RNAs, Phase Separation, and Membrane-Less Organelles: Are Post-Transcriptional Modifications Modulating Organelle Dynamics?

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Membranous organelles allow sub-compartmentalization of biological processes. However, additional subcellular structures create dynamic reaction spaces without the need for membranes. Such membrane-less organelles (MLOs) are physiologically relevant and impact development, gene expression regulation, and cellular stress responses. The phenomenon resulting in the formation of MLOs is called liquid–liquid phase separation (LLPS), and is primarily governed by the interactions of multi-domain proteins or proteins harboring intrinsically disordered regions as well as RNA-binding domains. Although the presence of RNAs affects the formation and dissolution of MLOs, it remains unclear how the properties of RNAs exactly contribute to these effects. Here, the authors review this emerging field, and explore how particular RNA properties can affect LLPS and the behavior of MLOs. It is suggested that post-transcriptional RNA modification systems could be contributors for dynamically modulating the assembly and dissolution of MLOs.

1. Introduction

How exactly the spatial separation and effective organization of the staggering diversity of molecular assemblies within cells is achieved has been a puzzling problem to biologists and chemists for a long time. Each cell contains an enormous number of different molecules, which all display different physicochemical properties but obviously manage to interact in a rather ordered fashion. To approximate the molecular load in eukaryotic cells, the total protein concentration has been estimated to be up to 300 milligrams per milliliter (mg mL$^{-1}$) while RNA concentrations can vary between 20 and 100 mg mL$^{-1}$.[1] Various concepts have been developed aiming to reconcile the high local concentrations of molecules in vivo with the limitations of biochemical experimentation and reduced in vitro complexities. For instance, the term “molecular crowding” has been introduced describing that the local concentration of particular molecules is often so high that the available physically occupied space becomes unavailable to other molecules, a situation that precludes random mixing, and allows specific reactions to take place.[2] However, individual molecules are often only present in small copy numbers,[1,4] which in combination with the stochasticity of molecular diffusion sets limits to the control of any system relying on chemical reactions between molecules. On the other hand, without control random fluctuations could become amplified during multi-step chemical reactions potentially interfering with the ordered fashion of molecular interactions that give rise to cell type-specific features and behaviors.[3]

An evolutionary conserved way to achieve such control is the creation of compartments allowing for passive and energy-efficient buffering of stochastic events such as transcriptional noise.[6] For instance, efficient separation of chemical reactions is accomplished through the creation of complex barriers (involving membranes) as is the case for nuclei, mitochondria, and various other organelles. For other, and usually smaller, compartments such as nucleoli, various nuclear bodies or speckles, centrosomes, cytoplasmic processing bodies and stress granules, separation of chemical reactions is somehow achieved without a discernible contribution of membranes.[7] For a long time, studying such membrane-less structures has largely been restricted to mere observation because of preparative hurdles connected to their biochemical isolation. Only recent progress in experimental methodology combining sophisticated in vivo labeling techniques, high-resolution microscopy imaging, mass spectrometry, and probing of highly concentrated protein solutions revealed that a physicochemical process called liquid–liquid phase separation (LLPS) dictates the efficient separation of macromolecular assemblies without the need for membranes.

2. Membrane-Less Organelles: Organizing a Universe of Ribonucleoprotein Particles

Formation of “colloidal” bodies that are separated from the rest of the cellular “protoplasm” had already been recognized as
subcellular organization principle more than a century ago. Prokaryotic cells form proteinaceous organelles called “bacterial microcompartments,” that concentrate enzymes of certain metabolic pathways. Eukaryotic cells create a multitude of membrane-less organelles (MLOs) with diverse physical properties, molecular composition, and subcellular localization; often displaying dynamic and even cell type-specific features. It has recently been suggested to rename MLOs as “biomolecular condensates” (BioMCs) throughout this article, please refer to Glossary for definition) because of their distinction from canonical macromolecular ribonucleotide particles (RNP) such as ribosomes. The most discernible BioMCs are spatially distinct sub-nuclear foci such as nucleoli, Cajal bodies (CBs), promyelocytic leukemia (PML) bodies, and various nuclear speckles. In addition, different cytoplasmic BioMCs exist that have either been named according to their resident molecules (i.e., glycine- and tryptophan-rich bodies, GW-bodies; uracil-rich bodies, U-bodies; Polycomb group bodies, PcG-bodies, etc.), a particular biological process (i.e., processing bodies, P-bodies; stress granules, SGs), through their association with other organelles (i.e., the nuage or inter-mitochondrial cement), or in correlation with particular disease-related intracellular inclusion bodies (i.e., Lewy bodies). Importantly, some BioMCs are continuously present with important functions under steady-state conditions (i.e., nucleoli, P-bodies), while others display dynamic behavior in response to various insults and stress conditions (i.e., DNA repair foci, SGs) (Figure 1).

Generally, BioMCs have been conceptualized as RNP-containing hubs in which complex biochemical reactions such as splicing, processing, and maturation of specific RNAs as well as metabolic turnover of various cellular components take place. However, particular BioMCs contribute also to specific biological processes such as polarity establishment and cellular identity in early embryos (i.e., P granules and RNP segregation in C. elegans), asymmetric cell division of stem cells in Drosophila, transcription, heterochromatin formation, DNA repair, genome integrity through nuage-affiliated processes, or the response to biotic and abiotic stress through the formation of SGs.

3. Dynamic Liquid–Liquid Phase Separation is Key for Proper Cellular Physiology

Germ granules in Caenorhabditis elegans (P granules) were the first BioMCs whose condensation and dissolution behavior was compared to liquids and characterized in vivo as flowing, deforming, coalescing, as well as fusion and fission of individual protein-rich droplets. Similar liquid-like properties have also been observed for highly concentrated preparations of proteins

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Figure 1. Membrane-bound cellular compartments contain various membrane-less organelles (MLOs) also called “biomolecular condensates” (BioMCs), which are either constituent structures fulfilling general functions under steady-state conditions (gray, black, and blue), or are newly formed or change their material properties during in response to particular stress conditions (red).
or RNAs in vitro.\textsuperscript{[24–26]} The process that influences the properties of fluids with different densities (i.e., oil-in-water emulsions) and governs their behavior is called liquid–liquid de-mixing or LLPS, which has gained a lot of attention recently. The interested reader is redirected to excellent reviews discussing the current knowledge, thermodynamic and physical properties as well as the implications of LLPS processes for biology.\textsuperscript{[10,27–29]}

A distinguishing feature of many BioMCs is their propensity to dynamically assemble and disassemble indicating changing physicochemical or material properties of their constituent molecules. Several parameters such as salt concentration, temperature, and molecular crowding agents affect the kinetics of LLPS processes both in vitro and in vivo. Furthermore, the flowing behavior of many BioMCs suggests fast internal molecular rearrangements. Indeed, constituent proteins and RNAs can be rapidly exchanged between BioMCs and their surrounding liquid phases, often in time frames of seconds to minutes, supporting the notion of constant access to and molecular turnover within these highly concentrated molecular assemblies.\textsuperscript{[30]} In addition, various BioMCs appear inherently structured with cores surrounded by shells, suggesting not just RNP aggregation but the existence and maintenance of higher-order structures as a result of LLPS of different components.\textsuperscript{[2,21,31]} Furthermore, particular nuclear BioMCs such as nucleoli and cytoplasmic BioMCs that are formed during stress conditions (i.e., SGs) share molecular content (i.e., splicing factors, specific heterogeneous nuclear RNPs, hnRNPs)\textsuperscript{[32]} indicating either relocation of BioMC content from one cellular sub-compartment to another or disassembly of BioMCs in one and use of their components in another compartment.

From these observations, a picture emerges suggesting that the material of and the interactions among the resident molecules in a given BioMC determines its biological functionality. Importantly, BioMCs can lose their liquid-like characteristics by transitioning into a more rigid and gelatinous state. Indeed, evidence is accumulating indicating that both aberrant formation of BioMCs and imbalances between liquid-like and solid-like states of particular BioMC components could be pivotal for disease etiology. For instance, many neurodegenerative diseases (i.e., Parkinson’s Disease, PD; Amyotrophic Lateral Sclerosis, ALS; Frontotemporal Lobar Degeneration, FTLD), amyloidoses, prion diseases as well as a number of inherited myopathies are presently characterized as stress-induced protein conformational disorders or proteinopathies.\textsuperscript{[33]} Importantly, various studies suggest that aberrant LLPS could be the culprit for initiating these disorders.\textsuperscript{[24,34–36]} Furthermore, deregulation of SG dynamics might represent a key step in the pathogenesis of some of these diseases, because familial mutations predisposing to the development of ALS, PD, FTLD, as well as to inclusion body myopathies (IBM), are detectable in genes important for SG dynamics (i.e., TIA1, TIAR1, ANG, and hnRNPA1).\textsuperscript{[24,35,37–39]} Furthermore, perturbation of LLPS by genetic manipulation resulted in less dynamic BioMCs, which acted as nucleation points for additional association-prone proteins (i.e., Tau protein) leading to the formation of insoluble protein deposits.\textsuperscript{[30,41]} A testable hypothesis therefore states that excessive gelation of intracellular compartments may result in the sequestration of key proteins and RNAs resulting in the inhibition of their normal function and hence detrimental effects on cellular physiology.

The principles and forces governing LLPS are currently extensively studied, not only by in vitro assays using purified BioMC components but also by using in vivo model systems.\textsuperscript{[26]} While many constituent molecules of particular BioMCs have already been identified\textsuperscript{[21,27,42,43]} and both overexpression as well as genetic deletion approaches of proteins and RNAs are being used to modulate the formation and dynamics of BioMCs, it still remains largely unclear which signals and specific factors trigger the separation of membrane-less macromolecular assemblies within complex environments. Equally unclear is how cells manage to avoid constant solidification of highly condensed matter in any intracellular compartment and which intracellular changes and molecular activities are required to efficiently maintain a liquid-like state while dissolving BioMCs when physiologically necessary (i.e., during stress recovery).

4. Liquid–Liquid Phase Separation is Impacted and Regulated by the Presence of RNAs

Proteins prone to undergoing LLPS are either multi-domain proteins or harbor intrinsically disordered regions (IDRs), often also referred to as prion-like domains (PLDs).\textsuperscript{[10]} Individual IDRs can self-associate and form oligomeric structures through multiple weak and adhesive interactions such as cation-pi, pi-stacking, or dipolar interactions between stretches of particular “disorder-promoting” amino acids (i.e., Q, S, N, Y, G), all resulting in phase separation of interacting proteins from surrounding solution.\textsuperscript{[24,30,44,45]} The ensuing multi-valency governs inter- and intramolecular interactions resulting in the assembly of oligo- and polymeric structures in which valency (the number of interaction modules) and affinity (strength of interactions of individual modules) are the key parameters controlling LLPS.

In addition, proteins with IDRs often also harbor RNA-recognition motifs (RRMs), and the same proteins are usually enriched in RNA-containing BioMCs. Interestingly, RNA-binding domains in IDR-containing proteins have been shown to be crucially important for undergoing LLPS, as multivalent interactions among, for instance, tyrosine residues in IDRs and arginine residues in RRMs appear to primarily govern the process.\textsuperscript{[46]} Of note, while most studies addressed the role of particular phase-separating proteins in both LLPS and BioMC formation, only very recently did RNA itself move into focus of phase transition research. Indeed, latest insights revealed profound roles for RNA in the regulation of LLPS processes.\textsuperscript{[36,47–49]}

5. Membrane-Less Organelle Dynamics: How Could RNA Act as Trigger, Scaffold, and Solubilizer of BioMCs?

How could RNA itself or RNA binding to other molecules affect the formation, maintenance, and even efficient dissolution of BioMCs? At least two roles can be considered that have already been experimentally verified.
5.1. RNAs Serve as Scaffolds During BioMC Formation

Many nuclear BioMCs form in coordination with transcription of specific RNAs. For instance, the assembly of nucleoli is coordinated by pre-rRNA transcription,\(^{[50]}\) nuclear paraspeckles emanate exclusively from NEAT1 transcription sites,\(^{[51]}\) and nascent small nuclear RNAs (snRNAs) serve as site-specific nucleators of CBs.\(^{[52]}\) The function of these RNAs in BioMC formation has been summarized with the term “architectural RNAs” (arcRNAs).\(^{[53]}\) Often, the transcription of arcRNAs occurs during specific developmental stages or is induced by specific stimuli, such as distinct stress conditions or upon viral infections.\(^{[53]}\) Furthermore, RNAs can also be recruited after initial BioMC formation through RNA binding to RRM and IDRs of BioMC-constituent proteins, which can in turn further sequester additional RNAs and proteins into the structural network.\(^{[50]}\) Of note, BioMCs forming around scaffolding RNAs often contribute to establishing gene expression patterns allowing stress evasion and maintenance of cellular homeostasis.\(^{[54,55]}\)

Hence, transcription of particular arcRNAs can provide a localized, site-specific, and rapid way of phase-separating proteins thereby initiating nuclear BioMC formation. Moreover, formation and dissolution of nuclear BioMCs could be achieved by dynamically regulating transcription and turnover of arcRNAs.

5.2. RNAs Influence the Material Properties of Established BioMCs

In contrast to transcription-dependent seeding of particular nuclear BioMCs, the formation of cytoplasmic BioMCs is probably and primarily governed by the availability of existing RNPs, proteins, or RNAs serving structural functions. However, regardless of whether RNA itself scaffolds the formation of BioMCs or becomes recruited into an existing proteinaceous network, RNA content impacted and even regulated the material properties of assembled BioMCs both in vitro and in vivo.\(^{[22,24,36,47–49,56]}\) More specifically, RNA facilitated LLPS of particular IDR-containing proteins by reducing the protein concentration needed for their phase-separation.\(^{[24,40,57]}\) Furthermore, RNA content of particular cytoplasmic BioMCs was crucial for maintaining the localization and material identity of assembled granules suggesting RNA-driven mechanisms that selectively recruit molecules toward BioMCs with different material content. Moreover, RNA appeared to “fine-tune” the dynamics of protein droplet formation by affecting material exchange rates as well as rigidity and shape of BioMCs.\(^{[22,47,59]}\) Importantly, overall RNA/protein ratios affected phase separation such that low RNA levels promoted LLPS and even caused excessive phase separation including the formation of solid-like structures in vivo.\(^{[30,36]}\) In contrast, high RNA/protein ratios prevented LLPS and promoted dissolution of particular BioMCs.\(^{[36]}\)

The regulating influence of local RNA concentrations on BioMC formation and dissolution was also observed in vitro and can be explained by a phenomenon known from polymer chemistry called “reentrant phase transition,” which is governed by charge inversion at threshold concentrations of mixing molecules.\(^{[60]}\) These combined observations indicated that local RNA concentration determines the material properties of BioMCs during their assembly (toward more solid, non-dynamic states) or disassembly (preventing gelation and supporting fluidity).

While many of the recent findings clearly documented the impact of RNAs on the formation, material state, and dynamics of BioMCs it remains largely unclear how the different properties of RNA molecules (i.e., charge, specific RNA sequence identities, particular RNA structures, protein binding, RNA modifications) contribute to these processes.

6. Which Biophysical Properties of RNAs Could Affect LLPS?

6.1. The Negative Charge of RNAs Impacts Electrostatic Interactions

Since the driving forces governing LLPS are mostly of electrostatic nature, high local concentration of charged molecules could affect the weak interactions between phase-separating proteins. Low-complexity RNAs (i.e., poly-U) alone can form coacervates, which, to some extent, mimic the behavior of phase-separating protein solutions.\(^{[25]}\) Shielding of net negative charge of RNAs through increasing ionic strength or addition of polyanines (i.e., spermidine or spermine) or arginine-containing dipeptides impacted the propensity of these RNAs to form coacervates\(^{[25]}\) while facilitating RNA-dependent seeding of BioMCs.\(^{[24,30,61]}\)

Curiously, adenosine-triphosphate (ATP), one of the basic building blocks of RNA, acts as a biological hydrotropic\(^{[62]}\) and influences LLPS and BioMC dynamics in vivo.\(^{[63]}\) In support of the notion that local concentrations of negative charge play major roles for LLPS, nucleic acid-mimicking biopolymers such as poly-ADP-ribose (PAR) induced phase transitions in vitro and in vivo\(^{[18]}\) and phosphorylation of low complexity regions in aggregation-prone proteins disrupted their phase separation.\(^{[20,64,65]}\)

Furthermore, also post-translational modifications (i.e., methylation) affecting the charge of particular amino acids in IDR-containing proteins disrupted cation-pi interactions thereby modulating LLPS.\(^{[66]}\) Moreover, introducing additional negative charge to PLD-containing proteins enhanced phase separation mediated by intermolecular interactions among PLDs and RRMs.\(^{[46]}\) These combined observations indicate that high local concentration of negative charge, which could be provided by any RNA, can modulate the electrostatic interactions with other molecules during LLPS.

6.2. RNAs Encode Specific Sequence Information and Often Sequence Repeats

Particular RNA sequences contributed to the formation of specific BioMCs both during physiological\(^{[47,56,58]}\) as well as during stress conditions.\(^{[21,42,67]}\) Of note, different mRNAs facilitated LLPS of the same protein albeit causing different dynamics and viscosities\(^{[47,56]}\) suggesting that RNA sequence identity can indeed determine the properties of formed BioMCs.
Furthermore, scrambling mRNA codon content abolished their partitioning in formed BioMCs, supporting the notion that sequence context of coding RNA can be of importance for LLPS. Interestingly, the presence of mRNA in SGs correlated with particular exposure to stress. For instance, SGs induced by UV stress or through inhibition of translation initiation did not contain mRNAs indicating that coding potential is sufficient but not required for mRNAs to be sequestered in particular BioMCs. Importantly, specific mRNAs are often expressed in rather low copy numbers. Hence, the number of potentially scaffolding mRNAs might be very low, especially when taking into account that mRNAs are usually translated and thereby sequestered into ribosomes. Thus, other RNA species might be better suited to act in LLPS and affect BioMC dynamics.

On the other hand, lncRNAs do serve as scaffolds in BioMC formation as is the case for several “RNA-seeded” nuclear bodies such as paraspeckles, nucleolar detention centers, or nuclear stress bodies; whose formation is dependent on various arcRNAs such as NEAT1, intergenic spacers (IGS), and satellite repeats (SatIII), respectively. Importantly, overall protein levels do not necessarily change during BioMC formation but rather the transcription status of particular genetic loci as exemplified by nascent NEAT1 transcription and seeding of paraspeckles during stress responses. In addition, particular sub-nuclear BioMCs, namely amyloid bodies (A-bodies), rapidly assembled in response to and disassembled after stress. Central for the formation of A-bodies was the expression of non-coding RNA from IGS within ribosomal DNA loci supporting the notion that transcription of particular RNA repeats contributes to transient BioMC formation. Of note, RNA expression from repeat regions (including transposable elements, TEs; and virus-derived sequences) can be linked to particular cellular states. For instance, stem cells express TEs and retroviral sequences over a spectrum of pluripotency states. LINE-1 (L1) elements are expressed during particular meiotic stages in the mammalian germ line and act as nuclear scaffolds for protein recruitment in embryonic stem cells; the de-repression of TEs and viral sequences correlates with the de-methylation of paternal genomes in the early zygote and often stress responses coincide with the expression of genomic repeat elements.

In addition to long RNAs, smaller RNAs were also able to affect LLPS and BioMC formation. Naturally occurring small RNAs such as snoRNAs, small nucleolar RNAs (snoRNAs), or tRNAs are highly abundant and can self-assemble, making them suitable for additional functions without affecting their canonical roles. For instance, CB assembly was shown to be connected to the interaction of snoRNAs with the key structural protein in CBs, collin. Furthermore, tRNA-derived small RNAs (tsRNAs) resulting from enzymatic cleavage of mature tRNAs in response to stress conditions, when transfected into cells, induced SG formation. Of note, a mutation affecting the catalytic activity of the RNase responsible for the production of tsRNAs (Angiogenin, ANG) abrogated SG assembly supporting the notion that high local concentrations of particular small RNAs such as tsRNAs can contribute to BioMC formation in vivo.

6.3. RNAs Form Structures That are Recognized by Proteins

RNAs form various structural elements, often in well-defined contexts, such as short and long stems, hairpins, helical regions, tetra-loops, or G-quadruplexes (G-quads), which contribute to the overall complexity of the three-dimensional space a given RNA can explore. In addition, these structural motifs also attract non-specific protein binders, potentially through interactions with IDRs and PLDIs. Importantly, since the number of putative RNA sequence motifs that could be recognized by proteins vastly exceeds the number of known RNA-binding proteins it is likely that RNA structure plays a more decisive role than sequence context in discriminating protein-RNA interactions. Indeed, particular protein domains that bind nucleic acids recognized structure over sequence identity. For example, particular conserved as well as repetitive sequences in the lncRNA NEAT1 were shown to be essential for protein binding resulting in nuclear paraspeckle formation. Moreover, structural changes in specific mRNAs can influence BioMC identity. More specifically, protein droplet identity was not only established through intermolecular mRNA-mRNA interactions, but particular RNA structures selectively exposed or masked RNA sequences capable of interacting with other RNAs, thereby directing mRNAs into specialized BioMCs. Formed BioMCs further became stabilized through additional interactions with RRM and IDR-containing proteins.

Of note, particular repeat-containing RNAs form structures that have been connected to the formation of BioMCs. For instance, biochemically purified SG cores were enriched in antisense RNAs capable of forming intramolecular duplexes, and these SG cores were resistant to high salt or aliphatic alcohol treatment, which disrupt protein-protein but not RNA-RNA interactions. Interestingly, some products of stress-induced tRNA metabolism, tsRNAs, can form homo- and heterodimers but also particular structures such as tetramers through G-quads which are important for SG formation. Of note, mRNAs containing sequences that can form G-quads, such as the GGGGCC (G4C2) intron-repeat expansion within the C9ORF72 locus, mediated LLPS both in vitro and in vivo. These observations support the notion that RNAs, especially carrying repeat sequences, facilitate intra- as well as intermolecular interactions and structures, which likely affect BioMC formation and dynamics. For example, sequence or structure-based interactions between small RNAs and other RNAs could bring about structural changes thereby affecting protein interactions and even RNA stability in BioMCs.

Importantly, a particular RNA structure is only partially determined by primary sequence context as it represents an equilibrium of possible structures, which can be affected by various parameters such as charge, temperature, ion concentrations, nucleotide modifications, and interactions with proteins as well as small ligands such as low-molecular-weight organic compounds (i.e., ATP, nicotine, thiamine pyrophosphate, amino acids) allowing major changes of RNA structure in response to, for instance, environmental signals or stress conditions. It follows that the propensity of RNAs to self-assemble and form higher-order structures is likely one of the
6.4. RNAs Can Be Chemically Modified

As of now, more than 150 different post-transcriptional RNA modifications have been identified. Most of these chemical modifications map to highly abundant and structured non-coding RNAs (i.e., tRNAs and rRNAs). However, recent years have seen a surge of data showing that RNA modifications can also be found in low abundance RNAs such as IncRNAs and mRNAs. The majority of modified residues features the addition of simple methyl groups (i.e., 2'-ribose-methyl, 2'-O-methyl; 6-methyladenosine, m6A; 1-methyladenosine, m1A; 5-methylcytidine, m5C) or are the result of base isomerization reactions (i.e., pseudo-uridine, Ψ) but some RNA nucleotides also carry rather complex modifications such as queuosine. While the existence of RNA modifications is now widely acknowledged, the biological function of most RNA modifications and their mechanistic consequences when occurring at specific positions in RNA remains largely unclear. However, RNA modification systems (a summary term for a particular chemical modification; the enzymes catalyzing this modification, “writers”; proteins binding to these modifications, “readers”; and the activities further modifying and even removing established modifications, “erasers”) carry all the hallmarks that allow tissue-, cell type- and even organelle-specific as well as time-resolved modulation of RNA sequence identity, charge of particular nucleotides, RNA structure and protein binding, all of which are inherent characteristics of BioMC formation and dissolution.

7. RNA Modifications: Potential for Dynamic Regulation of RNA Properties

7.1. RNA Modification Systems Respond to Environmental Conditions

Curiously, many RNA modification systems appear not to be essential for life since organisms with mutations in the responsible activities are viable and fertile. However, combining different RNA modification system mutations or exposing particular RNA modification system mutants to non-standard laboratory conditions (i.e., stress) caused observable phenotypes indicating that the biological function of many RNA modifications has to be sought in the response to environmental insults. Indeed, recent research showed that RNA modification patterns can change, especially during the response to particular stresses resulting, for instance, in the reprogramming of protein translation. In addition, nutrient deprivation or serum starvation induced U to Ψ conversion, heat shock-induced m6A (or m6Am) placement in 5' UTRs of mammalian mRNAs thereby promoting cap-independent translation and microbial-derived micronutrients linked RNA modifications to coordinated decoding of transcriptomes. Furthermore, the identification of RNA editing and RNA modification events in post-mitotic and adult tissues underscores the notion that RNA modification systems are not necessarily required for development and differentiation, but are dynamically placed, or can be further modified (i.e., Ψ by N1 methylatation, m6C to various oxidation products, 3-methylcytosine–3-methyluridine).

Importantly, subcellular re-localization of specific RNA modification system components in response to stress conditions has been reported. For instance, METTL14, part of the catalytic heterodimer that “writes” m6A, has a glycine-rich disordered domain, which might target this protein to specific subcellular regions under specific conditions. Also, m5C “writers” such as NSun2 or Dnmt2 re-localized between nuclear and cytoplasmic compartments during stress conditions or cell cycle stages. In addition, all m6A “readers” possess IDRs, which could facilitate targeting to P-bodies and SGs. For instance, during oxidative stress, the “reader” protein YTHDF1 localized to SG and promoted stress recovery by reinstating protein synthesis. Another “reader”, YTHDF2, localized to the nucleus during heat shock and promoted m6A methylation in 5' UTRs. These and numerous other observations indicated a highly dynamic interplay between RNA modification systems and specific environmental changes, the latter being also a hallmark for LLPS as well as specific BioMC formation and disassembly.

7.2. RNA Modifications Impact RNA Structure

The impact of particular chemical modifications on the physicochemical properties of RNAs has been studied for quite some time. RNA modifications can modulate charge, hydrophobicity, steric hindrance, base stacking as well as pairing potential of bases, all of which impact interactions with solvents and particular ions but also influence the formation of secondary and tertiary structural elements. Any RNA sequence can adopt several alternative conformations given that the Gibbs energy differences are sufficiently small. For instance, approaching the correct conformation represents a thermodynamic problem for highly structured RNAs (i.e., tRNAs), especially in complex cellular environments. RNA modifications can guide the tRNA folding process, thereby stabilizing RNA in a functionally relevant structure. Some modifications exert significant effects on the conformation of the ribose group and thereby the
overall structure of the RNA polymer. 2'-O-methylation (2'-O-me) of the ribose 2'-hydroxyl group favors the 3'-endo ribose conformation, thus minimizing the potential for steric clashes between methyl group and base.\[110\] 2'-O-me is most effective in pyrimidine nucleosides and the ensuing 3'-endo conformation translates into an overall thermodynamic stabilization and improved base stacking of 2'-O-me-modified RNAs.\[111\] Indeed, catalyzing 2'-O-me represents a powerful evolutionary strategy for stabilizing RNAs and RNA structures in organisms living at high temperatures.\[112\] Furthermore, addition of chemical groups (such as m5C) increases base stacking potential between adjacent bases by inducing dipole–dipole effects within the modified base. Since base stacking is one of the strongest interactions affecting RNA stability,\[113\] chemical modifications that influence base stacking exert powerful effects on overall RNA structure and rigidity. Besides chemical additions, the isomerization of canonical nucleosides also affects RNA structure. For instance, isomerization of uridine U to Ψ creates an additional hydrogen bond donor, resulting in the creation of a “water bridge” to the phosphate backbone and increased base stacking.\[113\] In contrast, isomerization of U to dihydrouridine (D) results in the reduction of the pyrimidine 5,6-double bond, thereby eliminating the characteristic planar conformation of the base.

Figure 2. Specific nucleoside modifications impact on secondary RNA structures (a, through m6A, thereby creating access sites for RNA-binding proteins to single-stranded RNA, RBP, or to m6A via YTH-domain binding proteins), on tertiary RNA structures (b, breaking of specific A–U pairing through m1A in mitochondrial tRNAlys), on stress-induced nuclease activity (c, through m5C, which negatively affects the extent of stress induced tRNA fragmentation into tRNA-derived small RNAs, tRFs), and on nuclease accessibility (d, through mcm5s5U, which positively attracts particular “killer” toxins to produce nicked tRNAs). Black circles: adenosine, A; gray circles: uracil, U; red circle: m1A; blue circles: m5C; green circles: m6A; yellow circles: mcm5s5U.
This causes the ribose to take on the 2’-endo conformation, resulting in poor base stacking and therefore in greater flexibility, especially in RNA loops, which seems functionally relevant in organisms living at low temperatures (psychrophiles).\[^{114,115}\]

RNA modifications can also disrupt the propensity of nucleosides to form hydrogen bonds by affecting the orientation of the N-glycosidic bond, thus limiting RNA duplex formation. For instance, methylation of adenosine at position 6 (m\(^6\)A) induces base rotation from the syn-conformation to the energetically unfavorable anti-conformation, leading to the destabilization of RNA duplexes in m\(^6\)A-U base pairs\[^{116}\] (Figure 2). On the other hand, m\(^6\)A in unstructured RNA regions (i.e., loops) causes considerably stronger base-stacking interactions because of its hydrophobic interaction with the phosphate backbone indicating that effects of chemical modifications on RNA structure can be highly context-specific\[^{116,117}\]. In contrast to m\(^6\)A, methylation of position N1 in adenosine (m\(^1\)A) introduces a positive charge, which promotes ionic interactions with the phosphate backbone resulting in effects on base pairing. For example, a single m\(^1\)A was crucial for shifting the RNA folding equilibrium toward the correct and functional tRNA structure by blocking a single A–U base pair\[^{118}\] (Figure 2). These observations indicated that a few RNA modifications can impact RNA structures globally, potentially resulting in major changes in RNA function.

### 7.3. RNA Modifications Impact RNA-Protein Interactions

RNA modifications can also directly affect protein binding by either attracting or repelling proteins.\[^{119}\] For instance, YTH domain-containing proteins are specifically attracted to m\(^6\)A in RNA.\[^{106}\] m\(^6\)A can act as a “molecular switch” by causing the disruption of particular structural motifs, thereby regulating the accessibility for RNA-binding proteins.\[^{120,121}\] For instance, U-rich sequences that interact with major constituents of SGs (i.e., hnRNPs)\[^{43}\] are frequently masked by poly-A stretches, which can be substrates for m\(^6\)A methylation. Indeed, m\(^6\)A destabilized RNA duplexes containing U–A and favored local duplex opening and hnRNP binding\[^{122}\] (Figure 2). In addition, transcriptome-wide mapping of in vivo RNA structures revealed that m\(^6\)A was indeed enriched in unstructured RNA regions,\[^{123}\] indicating the potential for particular RNA modifications in regulating structure. Of note, m\(^5\)C changes the dipole moment of the modified base, thereby impacting the aromatic amino acid-cation interactions, which have been shown to influence LLPS.\[^{66}\] Furthermore, particular methyl groups (i.e., m\(^5\)C or m\(^6\)A) introduce a hydrophobic moiety to the RNA major groove, which cannot be fully solvated. The ensuing “hydrophobic penalty” can be counteracted by non-specific interactions with hydrophobic amino acids in IDRs and PLDs of proteins lacking specific RNA modification-binding domains.\[^{124,125}\] Indeed, m\(^6\)A repressed the binding of several SG proteins to modified RNA\[^{126}\] supporting the notion that particular RNA modifications modulate binding of proteins to RNA, which could dynamically affect LLPS and BioMC biology.

### 7.4. RNA Modifications Affect RNA Processing

Because RNA structures can act as supra-molecular scaffolds in BioMC formation, it is highly likely that replacing, changing, and disrupting RNA-protein interactions will impact RNA processing. Disruption of these interactions and dissolution of BioMCs can be brought about through activities that target protein content including post-translational modifications (PTMs), chaperones or specific protein degradation, or that modulate RNA content including competition by additional RNAs, helicases, nucleases, or post-transcriptional modifications.
processing, or even degrading RNA content will affect BioMC architecture and might facilitate dissolution.

The probably best studied example for rapidly changing BioMCs is SG dissolution. SGs do rapidly disperse following stress cessation. SG dispersal is governed by pathways that are responsible for the general clearance of aggregated proteins. These include the activity of various chaperones (i.e., heat shock proteins), ubiquitination, and proteasome-proteins. These include the activity of various chaperones that are responsible for the general clearance of aggregated proteins and BioMC architecture could be neutralized and even reversed, especially when the dissolution of particular and transient BioMCs, once RNAs become associated with BioMCs, can determine the absolute number and identity of specific BioMCs. While regulation of RNA transcription and decay rates can determine the absolute number and identity of specific RNAs that actively contribute to seeding and scaffolding of particular BioMCs, once RNAs become associated with BioMCs, it remains unclear how their multivalent contribution to LLPS and BioMC architecture could be neutralized and even reversed, especially when the dissolution of particular and transient BioMCs (i.e., all stress-induced BioMCs) is required physiologically. Direct molecular “manipulation” of expressed RNA molecules and proteins is likely the means for achieving the

8. Conclusions and Outlook

RNA can serve as “trigger”, aggregating BioMCs, as “glue”, scaffolding BioMCs, as “exchange material” that associates with established BioMCs, and potentially as “access point” for activities changing BioMC architecture and possibly dissolving BioMCs. While regulation of RNA transcription and decay rates can determine the absolute number and identity of specific RNAs that actively contribute to seeding and scaffolding of particular BioMCs, once RNAs become associated with BioMCs, it remains unclear how their multivalent contribution to LLPS and BioMC architecture could be neutralized and even reversed, especially when the dissolution of particular and transient BioMCs (i.e., all stress-induced BioMCs) is required physiologically. Direct molecular “manipulation” of expressed RNA molecules and proteins is likely the means for achieving the
dynamic regulation of the multitude of non-membranous organelles (Figure 3).

Among the molecular activities directly affecting RNA structure and function, post-transcriptional RNA modifications are well suited for the dynamic modulation of sequence identity, charge, structure and protein binding of RNAs. However, analyzing the impact of RNA modifications on RNA function still suffers from low resolution, not only in terms of exactly mapping modified nucleotides in specific RNAs but also with regard to analyzing RNA content and modification status of sub-fractionated RNPs and BioMCs. Presently, only very few technologies allow separating specific RNAs and determining their modification status with reasonable certainty. Approaching sub-cellular organelle resolution (beyond separating cytoplasm from nuclei and mitochondria) has not yet been achieved in RNA modification research. Addressing RNA content, structure and modification status in individual BioMCs using improved biochemical and structural methodology might be an interesting path forward, especially for an emerging field called “epitranscriptomics” that is presently trying to reconcile the low stoichiometry of detectable RNA modifications, specifically in mRNAs, with the reported adverse effects of RNA modification system mutations on cellular and organismal physiology.

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Conflict of Interest
The authors declare no conflict of interest.

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