HHS Public Access

Author manuscript

Nat Rev Mol Cell Biol. Author manuscript; available in PMC 2021 April 01.

Published in final edited form as:

Nat Rev Mol Cell Biol. 2021 March ; 22(3): 183–195. doi:10.1038/s41580-020-0264-6.

RNA contributions to the form and function of biomolecular condensates

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Abstract

Biomolecular condensation partitions cellular contents and has important roles in stress responses, maintaining homeostasis, development and disease. Many nuclear and cytoplasmic condensates form from RNA and RNA-binding proteins (RBPs), which undergo liquid-liquid phase separation (LLPS). Whereas the role of RBPs in condensates has been well studied, less attention has been paid to the contribution of RNA to LLPS. In this Review, we discuss the role of RNA in biomolecular condensation, and highlight considerations for designing condensate reconstitution experiments. We focus on RNA properties such as composition, length, structure, modifications and expression level. These properties can modulate the biophysical features of native condensates, including their size, shape, viscosity, liquidity, surface tension and composition. We also discuss the role of RNA–protein condensates in development, disease and homeostasis, emphasizing how their properties and function can be determined by RNA. Finally, we discuss the multifaceted functions of biomolecular condensates, including cell compartmentalization through RNA transport and localization, supporting catalytic processes, storage and inheritance of specific molecules, and buffering noise and responding to stress.

Introduction:

The term liquid-liquid phase separation (LLPS) describes the formation of two immiscible fluids from a single homogenous mixture. Many, but not all phase-separated biological condensates arise from RNA and protein. Although proteins, lipids and DNA can undergo phase separation independently of RNA, in this Review we discuss only RNA-containing condensates. Many phase-separated condensates studied to date are enriched in and depend on RNA, and proteins involved in the formation and function of these condensates often

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Author contributions

The authors contributed equally to all aspects of the article.

Conflict of Interest:

The authors declare no competing financial interests

Peer review information

Nature Reviews Molecular Cell Biology thanks the anonymous reviewers for their contribution to the peer review of this work.

have canonical RNA-binding domains. Many properties of RNA can promote its phase separation with RNA-binding proteins (RBPs). The most basic property is the negative charge of RNA, which promotes complex coacervation — de-mixing of polymers owing to opposing charges and likely promotes non-specific RNA interactions that contribute to condensation[1]. However, sequence-specific features are also known or likely to be relevant to RNA-driven phase separation. For example, the number and spacing of RBP binding sites are analogous to the 'stickers and spacers' models describing peptides undergoing phase separation[2–4]. Other features encoded in RNA sequences that have been shown to, or likely influence the LLPS include RNA modifications and RNA–RNA interactions, which can be considered a source of multivalency[5, 6]. Finally, it is well appreciated that peptides in condensates contain low complexity sequences or intrinsically disordered regions **[G]** (IDRs) that enable the weak, multivalent interactions to promote liquid-like properties; a similar role for unstructured sequences in RNAs likely contributes to LLPS, but the role of disordered domains in RNA has not yet been shown. Thus, although various physiochemical properties of RNA support their widespread association with condensates, the molecular understanding of these properties is still limited.

In cases where LLPS has been reconstituted using RNA and RBPs, RNA had potent effects on phase behaviour, both controlling the phase boundaries and material properties of the condensed state. For example, proteins of P granules **[G]** in *Drosophila melanogaster and Caenorhabditis elegans*, and cytoplasmic condensates of the RBP Whi3 in *Ashbya gossypii* will undergo phase separation at lower protein concentrations and in more physiological buffers in the presence of RNA[7, 8]. This is thought to be owing to a combination of charge effects and the ability of a single RNA molecule to recruit multiple RBPs, bringing proteins into close proximity to promote phase separation. Interestingly, whereas low concentrations of RNA support the formation of condensates, RNA at a sufficiently high concentration can dissolve reconstituted condensates; indeed, the high concentration of RNA in the nucleus is thought to prevent certain proteins from spontaneously demixing[9]. This dissolving capacity potentially causes re-entrant phase behaviour **[G]** of components in nuclear condensates, which can be predicted from polymer chemistry for particular combinations of concentrations of components[10]. Furthermore, depending on the condensate, the presence of RNA can promote distinct material properties of the whole or a part of a condensate[5, 8, 11–13]. Thus, RNA can regulate the formation, material properties, composition and permanence of condensates. Akin to proteins, RNAs are likely to function as 'scaffolds', which are essential structural components of condensates, as well as 'clients', which are non-essential molecules that are recruited depending on the composition and function of a given condensate[14].

In this Review, we discuss the current knowledge of, and key open questions related to how RNA regulates the assembly, properties and functions of condensates. We first focus on how RNA affects the formation and biophysical properties of condensates, discuss considerations for using RNA in condensate-reconstitution experiments and finally highlight some of the functions of biomolecular condensates in vivo. In vitro reconstitution of condensates from purified RNA and proteins is an essential step in demonstrating their mechanism of formation (LLPS or otherwise) and minimal biochemical complexity. Reconstitution of

RNA–protein condensates can be achieved using recombinant protein and in vitro-transcribed RNA at appropriate ratios and buffer conditions. Considerations for the protein component in these reconstitution experiments have been extensively reviewed elsewhere[15, 16], so here we highlight RNA contributions for the formation of condensates. The composition, length, structure, modifications and expression level of RNA can all potentially contribute to the features of native condensates such as size, shape, viscosity, liquidity, surface tension, and composition, and thus should be considered in reconstitution experiments.

In the second part of the Review, we discuss key functional roles of RNA-based condensates. The physiological functions of some condensates have been inferred from loss of function studies of an essential component of the condensate at the cellular level and/or in whole organisms[17–23]. The functions of condensates can be classified into four categories, namely cell compartmentalization through RNA transport and localization, supporting catalytic processes, storage and inheritance of specific molecules, and buffering noise and responding to stress. Particular condensates may act by performing one or more of these functions and, in disease states; mutations may disrupt or create new, pathological functions. In section two, we will provide examples of the 4 roles for RNA-protein condensates in cell and organismal biology.

The formation of condensates

In vitro reconstitution is a powerful tool in the study of phase separation, as this simplification of the process facilitates the assessment of mechanisms and comparisons with computational simulations and physical modeling. In this section we discuss RNA features that have been demonstrated to have important roles in condensate formation. Although no in vitro experiment can perfectly recapitulate, measure or address all in vivo RNA features, we encourage the reader to apply the considerations presented in this section in their research, think critically about what RNA feature or features are most relevant to their studies and design their experiments in a way that minimizes artifacts and maximizes usefulness and physiological relevance.

Challenges in studying the role of RNA in condensates

RNA-rich condensates are often complex mixtures of many different RNA molecules, making it difficult to recapitulate their compositional complexity outside of the cell. It is a common practice in in reconstitution assays to use homotypic RNA polymers — molecules that consist of a single type of nucleoside such as poly(A), and which are shorter than native target RNAs (RNAs that are enriched in a particular granule), and tend to be single stranded and poorly structured. Importantly, different single-nucleoside polymers (poly(A), poly(G), etc.) and different polymer lengths can yield considerably different condensate properties [24] (Figure 1A). Another important consideration is the sequence binding preference of the RNA-binding domain of the target protein (protein that is enriched in a particular granule), as different RNA-binding domains may favour different sequences, for example purine-rich vs. pyrimidine-rich or single stranded vs. double stranded[14, 25]. By using non-native, homopolymeric RNAs, assays fail to capture the complex contribution of the diverse native

cellular RNA population, which can dramatically alter all aspects of phase diagrams **[G]**, material properties and molecular composition of condensates.

Thus, caution must be applied when interpreting the results of in vitro studies of non-native RNAs. Such experiments probe only a limited dimension of RNA molecules — their function as an anionic polymer — while neglecting the rich array of native RNA properties. Ideally, condensate reconstitution would employ native target RNA sequences, which can confer distinct biophysical properties to the condensates[8], but these might be difficult or expensive to synthesize in vitro in sufficient quantities for phase separation assays. Furthermore, RNAs of higher eukaryotes are often too long for in vitro transcription, particularly in un-spliced form. Finally, another complication is that most RNA–protein condensates likely arise from a collection of many hundreds or thousands of RNA species present in a single condensate (for example, in stress granules **[G]** [26] and P granules [27]). P granule RNAs appear to be arranged in specific spatial patterns[13, 28], and the protein component of stress granules (and likely also the RNA component) is assembled in a specific temporal order[29]. Reconstitution of the full cohort of recruited RNAs can only be realistically achieved using whole cell extracts and/or through isolation from condensates, which requires large amounts of biomass that can be obtained from *Xenopus laevis* oocytes[30], budding yeast[31] or cultured mammalian cells[32], but may be more difficult to obtain from condensates observed difficult to culture cell types such as neurons. It is important to characterize the RNAs present and enriched in condensates, and a number of techniques such as crosslinking immunoprecipitation followed by RNA-seq, physical condensate isolation, and cell free induction of condensate formation have been employed for recapitulating complex condensates such as stress granules[33], P bodies[32] and neuronal transport granules[34]. Profiling the variety of RNA targets of condensates may provide hints to condensate function, and is useful for identifying target RNAs for reconstitution experiments.

Despite the limited complexity of composition, there is tremendous value in employing reductionist approaches to reconstitute RNA-based LLPS. Because reconstitution is based on tailored experiments with either native sequences or carefully engineered RNAs that have particular sequence features, it is the most feasible way to begin to dissect the mechanisms by which RNA affects condensates. Next, we consider the distinct features of RNA that are functionally relevant for determining and informing about condensate properties, including nucleotide composition and sequence, polymer length, structure, modifications and higher-order assemblies.

Regulation through RNA sequence and length

RNA is a polymer consisting of purines (A and G) and pyrimidines (C, U). Analogous to the contribution of amino acid sequence to protein biochemistry, purines and pyrimidines confer different properties to the RNA molecule and in turn to the condensate, which is composed of various RNAs. It was first noted already in 1910 that guanosine in high concentration forms a gel [36]. In studies with homopolymers of RNA, condensate properties were conferred by RNA sequence, RNA–RNA interactions and RNA–protein interactions[2]. For example, condensates formed of poly(G) RNA form fractal-like and gel-like networks with proline–

arginine-repeats peptides, in contrast to other homotypic polymers (A, U or C), which form more liquid-like condensates (Figure 1A). Condensates consisting of poly(A) RNA are more viscous then those comprising poly(U) or poly(C) RNA, whereas mixtures of poly(A) and poly(U) RNA form more solid-like gels[24]. Thus, even artificial homopolymers can have considerably different effects on condensate properties depending on the nucleotide composition.

RNA polymers in cells are typically complex mixtures of nucleotides. Sequence (chemical) complexity in RNA is generated during transcription and processing. Variable sequences can be made from the same gene through use of alternative transcription start and termination sites, polyadenylation sites, alternative splicing (especially in higher eukaryotes)[37], or through degradation by RNAses[38] (Figure 1B). These changes could deliver an RNA to a particular condensate in one cell type but not in another cell type through as simple a mechanism as extending or shortening the length of the RNA. For example, in mammalian cells, relatively long and less translated mRNAs are thought to be favoured over short RNAs for phase separation in stress granules and possibly other condensates [33]. Another example of regulation of phase separation by RNA length is observed in mammalian cells with the long non-coding RNA *NEAT1* (nuclear enriched abundant transcript 1)[39]. *NEAT1* has two splice isoforms: the longer isoform is recruited to paraspeckles **[G]** whereas the shorter isoform is recruited into microspeckles **[G]** [40]. A particular sequence or structure in *NEAT1* that varies between isoforms may change specificity for particular compartments through its interactions with a specific RBP, or through changing physiochemical features of the RNA such as length or structure that could influence localization. Therefore, it is important to verify that the correct RNA isoform is present in a condensate of interest using techniques such as fluorescence in situ hybridization[41]. In vitro production of the particular condensate-relevant splice isoform could be ideal. Pure, full-length RNA must be used for reconstitution as contamination with shorter RNA fragments can alter phase separation in multiple ways, making it impossible to quantify the concentration of full length RNA and altering or abolishing phase separation by forming spurious RNA–protein or RNA–RNA interactions.

RNA sequence and length is tightly controlled; multiple condensate-associated diseases are caused by alteration of RNA primary sequences, independently of, or in addition to the alteration of the encoded proteins — the best example being alterations in *C9ORF72* in familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD)[42]. Alterations of the target RNA sequence can be divided into RNA mutations that modify the levels of transcript (phase separation is less likely to occur if RNA levels are reduced) or of translation (active translation shields RNA from phase separation). Alteration of the RNA sequence can cause RNA-dependent and protein-independent phase separation, for example in the disease-causing G_4C_2 repeat expansion in the *C90orf72* gene, where phase separation of RNA can occur independently of protein, likely through formation of G-quadruplex structures[42, 43].

Alteration of the RNA sequence can also remove or alter the protein component of condensates in disease and in normal physiology. Alternative splicing of mRNAs encoding RBPs that undergo phase separation can regulate phase separation of the corresponding condensates, potentially through changing both RNA and protein length. This could occur

through alternative splicing (inclusion or exclusion) of RNA-binding domains (although these exons tend to be constitutive) or, more likely, through alternative splicing of IDR exons. An example of regulation of phase separation through IDR sequences and their alternative splicing is found in fragile X mental retardation syndrome-related protein 1 (FXR1), the mammalian autosomal homologue of fragile X mental retardation protein 1 (FMR1). A mutation in the IDR of the muscle-specific exon 15 of FXR1 results in congenital minicore myopathy[44]. Multiple FXR1 splice isoforms are developmentally regulated and thus alter the IDR composition of FXR1 and modulate material properties of FXR1 condensates[45]. Overall, particular care should be taken to study physiologically-relevant RNA and protein sequences, as these are more likely to be representative of in vivo condensates.

Tuning condensates through RNA modifications

Analogous to post-translational modifications of proteins, RNA post-transcriptional modifications help regulate important aspects of RNA function. All four RNA nucleotides are chemically modified[46, 47]. Among the most abundant and well-studied RNA modifications are *N*^{6}-methyladenosine (m^{6}A), *N*^{6},2'-*O*-dimethyladenosine (m^{6}Am), deamination of adenine to inosine (I), 5-methylcytidine (m^{5}C) and 5-hydroxylmethylcytidine (hm^{5}C), *N*^{1}-methyladenosine (m^{1}A) and pseudouridine (Ψ). These modifications could influence phase separation in at least three ways: by altering the protein interactions of modified RNA, by altering the RNA secondary structure[48], and/or by inhibiting or enhancing RNA–RNA interactions. Although even for the most abundant modification (m^{6}A), the levels of modified RNA are much lower in cells then the levels of unmodified RNA, modifications can be enriched on particular transcripts and locations in the mRNA, for example m^{6}A modifications are enriched near the translation stop codon [49, 50]. This differential modification of RNAs suggests their role in condensate formation is restricted to a subset of target RNAs.

RNA modifications are deposited in a highly regulated manner by 'writer' enzymes, removed by 'eraser' enzymes and recognized by 'reader' proteins[38, 51]. Binding of m^{6}A by the reader YTHDF2 has been reported to induce LLPS in mammalian cells[6] and the m6A binding protein YTHDF which is found in clusters at the periphery of stress granules, potentially promoting their formation by reducing the activation energy barrier and critical size[52].

It is likely that other RNA modifications and their regulatory proteins can undergo LLPS or potentially block RNAs from undergoing LLPS (Figure 2A). RNA modifications may be primarily found within specific condensates. Mutations in the proteins that regulate RNA modifications, such as adenosine deaminase acting on RNA 1 (ADAR1; also known as DRADA) and APOBEC proteins, can cause human diseases [53–56]. Furthermore, RNA modifications are commonly mis-regulated in disease[57] and have important roles in the response to viral infection[58]. The presence or absence of a modification can alter RNA structure and sequence, as shown for ADAR1[59] and APOBEC proteins[53]. APOBEC proteins deaminate cytosine to uridine and ADAR1 deaminates adenine to inosine. Inosine is chemically similar to guanine (and is read as guanosine during translation), and is capable of

pairing with cytosine in forming RNA structures. A major unanswered question is how RNA modifications that tune RNA structure (Figure 2B) and RNA–RNA interactions (Figure 2C) can regulate the formation of condensates.

The difficulty of studying RNA modifications in vivo is that many thousands of RNAs may be modified in a particular cell type, and mutation in a regulatory protein (writer, reader or eraser) can affect most or all of them. How then does one assign a particular function to a particular modification of a particular RNA? Localization of modified RNA to, or absence from a condensate of reader proteins may be an interesting avenue for future functional studies. Another experimental option would be to change or remove the modified nucleotide or modification consensus site and assess the effect on RNA localization in condensates and their function.

In vitro transcription reactions typically include a small amount of a randomly incorporated fluorescently labeled nucleotide (usually uridine). The problem of studying RNA modifications in a cell-free system is thus fourfold: producing the modified nucleotide required for in vitro transcription reactions, efficiently incorporating the modified nucleotide into the nascent transcript, incorporating it in the correct location, and not incorporating it ubiquitously. Addressing all these issues can be difficult. To minimally demonstrate protein binding to modified RNA and their phase separation together, random replacement of modified bases could be a first step to analyze potential consequences of modified bases. Although this does not often faithfully recapitulate native modifications, it could show whether RNAs modified with particular moieties modulate phase separation of a target protein.

The main unanswered questions regarding the roles of RNA modifications in condensates are: do RNA modifications other than m^{6}A induce phase separation? Do modifications serve as a sorting mechanism for protein delivery into, or exclusion from condensates (through an RNA-structure dependent or independent manner)? And do condensates facilitate the addition or removal of RNA modifications?

Probing the roles of RNA structure

RNA structure can have a vital regulatory role in the formation and identity of condensates, both with and without proteins[5, 42]. Thus, any in vitro reconstitution experiment ideally should consider the structure of target RNAs. Whereas DNA favours the formation of Watson–Crick base pairings, RNA can form non-Watson–Crick base pairs, which are necessary for the formation of triplex and G-quadruplex **[G]** structures and give rise to other complex secondary and tertiary RNA structures[60]. Functional consequences of RNA structures on condensates in cells are discussed below.

Techniques to measure RNA structure (particularly of long, complex RNAs) are in their infancy relative to methods that determine protein structure, generally cannot capture 3D shapes and provide static reflections of structure ensembles (that is, they average a continuum of multiple RNA structures)[61]. Regardless of these limitations, methods such as SHAPE/DMS and mutational profiling (SHAPE, or DMS)[62, 63] and psoralen analysis of RNA interactions and structures (PARIS)[64] are available and in some cases have been used

to study how RNA structure contributes to condensate identity. The basis of structure-probing methods such as selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE), is that single-stranded RNA is more reactive to chemical modification than double-stranded RNA and that the modified nucleotides are mis-read during reverse transcription and produce detectable mutations. Mutation profiling data are fed into RNA structure-prediction computational pipelines to predict RNA folding patterns. Computational prediction of RNA structure can be undertaken independently of RNA structure probing; however, this approach may be less accurate[65–68]. When examining multiple RNA sequences, or highly conserved, simple RNA structures, computational prediction remains a viable alternative to RNA structure probing when the latter is unfeasible.

How should the experimental design take RNA structure into consideration? RNA structures are sensitive to many factors, so the buffer should be carefully selected with regards to salt concentration, pH, ion composition and inclusion of a crowding agent (Figure 3A). It is important to select a buffer that yields optimal behavior of both condensate components (RNA and RBPs) and, if possible, is physiologically relevant (considerations for proteins have been extensively reviewed elsewhere[69]). The species that the reconstituted condensate originates from is an important determinant in the selection of ion concentrations and no universal buffer exists for phase separation assays, because ion composition varies between different cells of different species. For example, the concentration of potassium ions (K^+) in budding yeast can reach 300mM[70], whereas most mammalian cells have half that concentration of K^+ (150mM). The concentration of K^+ and of sodium ions (Na^+), which are the most physiologically relevant monovalent cations, can stabilize G-quadruplexes [71, 72]. However, by far the most important criterion for salt selection when working with RNA is the inclusion or exclusion of magnesium ions (Mg^{2+}). Addition of the chelating agents like EDTA, will remove magnesium from the solution. Magnesium ions stabilize RNA structures and facilitate folding[73]. If Mg^{2+} is included in the buffer (in addition to K^+ and NA^+), it is important to consider in what form (buffered or unbuffered) and in what concentration. In the cell, magnesium is mostly bound to proteins or other biological molecules; therefore, for a buffer to be physiologically relevant, Mg^{2+} must be either present in very low concentration or buffered by another biological molecule such as glutamate[74]. As magnesium and manganese (Mn^{2+}) are chemically similar, manganese can effectively replace magnesium in many contexts, and so a similar caution should be applied when using this ion in phase separation buffers. Thus, the choice of salt is critical in reconstitution experiments, in which RNA structure is an important feature.

Another consideration for buffer selection is the inclusion and choice of crowding agent. Crowding agents mimic the dense intracellular environment and facilitate phase separation, but their use requires caution, as addition of crowding agent in sufficient quantities induces phase separation of any protein or RNA. Commonly used inert crowding agents include PEG, ficoll, dextrans and spermine; however, these compounds are unlikely to be truly inert. Addition of crowding agents stabilizes RNA structure in particular confirmations[75–77]. This effect has been observed in ribozymes (RNAs that catalyze biochemical reactions). Ribozyme activity is facilitated by addition of molecular crowding [78] and addition of crowding agents dampens the impact of structure-disrupting mutations on ribozyme catalysis[79]. Crowding agents likely have a similar effect on structured RNAs that undergo

phase separation; in agreement with this possibility, RNA structures can be different in the condensed phase **[G]** compared with the dilute phase **[G]** [5]. Thus, some condensates might stabilize or favour particular RNA structures, or promote specific inter-molecular interactions.

A final consideration for buffer selection is the pH and the buffering agent. Not only will the pH alter the phase separation of a protein depending on its isoelectric point, RNA stability and structure is also affected by pH. RNA is most stable at pH 4–5 and unstable at more alkaline pH[80]. Extreme pH levels can help melt RNA structures [81]. Many important downstream RNA experiments such as reverse transcription and RNA structure probing are optimized for pH 8 and the use of TRIS buffer[82]. Certain stress conditions can alter the pH of the cytosol[83] and thus pH can be an especially important component in studying RNA structure in stress-induced condensates.

The interplay between transcription kinetics and RNA folding can influence which secondary RNA structures will form out of a range of theoretically possible structures. This is evident in in vitro transcription reactions, which can give rise to a different RNA structure than that formed when RNA undergoes denaturation and refolding (Figure 3B). This is due to fewer sites being sampled for RNA–RNA interaction (in trans and in cis) at the 5’-end region of the RNA than at the 3’-end region, because sites at the 5’-end are transcribed first. Melting the RNA by heating it to 95°C and refolding it can allow the formation of RNA–RNA interactions between the 5’ and 3’ end regions, which would not be observed in vitro but may occur in a cell owing to the activity of RBPs. This effect is the most readily observed in the reconstitution of in vitro transcribed long, highly structured RNAs such as the *CLN3* mRNA, melting and refolding of which alters multiple condensate properties[5]. Thus, ideally the decision of whether or not to melt and refold the RNA would depend on which RNA production method provides RNA that most closely mimics the cellular RNA structure profiles. RNA structures can be measured in vivo using chemical structure probing as described above. RNA structure can vary between particular subcellular compartments. For example, in SHAPE data of RNA derived from the chromatin, nucleus and cytoplasm, the same RNA may have different reactivity, suggesting it is adopting different structures in different compartments[86]. Thus, it may be necessary to fractionate cells to probe structures at different cellular compartments.

How do different RNA structures fold from the same sequence? Just as chaperones regulate proteins folding, RBPs and helicases regulate RNA structure and function[87–89] (Figure 3C). RBPs can inhibit or promote RNA–RNA interactions in cis and in trans, and RNA helicases can rearrange RNA structures in vivo and in turn regulate condensate properties[90]. The extensive regulation of RNA structures by RBPs is thought to be a major cause of the discordance between cellular and cell-free RNA-structure profiling[85]. Therefore, reconstitution of native or native-like RNA structures in a cell-free or protein-free environment can be tricky. The longer RNA targets may sample more secondary structures than shorter RNAs, and have different structures in vitro and in vivo. Furthermore, more than one structural conformation could have a regulatory function (for example, an alternative RNA structure can regulate alternative splicing)[91]. It is also possible that a particular structure may be favoured for inclusion in condensates. Thus, the cellular

environment carefully regulates RNA structures, like protein structures, and this regulation may have a role in determining the formation and properties of condensates in vivo.

Although RNA structure remains one of the most difficult–to-measure features of RNA, it has the capacity to profoundly alter condensate properties. RNA structures can license specific RNA–RNA interactions that influence the molecular composition of condensates[5]; however, RNA structure could influence condensates also at biochemical and biophysical levels. RNA structures can change the affinity and valency of the RNA molecule to RBPs, which would change the stoichiometry of condensate components (see below). Structures can potentially change the flexibility of the RNA molecule, will influence the volume occupied by RNAs and direct the formation of higher-order RNA structures and long-range interactions[92].

RNA–RNA interactions

RNA–RNA interactions are likely an important driver of condensate formation and could control their composition[5, 92]. RNA features that can promote RNA–RNA interactions include length; high GC content; lack of RNA structure, binding by RBPs or RNA modifications; and poor translation. Thus, enrichment of these features is likely to promote RNA undergoing phase separation. A single RNA molecule can associate within itself (cis RNA interactions) or with another RNA molecule (trans interactions). Trans interactions can occur between two RNAs of the same type or between two or more different RNA types, and can be promiscuous (as in the case of stress granules) or specific, as in the case of the cytoplasmic Whi3 condensates of the multinucleate filamentous fungus *A. gossypii*, [5, 925, 925,905,905, 785, 785, 785, 785, 915, 905, 895, 88] where the *BNI1* and *SPA2* mRNAs assemble together[5, 92]. RNAs can (self-) associate on their own, or proteins can facilitate RNA–RNA interactions by bringing RNAs together, as in the case of Argonaute proteins that bring siRNA, shRNAs and microRNAs together with their target mRNAs. The organization of mRNAs in germ granules of *D. melanogaster* highlights roles of yet another type of RNA interaction. A recent study using super-resolution imaging in vivo showed that after being targeted to granules in a sequence-dependent manner, mRNAs of the same sequence cluster within the granule separately of other mRNAs[93]. This indicates the existence of a mechanism of spatial organization inside granules, which is dependent on the identity of the RNA. Interestingly, the study also revealed that such self-sorting is based on a sequence-independent RNA–RNA interaction; thus, such like-RNAs self-assemble, but in a manner that does not require specific sequence elements.[93] This study suggests that RNA organization in germ granules, and perhaps in different RNA-rich condensates, is the result of an ensemble of properties including bound proteins and length, modifications and structures of RNAs, which results in homotypic condensation of RNAs within a larger condensate.

Crosslinking of RNAs inside or outside cells (using psoralen[64], AMT[94, 95] or dimethyl sulfide[96]) can be used to identify RNA–RNA interactions without *a priori* knowledge. RNA-RNA interactions likely have a specialized role in condensate formation in at least four ways: lowering the threshold for phase separation (nucleation), controlling condensate growth rates, sorting particular RNAs to a particular condensate, and creating a meshwork of

interconnected RNAs that scaffold condensates and confer important material properties[92]. With regard to facilitating nucleation, RNA dimerization or multimerization could promote phase separation by concentrating RBPs. Independently of nucleation, RNA–RNA interactions can promote condensate growth by providing more sources of multivalency. Interestingly, the RNA–RNA interaction can regulate sorting of particular RNAs into either the same or different condensates, with RNA structure masking or revealing sites of interaction[5].

Like RNA structure, measuring RNA–RNA interactions accurately is challenging. RNA–RNA interaction is easiest to prove between RNAs of two different types, as most crosslinking approaches rely on the sequencing of chimeric reads to identify interactions between two RNAs. RNA–RNA interactions can be estimated using RNAhybrid[97] or similar programs. The role of RNA–RNA interactions in phase separation has also been discussed elsewhere[92], but this understudied feature of RNA is an important emergent property that is informed by RNA sequence and structure.

Balancing the stoichiometry of RNA and protein in condensates

RNA expression levels are tightly regulated through transcription, stabilization and degradation, and RNA levels are important for the formation and maintenance of condensates. For example, transcription of rRNA can induce nucleoli formation[98], and transcription of *NEAT1* leads to paraspeckle formation in mammalian cells. Additional RNA regulation occurs at the level of cellular localization and interactions with RBPs. For example, concentrations of soluble RNA are typically higher in the nucleus than in the cytoplasm and consequently multiple RNA–protein condensates (paraspeckles, speckles **[G]**, Cajal bodies **[G]**, nucleoli, etc.) can be found in the nucleus. However, RNA in the nucleus has also been proposed to solubilize some phase-separating proteins and thus prevent pathological aggregation in mammalian cells [9]. Many diseases are associated with a failure of phase-separating RBPs to localize to the nucleus[9], because in the cytosol they assemble aggregates at least in part due to the lower levels of RNA[99]. Cytoplasmic RNA–protein condensates may be less common than nuclear condensates, because cytoplasmic RNA is protected from phase separation by translation and free RNA is destroyed by antiviral sensing mechanisms. Thus, RNA and RBP components of condensates must be tightly regulated in abundance and location.

When reconstituting phase separation in vitro, it is therefore important to consider physiological RNA and protein concentrations and ratios, as inferred from cellular measurements, to ensure that the cell-free observations are relevant. Particular care must be taken when considering the required concentrations of RNA and protein: if cellular concentrations are insufficient to promote phase separation, it is likely that additional regulation of LLPS is at play in cells. Altering RNA and protein ratios can result in more liquid-like or more gel-like condensates, which is similar to disease condensate states resulting from imbalance of RNA or protein components that lead to condensate ‘hardening’[100–103]. Therefore, in vitro reconstitutions must be informed by in vivo observations of RNA and RBP concentrations measured using methods such as mass

spectrometry, quantitative western and northern blotting or fluorescence correlation spectroscopy.

Another factor to consider when choosing RNA and protein concentrations is valency, or the number of protein contact sites for a given RNA. If the RNA-binding domain binds highly specific RNA motifs, valency can be estimated by counting the number of times the sequence appears in the RNA (Figure 4A and B), although interactions of RNA with disordered, low complexity domains of RBPs are more difficult to assess. For example, disordered RNA-binding domains such as RGG (Arg–Gly-rich) regions may bind a variety of different RNA sequences —RGG regions were shown to bind to poly(G), poly(A), poly(U) and weakly to poly(C) sequences[104]. Altering the number of interactions between RNA and RBPs may shift the phase diagram with increasing valency, thereby reducing the saturation concentration (Figure 4C and D) of RNA for phase separation and vice versa (Figure 4E, F and G), but specific situations might deviate from this simple prediction. Ideally, the binding of RNA to an RBP will be assessed first in vivo using crosslinking to identify genuine sites of contact, and if possible by removing the RNA-binding sites (through mutation or deletion) to abolish phase separation[105]. Although the valency of a specific RNA is unlikely to be variable in a given species (unless the RNA undergoes modification), it may be subjected to evolutionary selection, thereby enabling interesting analysis of condensates across species.

In summary, the physiochemical role of RNA in condensate formation remains an important understudied topic. Many challenges remain in studying the role of native and native-like RNA targets in a cell-free context, including chemical complexity (RNA sequence, length, variability, modifications) and sequence-dependent properties such as RNA structure, RNA–RNA interactions and RNA–protein interactions. Incorporation of these considerations into experimental design will result in reconstitution of more native-like condensates and improve our understanding of how the biophysical properties of condensates inform in vivo functions.

Physiological roles of RNA condensates

Condensates can regulate the localization and function of cellular molecules in space and time. In this section, we provide examples of RNA-rich condensates and their cellular functions.

Cell compartmentalization through RNA transport and localization

Cell size varies between and within organisms. Regardless of size, all cells must compartmentalize their contents to optimize their function, and one mechanism for achieving this is by compartmentalizing processes in different condensates[106, 107] (Figure 5). Cells of many sizes form condensates; however, diseases with condensate involvement seem to be associated with large cells such as neurons (Figure 5A), oocytes (Figure 5B) and syncytial cells such as muscle cells and filamentous fungi (Figure 5C). This association likely exists because large cells must expend more effort to actively compartmentalize their cytoplasm than smaller cells. In large cells, it is crucial to enclose mRNAs in condensates for long-range transport and for the often ensuing localized, on-demand translation. RNA

compartmentalization is conserved across multiple cell types and species. For example, the Staufen family of RBPs compartmentalize both oocytes and neurons in fruit fly and mouse[108109]. With regard to active RNA transport, Staufen proteins (Staufen in the fruit fly and STAU1 and STAU2 in humans) bind both double-stranded RNA and microtubules, and thus provide both RNA-target specificity (through the preference of their RNA-binding domain) and a mechanism of transport (microtubules), which is conserved from fruit flies to humans[108, 110]. Condensates can also 'hitchhike' on other cytoplasmic components. For example, annexin A11 helps transport RNA granules in neurons by tethering them to moving lysosome[111].

In neurons, active transport of RNA–protein condensates in the axon[111–113] is crucial for neuronal function, and multiple neuron-specific RBPs are localized to condensates that deliver RNA to sites of local translation to enable rapid cellular responses (Figure 5A). Mutations in proteins that are enriched in RNA transport condensates can lead to neurodegenerative diseases such as ALS[114]. Notably, RNA condensate transport is evolutionary ancient and used in filamentous fungi to transport specific mRNAs to the apical-tip cell using the same machinery as in neurons. For example, mRNP condensates in corn smut (*Ustilago maydis*) require the RBP Rrm4 and microtubules for establishment of polarity[115–119]. Thus, active transport of condensates has essential and conserved roles in compartmentalizing the cell cytoplasm.

A special case of compartmentalization occurs in syncytial cells, which comprise several nuclei sharing the same cytoplasm. These cells can form as a result of cell fusion, such as in muscle cells or syncytial trophoblasts, or through endocytosis without cytokinesis. In syncytial cells, each transcriptionally active nucleus sets a sphere of influence within the cytoplasm, and the different nuclei communicate to perform normal cellular functions. Condensates pattern the cytoplasm of syncytial cells, for example in muscle cells (formed by cell fusion[45, 120]) and in *A. gossypii*, which is a multinucleate filamentous fungus (formed by asynchronous endomitosis) (Figure 5C). Many other polyploid and syncytial cells exist[121], which may utilize condensates to compartmentalize their contents[109].

Supporting catalytic processes

Almost all eukaryotic cell types concomitantly form multiple RNA–protein condensates, such as P bodies, Cajal bodies, PML bodies **[G]**, paraspeckles, speckles, nucleoli, nuclear speckles, and transcription-related condensates[122, 123]. Many of these condensates form in interphase nuclei and dissolve during mitosis. Why form multiple condensates in the same nucleoplasm or cytoplasm? One reason is that different RNAs perform different functions in the cell and, in the absence of regulatory proteins, RNA has the tendency to aggregate in cellular salt concentrations in a cell free system[26]. Thus, one function of these condensates may be to sequester RNAs of different types and prevent irreversible aggregation. Indeed, recent work on the conserved stress granule protein G3BP1 provides evidence that it may have a role in limiting entanglement of long RNAs[124]. It is likely that another function of RNA condensates is to increase the rate of biochemical reactions involving RNA or the condensates RNAs help seed, by locally increasing the concentration of reaction components. This has been most easily ascribed to condensates that perform a specific

catalytic functions on target RNAs such as transcription[125], splicing in the spliceosome (not to be confused with nuclear speckles, which function to store and modify splicing factors)[126], ribosome assembly in the nucleolus[127], mRNA polyadenylation[128], and mRNA decay in P bodies[129]. Condensates can also concentrate essential non-condensate components [130, 131] and regulate and localize translation[132]. Emergent RNA-encoded biophysical properties are essential for sorting RNAs to distinct compartments in some cases[5]. Further work is needed to understand how condensate identity and sorting is achieved and maintained in the same cell compartment, given the liquid-like nature of many condensates.

Storage and inheritance of specific molecules

In germ cells, condensates can store RNA and proteins temporally or propagate condensate formation in daughter cells through multiple rounds of division. Germline granules, which have been reviewed extensively elsewhere, are an excellent example of storage of RNAs for propagation through cell divisions[133, 134]. Long-term storage is useful in cell types that do not frequently divide, as frequently dividing cells may rapidly dilute condensate contents. The influence of cellular lifespan on condensate lifespan is of particular interest given the wide variety of cellular lifespans that exist in different cell types in the same organism, from neurons that persist for decades to neutrophils, which have a half-life of only 6–8 hours. In long-lived cells, condensates were proposed to act as a ‘wastebasket’ for storage of problematic proteins and RNAs[135].

The role of condensates in long-term storage within a single cell has been most commonly observed in oocytes. In mammals, oogenesis is completed during development and the oocytes must be stored until puberty and fertilization, which can take decades. Maternal RNA must be preserved during this time and until the activation of zygotic gene expression following fertilization. One way to preserve the maternal RNA is through storage in condensates. The RBP FMR1 was proposed to perform this function in different organisms. In *D. melanogaster* oocytes, FMR1 may form condensates consisting of long maternal RNA, and loss of FMR1 reduces the functionality lifetime of oocytes[136] (Figure 6A). Similarly, in humans, FMR1 mutations, which cause fragile X syndrome are associated the formation of FMR1 inclusions and with infertility in women[137, 138], suggesting that RNA storage in oocytes is a conserved regulatory mechanism (Figure 6B). Finally, in *X. laevis*, the IDR- and prion-domain-containing protein Xvelo (also known as velo1) is crucial for the formation of the Balbiani body (a condensate containing proteins, RNA, mitochondria and other organelles) in the oocyte through assembly of an amyloid–like network with RNA and mitochondria[139] (Figure 6C). What governs the dissolution of long-term storage condensates is still poorly understood, but some examples include the disruption of interactions between condensate components[140], modification of the scaffolding RBP[141–143], and engagement of a chaperone[144].

Buffering noise and responding to stress

Condensate formation can help cells to rapidly respond to environmental cues and thus promote survival. The buffering capacity of condensates can serve two purposes: suppressing naturally occurring transcriptional and translational heterogeneity between cells,

and responding to acute stress. Single cell RNA-seq and proteomics analyses have revealed that no two cells are exactly identical and that the transcriptional profile of the cell often has little to do with its protein expression profile[145]. How do cells retain their identity in conditions of transcriptional and translational noise? One mechanism may be through the formation of RNA–protein storage condensates[146]. If cellular condensates obey physical principals of LLPS, in the case of a simple two-component system, then above a critical concentration for demixing, the soluble concentration will remain constant while the volume fraction of the condensed phase continues to grow[7]. However it is important to note, that in cells, such a simple prediction is unlikely to be correct because of the compositional complexity of condensates[147]. In this way, condensate formation could ensure maintaining a constant concentration of soluble molecules despite fluctuations in their number. Alternatively, excess of cellular components could be stored in condensates, thereby allowing responding to cues more rapidly than can be achieved by changes in transcription and/or translation. Indeed, properties of a long-lived condensate can be altered rapidly following the recognition of a specific stimulus; for example, nucleic acid cues facilitate LLPS of the innate immunity factor cGAS and thus promote its activity[148]. Finally, condensates can be formed to deal with acute stress. For example, acute cellular stresses such as heat shock, trigger translation shutdown, release of mRNAs from the translation machinery and the formation of mRNA-containing stress granules, which sequester mRNA and proteins until the stress is resolved[149, 150] (Figure 5D). P bodies are another type of RNA–protein condensate that forms in response to acute cellular stresses[129]. Condensates can also form from RBPs that are removed from RNA during stress, as in the case of polyadenylate-binding protein in budding yeast[151].

Conclusion and future directions

The formation of membraneless, RNA-dependent condensates is a tightly regulated process that depends on RNA sequence (identity, length, modification), on various sequence encoded properties (RNA structure, RNA–RNA interactions, RNA–protein interactions) and on RNA and protein expression levels. Collectively, these RNA encoded features confer specific condensate biophysical properties, which are essential for condensate functions in homeostasis. The consequence of altering condensate biophysical properties can be loss of cell function and disease. Following a decade of research focus on the protein components of condensates, many exciting, unexplored avenues exist for future study and methodological advances. Although RNA is challenging to study, we hope to have inspired the reader to engage with the expansive potential of RNA in condensate biology.

Acknowledgments:

We thank Dr. Christiane Iserman for critical review of this manuscript. This work has been funded by: R01-GM081506 (NIH-NIGMS) (A.S.G.), HHMI Faculty Scholars program (A.S.G.), T32 CA 9156–43 (C.R.), F32 F32GM136164 (NIH-NIGMS) (C.R.) and the L’Oreal Women in Science Fellowship (C.R). The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

Glossary:

P granules

presumptive germ cells granules; a non-membrane bound.

Stress granules
a membraneless condensate consisting of RNA and protein that appears in cells in conditions of stress.

G-quadruplex
a nucleic acid four-stranded structure formed by guanine-rich sequences.

Paraspeckles
a membraneless condensate consisting of RNA and protein in interphase nuclei; scaffolded by the long non-coding RNA *NEAT1.*

Microspeckles
a membraneless condensate consisting of RNA and protein in interphase nuclei; scaffolded by the long non-coding RNA NEAT1 splicing isoform 1–1.

Speckles
A membraneless condensate in the nucleus that stores and modifies splicing factors.

Cajal bodies
also known as coiled bodies. Membraneless nuclear bodies containing RNA and protein that are sites of post-transcriptional modification of small nuclear and nucleolar RNAs.

PML bodies
membraneless bodies consisting of RNA and protein and scaffolded by the promyelocytic leukemia (PML) protein. May have roles in apoptosis, cell division and response to viral infection.

Phase diagrams
a chart used to show the state of a mixture at equilibrium, depending on particular conditions; for biomolecular condensates, this state is often a function of concentration of mixture components such as protein and RNA.

Condensed phase
Refers to solid or liquid condensates, which form by electrostatic interactions and de-mixing from solution.

Dilute phase
The molecules remaining in solution, unincorporated into either solid or liquid condensates.

Re-entrant phase behaviour
This behavior is seen when sufficiently high concentrations of a component, such as RNA, prevents condensation, in many cases due to charge repulsion effects.

Intrinsically disordered regions
(IDRs) Sequences of RNA or protein that lack defined three-dimensional order or structure.

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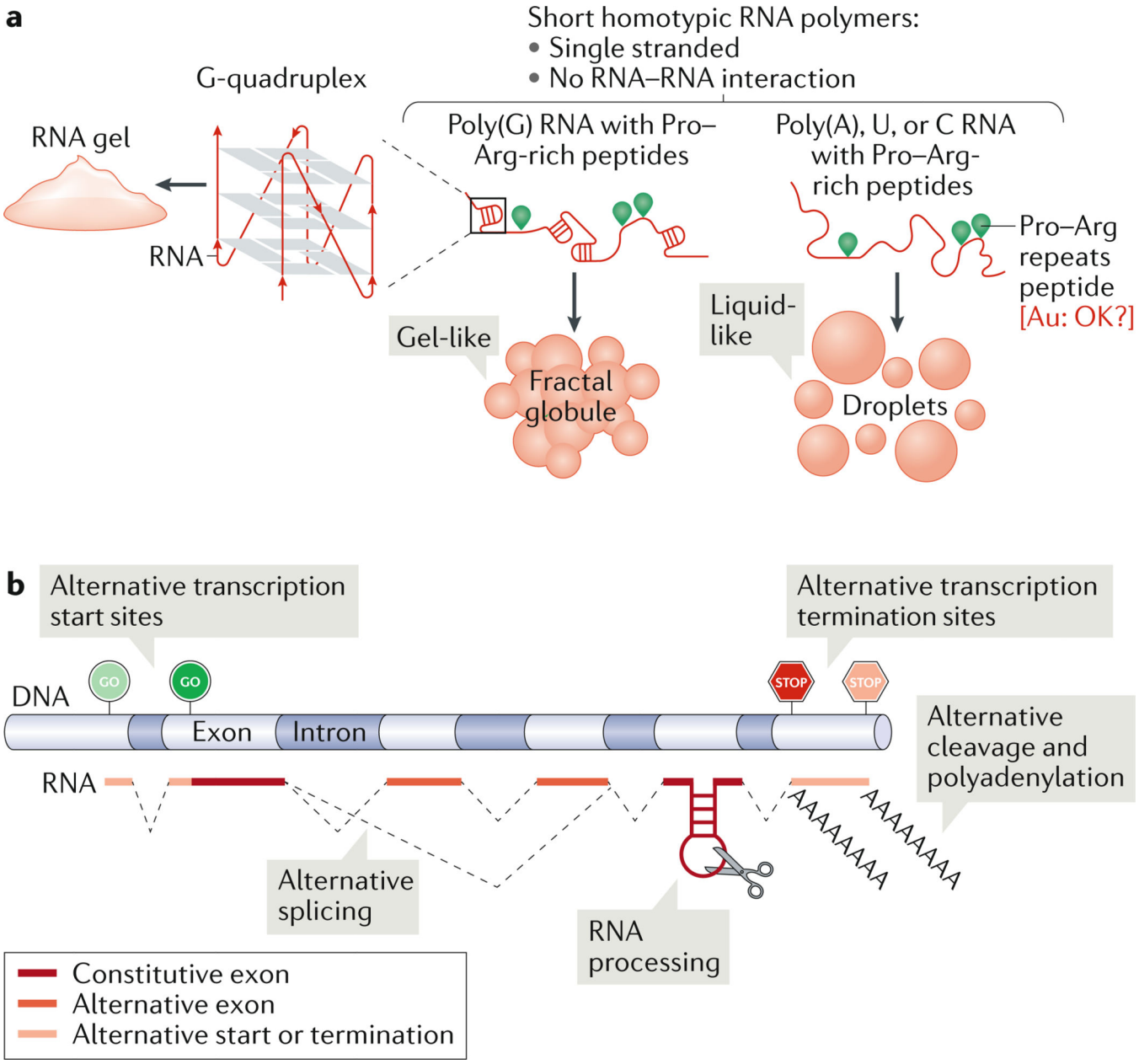

Figure 1: Regulation of condensate properties through RNA sequence and length.

(A) Homotypic RNA polymers mixed with proline–arginine-repeats peptides yield either liquid-like (poly(A), U or C) or gel-like (poly(G)) condensates. At high-enough concentrations, poly(G) RNAs can form G-quadruplexes and RNA gels. **(B)** Generating RNA sequence (chemical) complexity. RNA sequence length can be regulated by choice of transcription start or termination sites, alternative splicing, RNA processing or alternate cleavage and polyadenylation.

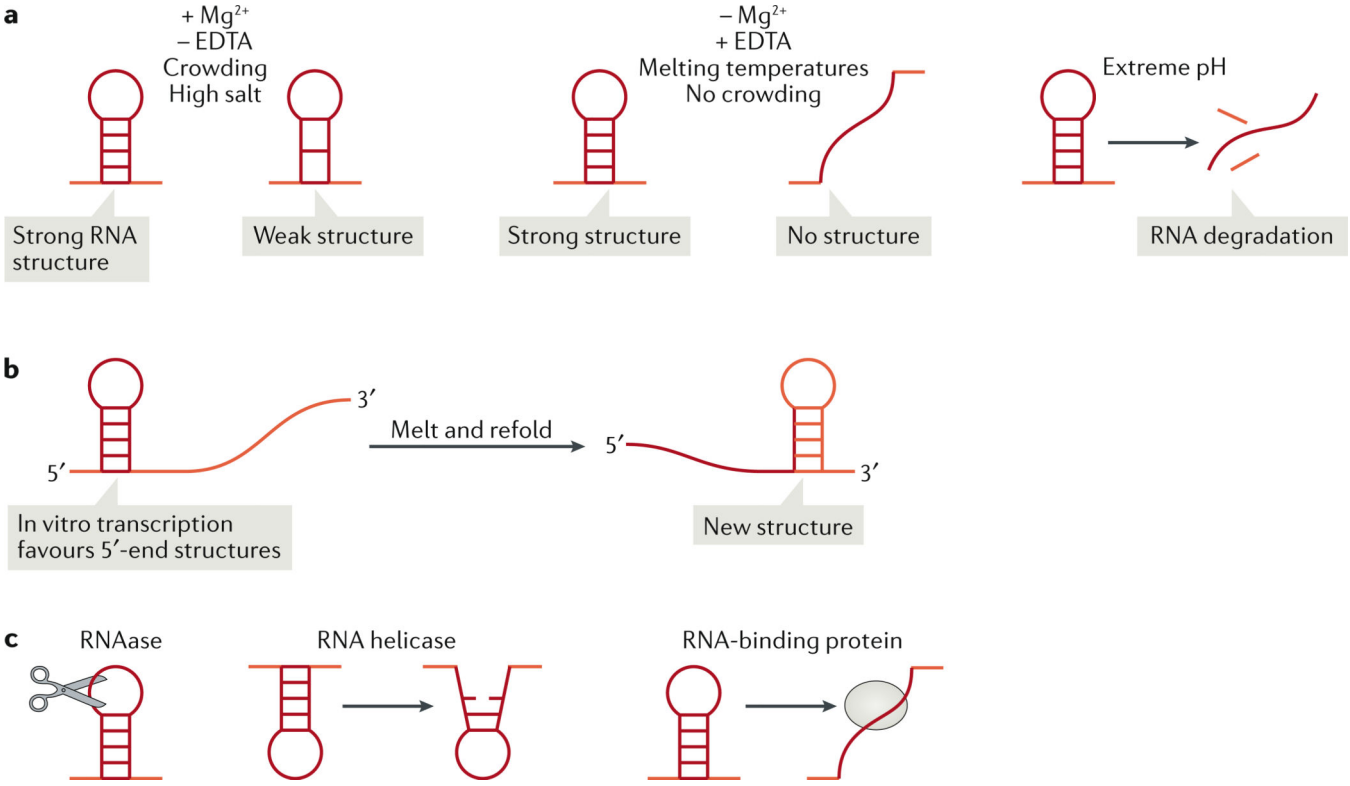

Figure 2: Tuning condensate properties through RNA modifications.

(A) RNA modifications are recognized by ‘reader’ proteins. Interaction with a reader can enhance or inhibit RNA undergoing liquid–liquid phase separation (LLPS). **(B and C)** RNA modifications can tune RNA structure by blocking or promoting RNA–RNA interactions in cis, within the same RNA molecule **(B)** or in trans between different RNA molecules**(C)**. Modification of RNA sequence and structure could alter phase separation of target RNA.

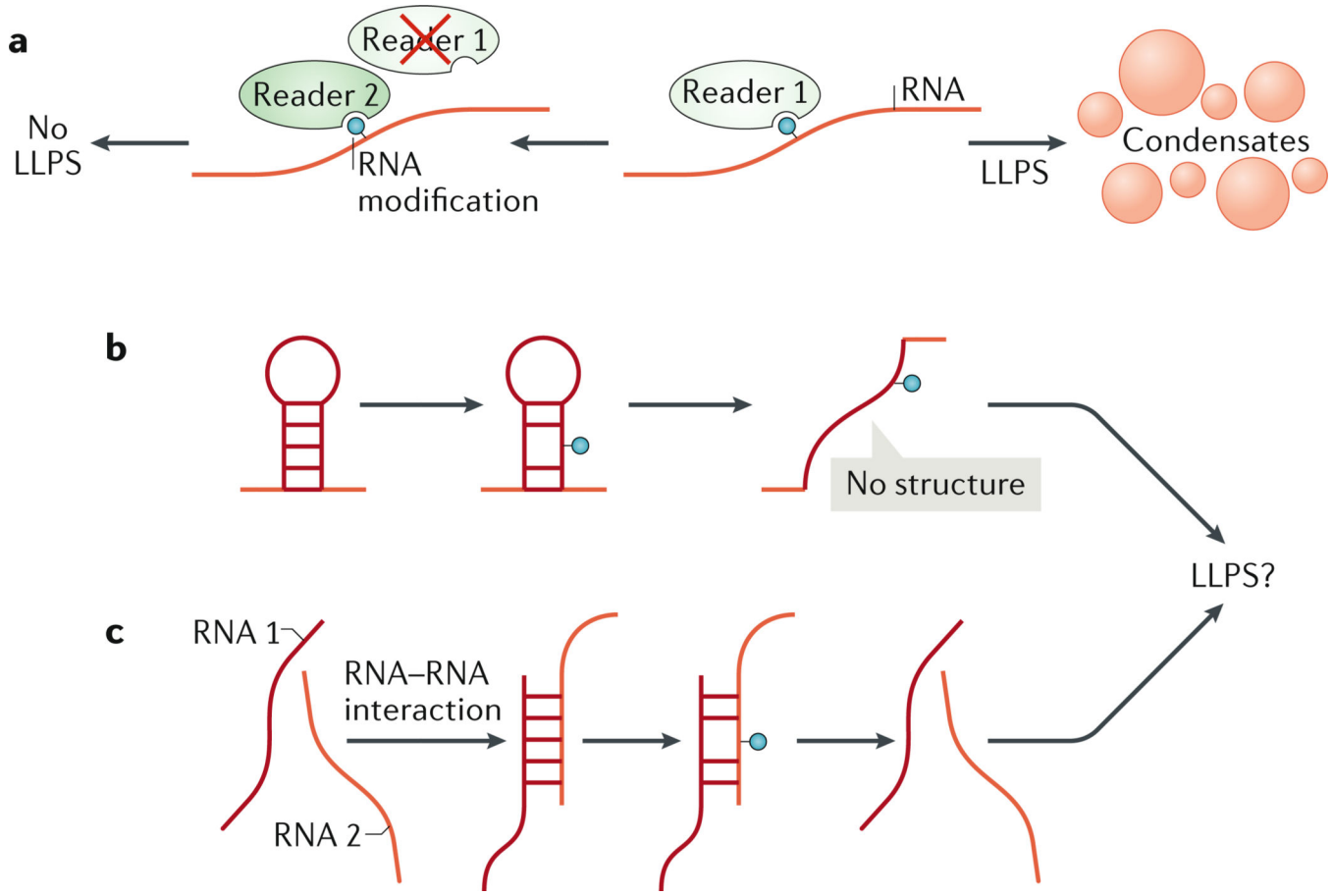

Figure 3: Modifying RNA structure in vitro and in vivo.

(A) In vitro, buffers that include magnesium ions (Mg^{2+}) and a crowding agent and exclude the chelating agent EDTA, will support the formation of both strong RNA structures (structures with many base pairs) and weak RNA structures (fewer base pairs) at lower temperatures (left), whereas in the opposite buffer conditions or in higher temperature, the weaker RNA structures will unfold (middle). Extreme pH will denature and cause the degradation of even strong RNA structures (right). **(B)** In vitro transcription favours interactions at the 5'-end region, as this region is transcribed first. Melting and refolding of the in vitro transcribed RNA will allow and the formation of interactions between the 5' and 3' regions of the molecule. **(C)** In vivo, RNA structures are modulated by cellular RNAses, which actively remove particular RNA sequences and structures; by RNA helicases, which unwind double-stranded RNA; and by RNA-binding proteins, which can shield RNA sequences from RNA–RNA interaction or block structure formation. The cellular environment helps to explain the discrepancy between RNA-structure probing data obtained in vitro or from cells. This discrepancy may be explained also by RNA modifications (Figure 2).

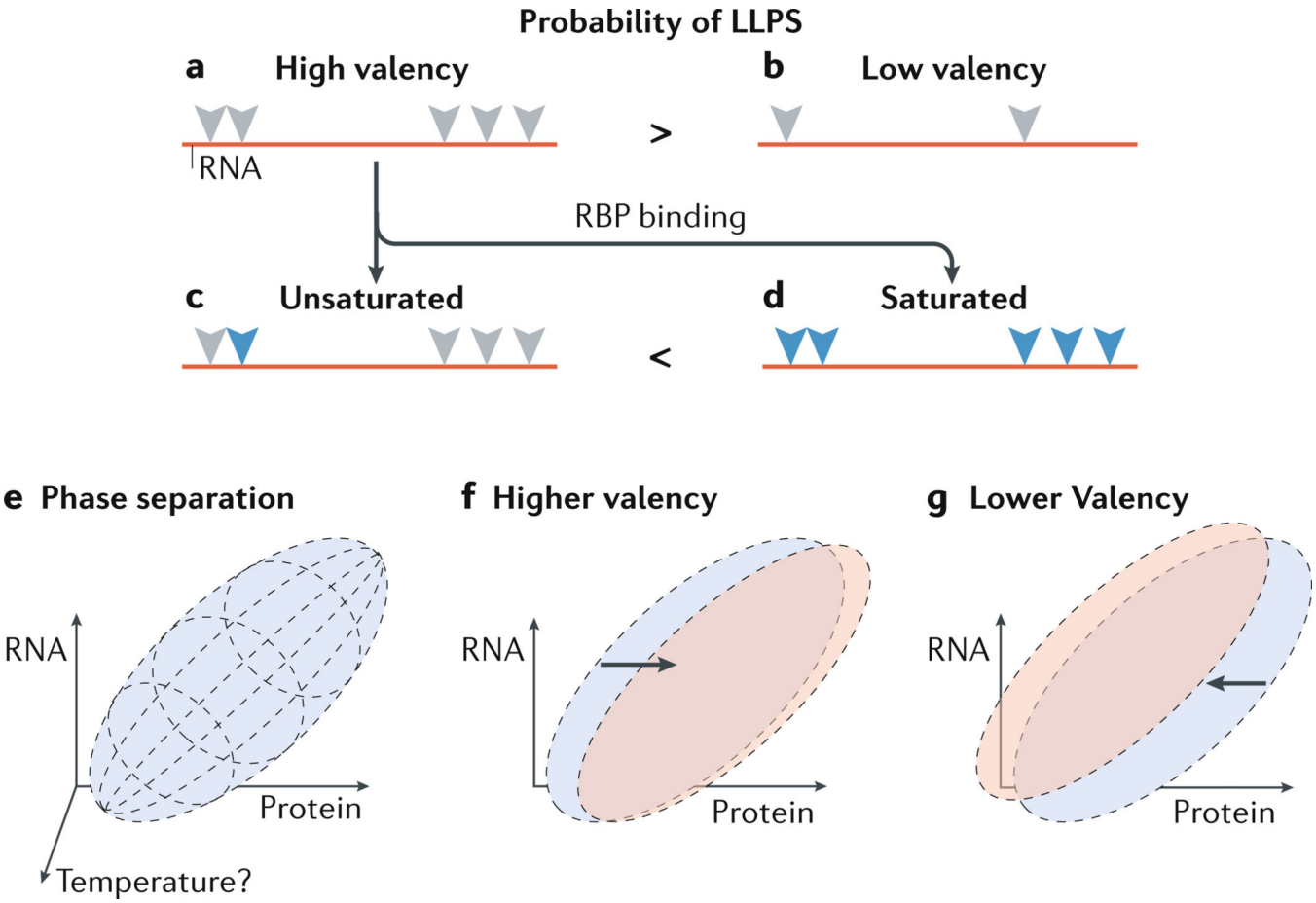

Figure 4: Balancing RNA and protein ratios in condensates.

(A and B) RNA with high valency **(A)** refers to an RNA molecule that contains multiple sequences and/or structures (grey triangles) that can be recognized by an RNA-binding protein (RBP). Low valency **(B)** refers to an RNA with few such sequences or structures. High valency may favor liquid–liquid phase separation (LLPS) over low valency. A fraction, or all of the potential binding sites for RBPs could be actually bound by RBPs (blue triangles), resulting in unsaturated **(C)** or saturated **(D)** RNA to protein ratios, respectively. Saturation may favor LLPS. **(E)** A possible phase diagram of a mixture of RNA and protein at a particular temperature. Phase separation between the RNA and protein occurs within the parameters of the purple ellipsoid and is a factor of the concentrations of RNA, of protein, and of buffer (not shown). **(F)** Increasing the valency of the RNA target may shift the phase diagram to the right (red ellipse), indicating that a lower RNA concentration is required to induce LLPS. **(G)** Reducing RNA valency may have an opposite effect.

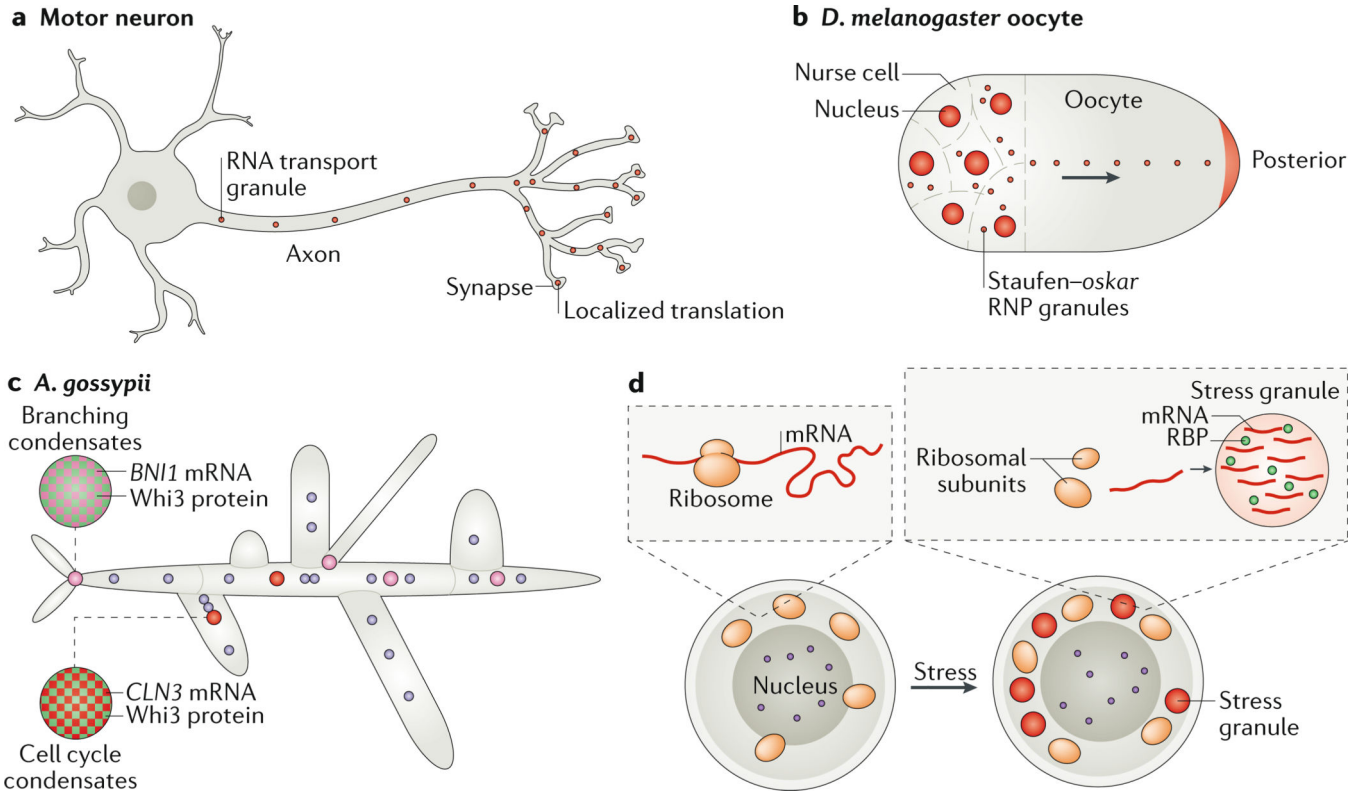

Figure 5: Examples of cytoplasmic RNA–protein condensates.

Compartmentalization of cytoplasmic RNA is prevalent in large **(A and B)** or multinucleate cells (**C and D). (A)** In motor neurons, RNA transport granules move mRNAs on microtubules (not shown) towards synapses, to allow their localized translation away from the cell body. **(B)** Oocytes compartmentalize and store maternal RNA. For example, in *Drosophila melanogaster* oocytes, the *oskar* mRNA is transported along microtubules to the posterior pole of the embryo by the RNA-binding protein (RBP) Staufen. **(C)** *Ashbya gossypii* is a multinucleate filamentous fungus that utilizes condensates to compartmentalize its cytoplasm. The RBP Whi3 is used to define sites of branching, by binding to the SPA2 and BNI1 mRNAs, and to control nuclear cell cycle asynchrony by binding G1 cyclin RNA (CLN3). CLN3 and BNI1–SPA2 condensates are immiscible; condensate immiscibility is controlled by RNA sequence and structure. Note that the nuclei are not drawn to scale — condensates are roughly 1/10–1/5 the size of nuclei. **(D)**Cells in conditions of acute stress undergo translation shutdown and release mRNAs from the translation machinery. The mRNAs form stress granules with RBPs. The purple circle is the nucleus.

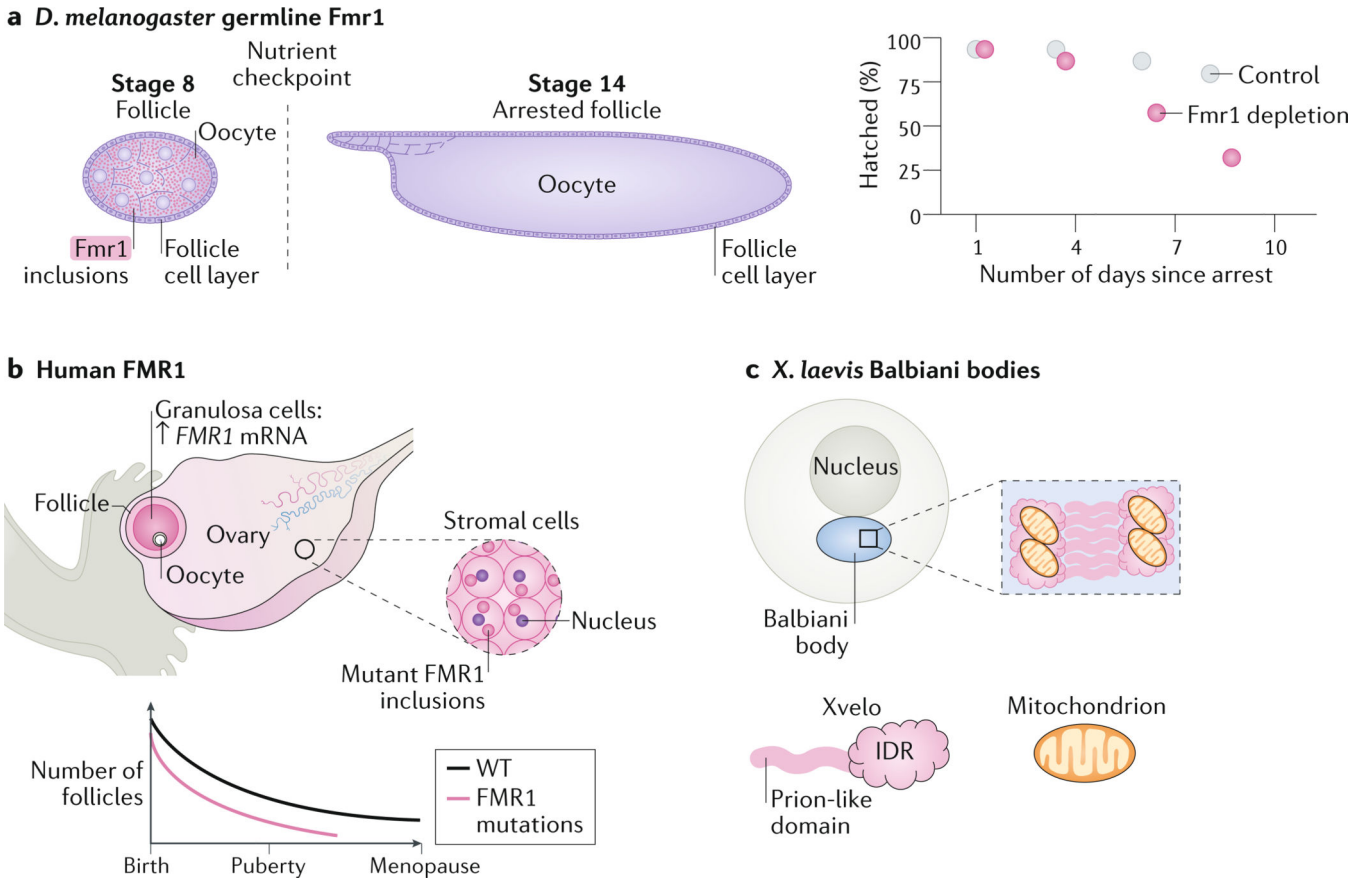

Figure 6: Oocytes may use condensates for long-term RNA storage.

(A) Loss of fragile X mental retardation protein 1 (Fmr1) in *Drosophila melanogaster* oocytes accelerates the reduction in hatch rate in arrested oocytes over the fly life time. Fmr1 is expressed and forms condensates in the cytoplasm of stage 8 oocytes. Starvation of female flies leads to arrest of oocyte development at stage 14. Fmr1 depletion leads to a reduction in hatch rate over time.

(B) In humans, mutations in FMR1 that cause fragile X syndrome are associated with female infertility. The FMR1 protein forms inclusions in ovarian stromal cells. The number of follicles in women with fragile X syndrome decreases overtime more than in healthy women, leading to premature ovarian failure.

(C) In *Xenopus laevis* oocytes, the protein Xvelo, which contains an intrinsically disordered region (IDR) and a prion-like domain is required for the formation of Balbiani bodies through assembly of an amyloid–like network with RNA and mitochondria.