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Liquid–Liquid Phase Separation in Biology: Specific Stoichiometric Molecular Interactions vs Promiscuous Interactions Mediated by Disordered Sequences

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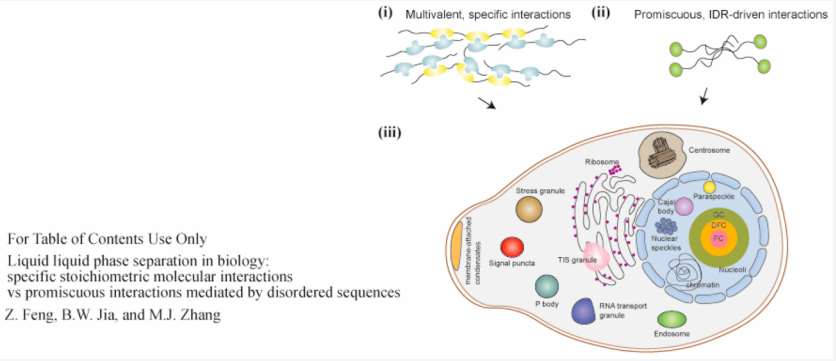

ABSTRACT: Extensive studies in the past few years have shown that nonmembrane bound organelles are likely assembled via liquid–liquid phase separation (LLPS), a process that is driven by multivalent protein–protein and/or protein–nucleic acid interactions. Both stoichiometric molecular interactions and intrinsically disordered region (IDR)-driven interactions can promote the assembly of membraneless organelles, and the field is currently dominated by IDR-driven biological condensate formation. Here we discuss recent studies that demonstrate the importance of specific biomolecular interactions for functions of diverse physiological condensates. We suggest that phase separation based on combinations of specific interactions and promiscuous IDR-driven interactions is likely a general feature of biological condensation under physiological conditions.

Cells utilize lipid membranes to compartmentalize their reaction machineries for unique biological functions with precise spatiotemporal controls. The presence of a physical barrier restricts molecules from freely moving into and out of a defined organelle, and movements of molecules require specialized transport machineries to control the organelle composition. However, many cellular compartments are not enclosed by lipid membranes (Figure 1), and yet, they can selectively concentrate molecules in defined space and time to execute desired functions. Examples include ribonucleo protein (RNP) granules in the nucleus and the cytoplasm, signaling complexes, and transport granules. These structures can rapidly assemble and be stably maintained, even though the interior components are not physically separated from the surrounding medium. For decades it has remained elusive that how nonmembrane bound organelles can selectively concentrate molecules, dynamically regulate reaction components, and precisely modulate internal biochemical activities. An important clue toward solving this long-standing mystery came from the discovery that P granules in the germ cells of *Caenorhabditis elegans* are liquid-like condensates formed through liquid–liquid phase separation (LLPS).[1] Later studies revealed that LLPS might be a common mechanism that underlies the assembly and organization of membraneless organelles. A new name was given to these condensed molecular assemblies that are not demarcated by lipid membranes as biological condensates.[2−5] The concept of phase separation has been well studied in the polymer science field. It is a spontaneous process when the polymer chains segregate from a homogeneous mixture into a more concentrated phase coexisting with a dilute phase with a clear boundary in between. In biological systems, phase separation happens when the interactions between macromolecules are sufficiently stronger than the interactions between macromolecules and the surrounding solvent, and eventually macromolecules gain the tendency to phase separate

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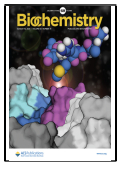

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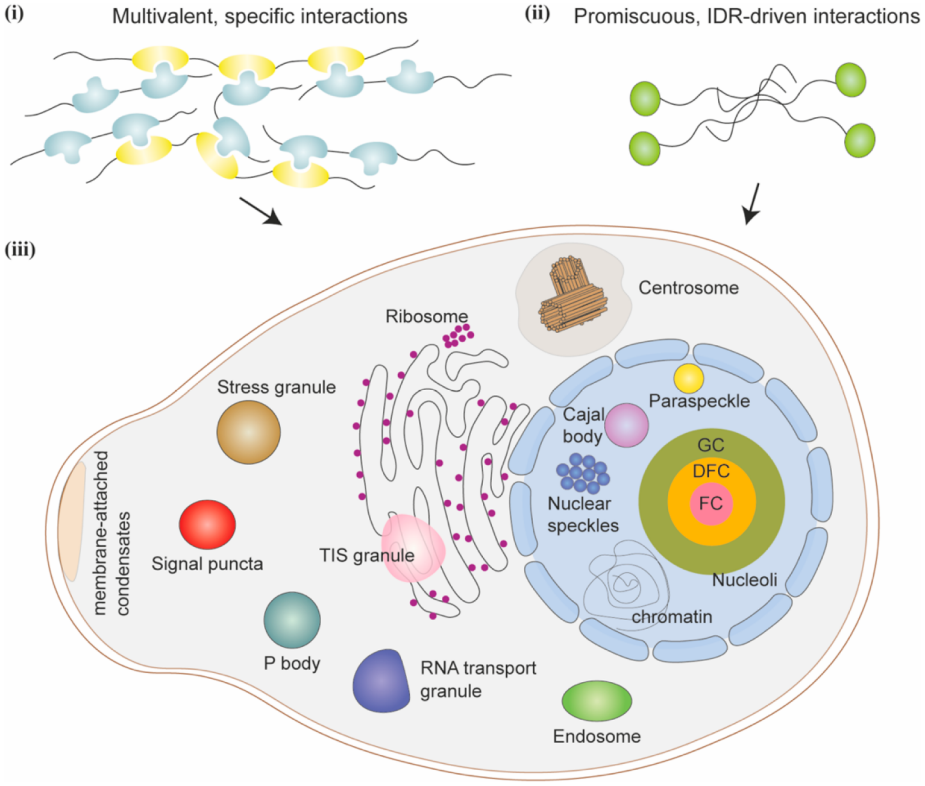

Figure 1. Biomolecular condensates in eukaryotic cells. Schematic of the biological condensates existing in the nucleus, the cytoplasm, and membranes of a eukaryotic cell. Multivalent interactions drive phase separation in three potential manners: (i) modular domain-mediated, specific stoichiometric interactions; (ii) intrinsic disordered region (IDR)-driven, nonspecific interactions; (iii) combination of specific and promiscuous interactions.

from the bulk solution forming a condensed liquid phase coexisting with a dilute liquid phase. The configurational entropic loss within the system upon forming two immiscible phases is compensated for by the gain in favorable free energy from macromolecule-macromolecule interactions (i.e., the free energy gain due to increased molecular interactions in the condensed phase offsets the unfavorable chemical potential generated due to the concentration gradient of molecule(s) between the condensed and dilute phases).

In the past few years, there has been intense interest in the idea of biomolecular condensation, and numerous studies have shown the connection between phase separation and biological functions. The assembly of these condensates is often driven by proteins, sometimes in association with nucleic acids, that contain intrinsically disordered regions (IDRs) for promiscuous interactions with poor stoichiometries and/or specific interactions with defined stoichiometries (e.g., interactions mediated by modular domains in proteins or by multimeric proteins) (Figure 1i,ii). Stoichiometric molecular interactions often occur between folded proteins/modular domains or between folded proteins/modular domains with extended linear motifs in a stereospecific manner, and thus we refer these interactions to specific interactions. In contrast, IDR-driven interactions are often of weak binding affinity and with poorly defined stoichiometries lacking stereospecificities. Interactions can occur between IDRs of many different proteins with poor discrimination between binding partners, and thus we refer these interactions to promiscuous interactions. Proteins enriched in biological condensates often contain multiple modular domains or repeats of IDRs for multivalent, intra-, or inter- molecular interactions. RNA and DNA molecules contain multiple regions that can bind to other nucleic acids or proteins. Using engineered proteins that consist of tandem repeats of modular domains, it has been demonstrated that valence of interaction systems and interaction affinity between molecules are important parameters that control phase separation.[6,7] There are now substantial mechanistic insights into how protein−protein/nucleic acid interactions contribute to phase separation, and we refer the interested readers to several recent reviews.[8−14] In principle, phase separation can be driven by three possible interaction manners: (1) specific interactions with a set of defined components; (2) weak, promiscuous interactions driven by IDRs; (3) a combination of specific and promiscuous interactions. The field seems to heavily favor an assertion that IDR-driven interactions underlie the assembly of biological condensates. Given the promiscuous nature and the weak affinity of most of IDR-mediated interactions, IDRs alone are often insufficient to drive biological condensate assembly under physiological conditions. Specific molecular interactions and IDR-driven interactions both provide binding energies that promote phase separation, but specific interactions also provide specificity for condensate formation and more space for regulation. In this Perspective, we discuss the critical roles of specific multivalent interactions in the formation of compositionally and functionally specific biological condensates.

WHY NEED SPECIFIC INTERACTIONS?

Compositional Specificity. In membrane-bound organelles, molecules within each compartment are physically separated from the surrounding medium. Lipid membranes are largely impermeable to most biological molecules and thus

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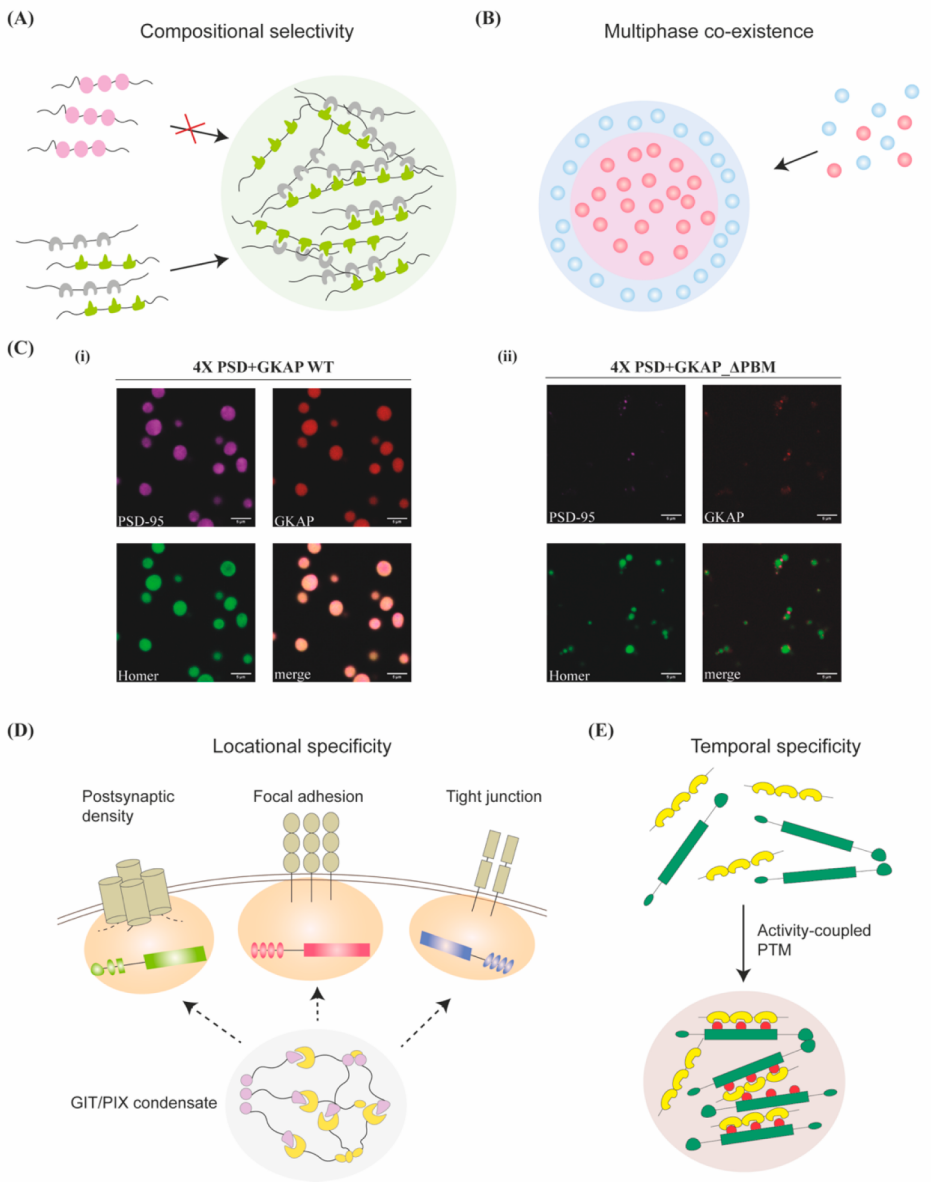

Figure 2. Functional requirements of specific interaction-driven phase separation. (A) Biomolecular condensates are selective for residential constituents. Specific stoichiometric interactions drive protein enrichment into droplets, whereas molecules that do not interact are excluded from condensates. (B) The two or more types of biological condensates can form independently, but they do not fuse when mixed together. (C) Multiphase segregation can be induced when the linkage between subcompartments within a condensate is disrupted. In in vitro reconstituted PSD condensates, major scaffold components show strong colocalization (i). Deletion of the PDZ-binding motif from GKAP (refers to as GKAP_ΔPBM), a linkage protein in PSD assemblies, leads to segregation of multiple phases (the NR2B, PSD-95 and GKAP_ΔPBM phase shown in cyan and the Homer and Shank3 phase shown in green; ii). 4XPSD refers to the mixture of NR2B, PSD-95, Shank, and Homer proteins. (D) Condensates can be recruited to different cellular localizations depending on their upstream or downstream regulators. Here the targeting of GIT/PIX condensates to various cellular compartments is shown for illustration. Interactions with scaffolding proteins in different signaling pathways determine the versatile functions of GIX/PIX condensates. (E) Post-translational modifications (PTMs) coupled with cellular activities can modulate protein−protein or protein−nucleic acid interactions to induce or remove binding interfaces that drive phase separation.

the compartment composition is controlled via specialized membrane transport machineries. In nonmembrane bound organelles, proteins and nucleic acids spontaneously assemble into discrete foci once the threshold concentration for phase separation is reached. The composition of biological condensates is specifically defined and is tightly coupled with cellular activities, whereby some components are constitutively retained and others are only recruited transiently. Different condensates can contain tens to hundreds or even thousands of types of proteins and nucleic acids. Some components might be shared between different condensates, while many are uniquely localized in specific condensates. What are the underlying mechanisms for enrichment of molecules into, or exclusion from, condensates? A general framework was developed trying to explain the condensate composition on the basis of two classes of residential molecules, scaffolds and clients.[7,15] Scaffold proteins often possess higher valence of interactions and are essential for the formation of condensates. Client molecules are recruited via interacting with scaffolds and do not significantly affect condensate formation. Specific binding interactions determine the selective client recruitment into a condensate (Figure 2A). For instance, in vitro

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reconstituted postsynaptic density (PSD) assemblies specifically enrich the auxiliary subunits of AMPARs via interactions with submicromolar affinities[16] or synaptic enzymes such as SynGAP with defined stoichiometry and high specificity.[17] Nephrin and LAT, which are transmembrane proteins for slit diaphragm formation and T cell activation, respectively, undergo phase separation following phosphorylation of multiple tyrosine residues on their cytoplasmic region that provides binding sites for cytoplasmic, multivalent adaptor proteins again with quite strong binding affinities and high specificities.[18−21]

RNP granules represent an abundant class of cellular assemblies that are organized by phase separation. RNPs include processing bodies (P bodies), stress granules (SGs), germ granules, nucleoli, Cajal bodies, etc.[22−24] They often require specific RNA species and RNA binding proteins (RBPs) for assembly and subsequently for diverse functional roles. Proteins within RNP granules are often enriched with IDRs for weak electrostatic, dipole−dipole, $\pi-\pi$, and cation-π interactions.[25−33] These low complexity regions were shown to drive phase separation of RBPs in vitro under various conditions. Later studies revealed that IDRs are necessary but not sufficient to drive phase separation under physiological conditions, and RNAs play critical roles in the granule formation both in vitro and in vivo. RNAs seed condensation by providing a multivalent scaffold to recruit multiple RBPs via association with RNA recognition motifs (RRMs). This dramatically lowers the protein concentration required to induce phase separation and allows condensation to occur in more physiological buffers.[30,34−40] The number and spacing of RRMs can influence the connectivity of molecules, the valence of interactions, and the binding strengths between proteins and RNAs, all of which are primary determinants of phase separation.[35] Sequence-specific RNA interactions likely offer substrate specificity for RNP granules. For example, polar positioning of P granules in the *C. elegans* embryo is determined by a competition mechanism for RNA binding.[41] PGL-3, a P granule protein, can phase separate in vitro and bind to long mRNA molecules with low sequence specificity.[41] MEX-5, an RBP that resides predominantly at the anterior pole of embryo, could therefore compete with PGL-3 for mRNA binding and sequester PGL-3 condensate formation in the presence of RNAs.[41] More recently, another study demonstrated that engineered condensates (ArtiGranule/ArtiG) of a synthetic protein scaffold, which consists of a self-interaction domain coupled with an RNA-binding domain isolated from a repressor protein accumulating specifically in P-bodies, can recruit a particular subset of RNAs that further localize endogenous P-body components to the condensates.[42] Alternatively, interactions between RBPs and RNAs may happen in a sequence-independent manner but show preferences for mRNA single-strandeness, length of RNAs, and availability of RNA−RNA interactions that can also confer compositional specificity for a condensate.[5,38,43−45]

Apart from selective enrichment of clients into condensate droplets, specific binding interactions also regulate the exit/unload of clients from condensates. For example, high affinity binding of Kapβ2, a nuclear import receptor (NIR), to the nuclear localization signal (NLS) of hnRNPA1, a key nuclear RBP involved in gene regulation and RNA metabolism, can rapidly dissolute hnRNPA1 droplets.[46] Similarly, Kapβ2 was also found to disperse FUS condensation via weakly binding to multiple unstructured regions, in addition to the high affinity interaction with the FUS NLS located outside the IDR.[46,47] Heterotypic Kapβ2-FUS interactions compete with homotypic FUS-FUS interactions and hence disrupt FUS phase separation. In the absence of a specific NLS-NIR interaction, FUS condensation was unaffected despite the presence of other weak interactions. Specific interactions of Kapβ2 with RBPs bearing an NLS prevent their accumulation in cytoplasmic SGs, restore their nuclear localization and function, and reverse the aberrant phase separation associated with disease conditions.[46] Another representative example has been proposed to promote PSD condensate dissolution by a specific scaffold binder that lacks multivalency.[48] Homer1a, a monomeric splicing variant of tetrameric Homer1c, can disperse the reconstituted PSD assemblies via direct competition for binding to Shank3, a scaffold protein participating in PSD condensation. This in vitro observation could be physiologically relevant, since an increased expression level of Homer1a is reported to be correlated with global downscaling of PSD sizes in cortical neurons of mice during sleep.[49,50]

These observations above suggest that specific protein−protein/nucleic acid interactions determine the selective enrichment of clients into biological condensates. In some systems, however, promiscuous IDR interactions could also serve similar roles. One example came from the observation that Ddx4 droplets and shows differential partitioning with nucleic acids. Ddx4, a primary constituent of nauge, utilizes its disordered C-terminal tail for liquid condensation. Intriguingly, Ddx4-containing nauge condensates specifically enrich single-stranded DNA, but not double-stranded DNA.[28] However, more often, nonspecific, IDR-driven interactions themselves are not sufficient, but act in concert with specific interactions, to drive selective condensation of biomolecules. For instance, fusing the IDRs from various RNP granule proteins to Cry2 can induce light-inducible LLPS driven by specific interaction-mediated oligomerization.[51] Similarly, tethering the IDRs of these proteins to an RNA-binding protein leads to phase condensation in the presence of RNA.[37] In both cases, the IDRs alone could not undergo phase separation. The extensive weak interactions provided by IDRs significantly promote LLPS driven by specific interactions, and thus the two types of interactions could work together to drive macromolecular assembly.

Formation of condensates mediated by specific and strong interactions dramatically increases the local concentration of residential components. The most striking enrichment was observed with chromatin condensates. Quantification of nucleosome concentration in condensate droplets and in the dilute solution showed a >10 000-fold increase in concentration following phase separation.[52] Similarly, it was reported that the formation of pre- and postsynaptic protein assemblies driven by specific protein−protein interactions among scaffold proteins lead to >1000-fold protein enrichment in the condensed phase.[48,53] The massive increase in molecular concentration via condensation reduces energy and material burdens for the cell, since much less material needs to be synthesized in order to reach similar concentrations required for biochemical activities when compared to reactions that take place in homogeneous solution state. The increase in concentration would also promote enzymatic activities and reaction kinetics when both enzymes and substrates show enrichment in condensates.[54−56] On the other hand, sharp concentration gradients between the condensed phase and the

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dilute phase avoid unwanted reactions to occur under concentration fluctuations.[57] In IDR-driven phase separation systems, however, the enrichment of residential species is much less significant and thus may have less profound effect on the robustness of biological systems.

In a single component system, the concentration of biomolecules within a condensate will not change, once above a threshold concentration (C_{sat}) that drives phase separation, and only the volume of droplets continues to grow.[29] Nevertheless, under physiological conditions, molecules are not isolated, but exist in a crowded multicomponent environment. Thus, compositional complexity of condensates certainly needs to be taken into consideration for thermodynamics of phase separation in living cells. A recent study measured the free energy of transfer ($\Delta G^{transfer/tr}$) associated with strong versus weaker RNA binders upon their enrichment into nucleolar condensates when the concentration of a key nucleolar protein nucleophosmin (NPM1) was manipulated.[58] As the concentration of NPM1 was increased, its associated ΔG^{tr} became unfavorable, and its nucleolar partitioning was reduced. This observation suggested that heterotypic interactions are dominating over homotypic interactions in multicomponent systems. For stronger and specific RNA binding partners, their ΔG^{tr} values were insensitive (or at least less sensitive) to variations in the NPM1 concentration. However, for weaker and promiscuous binding partners, their enrichment was hindered with the increasing NPM1 concentration likely due to direct competition for substrates. These quantitative measurements of the interplay between composition-dependent thermodynamics and the free energy change associated with condensate assembly indicated that IDR-based interactions are likely to be interfered by other competing proteins in the context of a rich protein environment.

Multiphase Organization. IDR-containing proteins often harbor repeating sequence elements for multivalent weakly adhesive intermolecular interactions. It is hard to envision that functionally and compositionally specific condensates are predominantly dictated by promiscuous IDR-based interactions, especially in an immiscible multiphase system wherein two or more types of biological condensates show unfavorable interfacial contacts and stay as immiscible phases (Figure 2B). Molecular mechanisms that drive condensate immiscibility are still poorly understood, but studies in recent years started to unravel that specific interactions may play pivotal roles in driving the incompatibility of liquid phases.

Nucleolus displays internal organization comprising multiple coexisting subcompartments, known as the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC) (Figure 1iii). The formation of each subcompartment is driven by LLPS and contains distinct molecular compositions revealed by immunofluorescence microscopy.[9,59] The key nucleolar proteins of the DFC and GC subcompartments, FIB1 and NMP1, respectively,[60,61] could undergo LLPS individually,[62−64] but when mixed together, they assembled into separate droplets with one encapsulating the other that recapitulated the "core−shell" architecture of nucleoli.[65] Via further domain truncations, it was demonstrated that the IDRs of FIB1 and NMP1 were totally miscible but the RRMs drove the mutual exclusion of the two liquid phases owing to their distinct RNA substrate specificities. The involvement of RNA binding domains in driving the maintenance of multiphase assemblies is further demonstrated in a recent study regarding the SG assembly.[66] G3BP, together with its high-affinity binding partner UBAP2L, serve as interaction nodes to recruit more RBPs, via specific domain interactions, needed to form a condensed RNP network following the influx of RNAs. Using reconstituted cytoplasmic SGs in human cells, it was shown that when the RNA binding domain of GB3P coupled with a light-inducible oligomerization domain was coexpressed with its full-length SG binding partners, both proteins assembled into immiscible condensates. In contrast, when its specific protein interaction region was retained but the RNA binding domain was removed, assembled condensates showed strong colocalization. The immiscibility of two or more phases is dictated by the values of surface tensions between condensates,[67] findings from above studies suggested that specific interactions between RNA binding domain and RNA are critical for tuning the surface tensions of different phases.

Apart from protein−protein/RNA interactions, RNA−RNA interaction is also a key driver for condensate formation and controls the residential biomolecular composition. Whi3 is a polyQ-containing RBP that functions in morphogenesis, memory of mating and stress responses, where it forms aggregates with RNA-processing bodies.[35] It possesses an RRM and an expanded polyQ tract, and both regions promote LLPS. In *Ashbya gossypii* cells, Whi3 can form condensates with specific mRNAs encoding either cell cycle regulators (e.g., the *CLN3* mRNA) in the perinuclear region or regulators for actin cytoskeleton organization (e.g., the *BNI1* and *SPA2* mRNAs) at cell tips.[38] Different subcellular localizations of its mRNA targets determine the distinct composition of Whi3 condensates. Additionally, self-interactions between mRNAs are relevant for the sorting of specific RNAs and thus for driving the compositional specificity of condensates. The secondary structure of mRNAs determines sequence complementary between RNA molecules and thus allows those encoding functionally related proteins to colocalize but excluding those encoding functionally unrelated proteins. In the presence of Whi3, protein binding further alters structural dynamics of target mRNAs, which in turn helps maintaining condensate immiscibility induced by RNA complexing.

In a multicomponent condensate, which is driven by extreme specific and multivalent interactions, multiphase phenomenon can be induced when the linkage between subcompartments within a condensate is disrupted. For example, in a five-component reconstituted PSD condensate, NR2B, PSD-95, GKAP, Shank, and Homer are involved in extensive, specific interactions, and all five components are well-colocalized in a condensed droplet. However, a GKAP mutant with the deletion of the PDZ-binding motif (PBM) becomes co-condensate with NR2B and PSD-95 but is segregated from and is immiscible to Shank/Homer condensate (Figure 2C). Manipulation on a single interaction node can, therefore, significantly alter the miscibility of condensate constituents, and this is hard to achieve if phase separation is dominated by low-specific interactions.

Nevertheless, recent simulation and theoretical studies propose that IDRs could also contribute to multiphase immiscibility. For instance, the matching of charge patterns between participating IDRs shows correlation with the tendency of proteins to demix or co-condense in coexisting phases.[68,69] In addition, interaction heterogeneity in general favors the segregation of multiple phases.[70,71] Although our understanding toward multiphase organization is still very

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rudimentary, we hypothesize that promiscuous and specific interactions are likely combined to achieve the coexistence of different biomolecular condensates each with distinct compositions.

Localization Specificity. The previous sections describe how specific protein−protein interactions offer specificity for condensate compositions. In this section, we discuss how specific, strong protein interactions determine unique localizations of condensates for broad cellular functions. One example is specific targeting of GIT/PIX condensates to distinct cellular compartments via different partner proteins in response to various signals[72] (Figure 2D). GIX and PIX are Arf-specific GTPase-activating proteins (GAPs) and Rho-specific nucleotide exchange factors (GEFs), respectively. Unexpectedly, the two enzymes bind to each other stoichiometrically with a nanomolar affinity, and the complex can undergo LLPS in vitro and in living cells without the help of additional scaffolding molecules. Depending on their interactions with specific upstream adaptor proteins, GIX/PIX condensates can be recruited to focal adhesions, tight junctions, or synapses for diverse functions. This mechanism allows a limited amount of material to be dynamically concentrated in different subcellular locations for specific processes.

Another example is illustrated by specific targeting of condensates for autophagic degradation. During selective autophagy, autophagosome is formed around cytoplasmic components for degradation by fusion with lysosome. In yeast, Ape1, a well-characterized target of selective autophagy, can assemble into liquid-like droplets that recruits Atg19, an autophagosome receptor, to the surface of condensates via specific protein interaction.[73] Using giant unilamellar vesicles (GUVs) that comprise similar lipid compositions to endomembranes in yeast, it was demonstrated that Ape1 droplets could be tethered to Atg8-anchored GUVs in an Atg19-dependent manner and this tight association between condensates and membrane drives membrane invaginations into the lumen of GUVs mimicking the process of autophagosomal engulfment. Similarly, the site of preautophagosome structure (PAS) formation is also determined by high affinity interaction between condensate and a vacuolar membrane protein (e.g., Vac8).[74] The PAS initially comprises Atg1 complexes consisting of Atg1, Atg13, Atg17, Atg29, and Atg31.[75] This early PAS then recruits downstream Atg proteins and vesicles during maturation and targets proteins from cytoplasm to vacuoles.[76,77] PAS is a biomolecular condensate with liquid-like properties both in vitro and in vivo. Its assembly is driven by multivalent specific interactions between Atg13 and Atg17, which together form a scaffold structure to recruit other Atg proteins in a stoichiometry-dependent manner.[74] Biochemical reconstitution using GUVs further demonstrated that PAS droplets could be tethered to Vac8-anchored GUVs via 1:1 interaction between Vac8 and Atg13 with sub micromolar binding affinity.[74,78]

Furthermore, long-distance transport of RNA granules is recently found to be governed by specific tethering to moving lysosomes in neurons.[79] Protein translation often occur locally in neurons to ensure precise spatiotemporal signaling. This means numerous mRNAs and ribosomes need to be transported from the nucleus to distal ends of axons and dendritic arbors that can locate meters away. It was recently demonstrated that an ALS-associated protein annexin A11 (ANXA11) acts as a molecular tether between RNA granules and lysosomes.[79] ANXA11 contains an IDR, which facilitates its enrichment into RNA granules, and a membrane binding domain, which binds to $PI(3,5)P_2$-containing membranes in the presence of Ca^{2+} ions. Neurodegenerative disease-associated mutations in ANXA11 altered biophysical properties of its associated RNA granules and disrupted its lysosome interactions, leading to defects in delivery of mRNAs to distal regions of neurons.

Many biomolecules in subcellular compartments need specific orientation to perform their biofunctions. For example, PSD is a semienclosed condensate with one side being attached to the postsynaptic membrane and another side facing the cytosol. In PSD condensate, MAGUKs are responsible for enriching and stabilizing receptors on the membrane, whereas Shank is associated with cytoskeleton-binding proteins. Previous studies demonstrated this orientation specificity either by immunogold EM[80,81] or by super-resolution imaging.[82] In vitro reconstitution of PSD assemblies on a supported lipid bilayer also demonstrated specific layered organization of condensate components.[48] We believe that this orientation specificity is governed by a specific interaction network so that biomolecules are well-organized layer by layer to bridge upstream receptors to a downstream actin cytoskeleton network.

Temporal Specificity. One advantage of phase separation systems is that condensate functions can be easily tuned through condensation or dissolution of internal components for optimal and precise cellular reactions. Post-translational modification (PTM) is one of the most important regulatory mechanisms and is often coupled with different stages of cellular events. One can envisage that protein−protein or protein−RNA interactions might be introduced or removed though modification (Figure 2E). For instance, chromatin acetylation creates new interaction interfaces for bromodomain-containing proteins, and multivalent interactions could induce phase separation of acetylated chromatin that forms a distinct phase condensate with unmodified chromatin structures.[52] This creates an example that is believed to be compatible with other covalent modifications of histone tails and thus to establish different regulatory condensates for diverse functional aspects. Similarly, cGAS exhibits cell cycle-dependent activity based on its PTM status and subsequently the biomolecules it binds to.[83,84] cGAS binds to double-stranded DNAs via its positively charged N-terminal region and its catalytic domain. During interphase, multivalent interactions between cGAS and DNA lead to their phase separation and cGAS activation in the cytoplasm to trigger immune responses. At the G2/M transition, phosphorylation at the N-terminus of cGAS disrupts DNA binding but induces its binding to chromatin instead. Chromatin-associated cGAS could not phase separate and leaves the protein in an inactivated state. Analogous to PTMs of proteins, PTMs can also occur on RNAs to regulate their functions. Due to difficulties working with RNA PTMs in vitro and in vivo, much less is known about how PTMs can regulate condensate formation and sorting of RNAs to different condensates. However, one can imagine that modifications can alter phase separation by altering RNA structures, adding or removing protein binding sites or modulating RNA−RNA interaction strengths.

Transmembrane signaling events are regulated in response to extracellular signals. In excitatory synapses, the arrival of action potential at the presynaptic terminal triggers the release

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of neurotransmitters that bind to and activate receptor channels located on the postsynaptic membrane. After depolarization, the subsequent influx of Ca^{2+} ions activate CaMKII, the most abundant protein in PSDs, and induces its association with PSD-95/NR2B condensates.[85] Upon the removal of Ca^{2+}, CaMKII reverts to its autoinhibited conformation upon dephosphorylation by protein phosphatases such as PP2A and dissociates from the PSD-95/NR2B phase condensates. Alternatively, it binds to and forms condensates with Shank3, another major scaffold protein in PSD. The switch of specific binding partners, in the presence or absence of Ca^{2+}, allows CaMKII to shuttle between different layers (also known as different nanodomains) of PSDs for scaffolding and kinetic functions, under excitatory conditions, or as a pool for storage, under basal conditions. The Ca^{2+}-dependent lipid binding activity of ANXA11 for RNA granule/lysosome docking,[79] which is mentioned in the previous section, provides another example to illustrate how extracellular signals can modulate precise spatiotemporal recruitment/release of condensates.

CONCLUDING REMARKS

Here in this Perspective, we highlight examples to illustrate how specific protein−protein/nucleic acid interactions dictate condensate composition, multiphase immiscibility, subcellular localization, and subsequently their functional roles. In IDR-driven phase separation systems, the molecular mechanisms governing their compositional and functional specificity have been poorly characterized. The purpose of our review is not to argue that weak, promiscuous IDR-driven interactions are not critical to drive phase separation, or they cannot contribute to the specificity of condensate assembly. Some IDRs can provide specific interactions, for examplem via formation of local structures such as LARKS or cross-β interactions.[27,30,39,86] Additionally, when large concentrations of IDRs are synthesized/concentrated in locally defined regions such as protein translation factories, RNA processing machineries, RNA storage depots, etc., such IDRs via interacting homotypically and/or heterotypically can form functionally specific condensates. The charge distribution and the spacing between charged clusters could also afford sequence specificity.[87] Small changes in promiscuous interaction strengths might have additive effects to modulate the phase separation capability. However, IDRs alone, in our opinion, are often not sufficient to drive LLPS in cells under physiological conditions. This is not unexpected, since IDRs can bind to itself or other IDRs in many other proteins. In a crowded protein environment, other proteins may compete for binding to IDRs and thus disrupt IDR-driven LLPS. In some systems, the IDRs from any generic proteins are interchangeable, suggesting that IDRs promote LLPS through a variety of weak, nonspecific interactions. However, IDR-based interactions can lower the threshold concentration for phase separation in conjunction with specific molecular interactions. In addition, the nonstochiometric nature of IDR interactions enable recruitment of many proteins to the condensate. Thus, the two types of heterotypic interactions act cooperatively to promote LLPS, and the combination of specific and promiscuous interactions is likely to provide a general mechanism that governs biological condensation.

Numerous studies over the past few years have demonstrated the significance of phase separation in driving biological condensate assembly and function. This fast-growing research field has attracted huge crowds of researchers, and we are beginning to unravel some of the general principles behind biological phase separation. As the appreciation of the significance of this phenomenon increases, it is important to keep in mind that physiological conditions and full-length proteins should be used whenever possible for all experiments. Nonphysiological buffer conditions, such as low salt concentrations, extreme temperature range, the presence of high concentrations crowding reagents, etc., relatively high protein concentrations, and the inclusion of large fluorescence tags could all lead to nonphysiological phase separation in vitro and in living cells. And in some cases, the requirement of extreme assay conditions to drive phase separation might in fact hint at a lack of specific molecular interactions within the system.

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