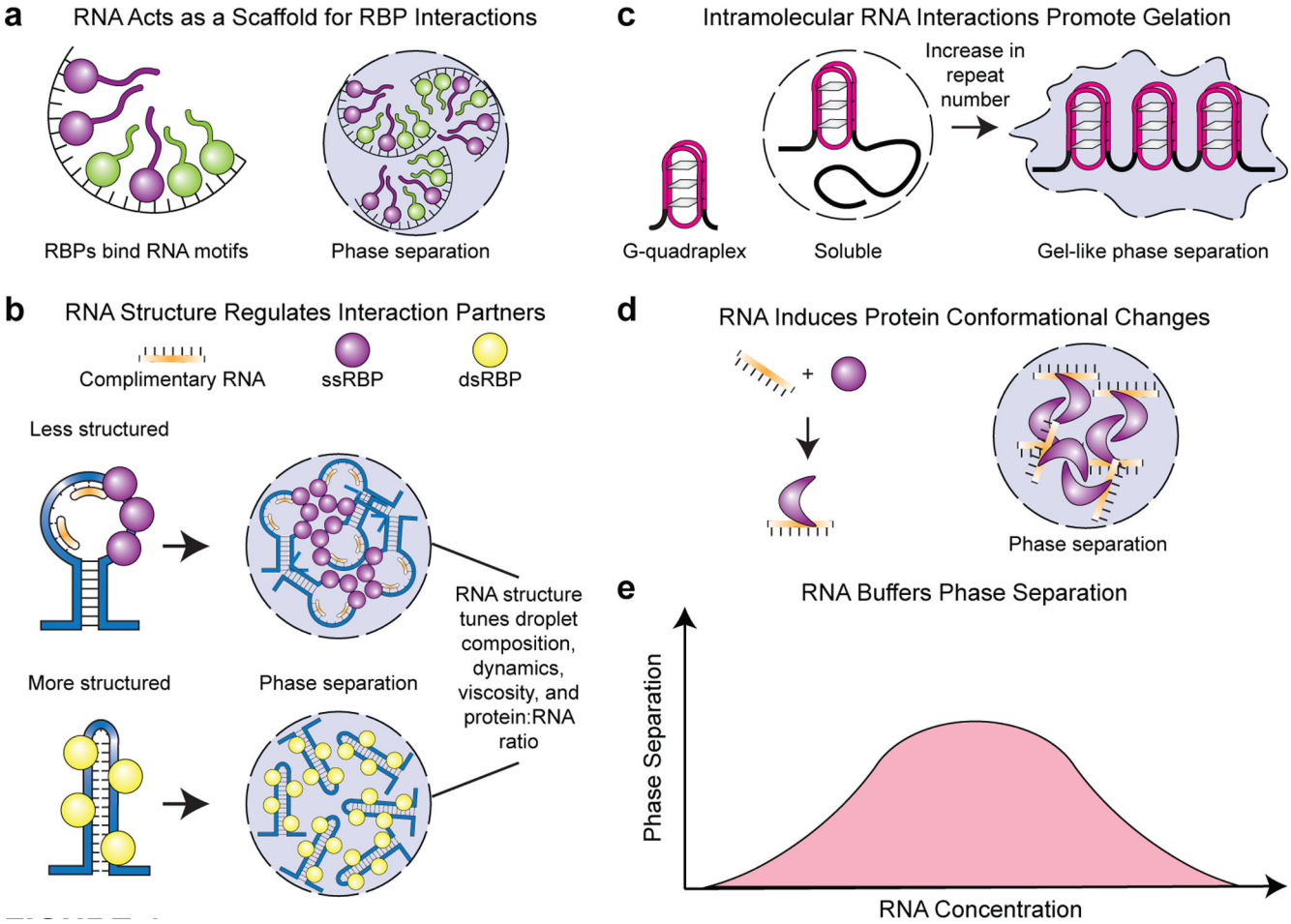


Figure 4. RNA influences phase separation and condensate composition through inter- and intramolecular interactions.

a, RNAs orchestrate the assembly of multiple RBPs to concentrate proteins, favoring multivalent interactions. **b**, Secondary RNA structure, such as RNA hairpins, can prevent binding of complementary RNAs and single stranded RNA binding proteins (ssRBPs) while promoting interactions with double stranded RNA binding proteins (dsRBPs). Therefore, RNA structure influences the composition of condensates and tunes their biophysical properties. **c**, G-quadraplexes form in guanine-rich sequences. Repeated poly(G) RNA sequences form gel-like condensates in the absence of protein. **d**, Protein-RNA interactions can alter protein conformation, allowing for additional interactions that promote or inhibit phase separation. **e**, RNA can increase the propensity for RBPs to form condensates, but high RNA concentrations inhibit phase separation.