Review Article

Advances in the phase separation-organized membraneless organelles in cells: a narrative review

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Abstract: Membraneless organelles (MLOs) are micro-compartments that lack delimiting membranes, concentrating several macro-molecules with a high local concentration in eukaryotic cells. Recent studies have shown that MLOs have pivotal roles in multiple biological processes, including gene transcription, RNA metabolism, translation, protein modification, and signal transduction. These biological processes in cells have essential functions in many diseases, such as cancer, neurodegenerative diseases, and virus-related diseases. The liquid-liquid phase separation (LLPS) microenvironment within cells is thought to be the driving force for initiating the formation of micro-compartments with a liquid-like property, becoming an important organizing principle for MLOs to mediate organism responses. In this review, we comprehensively elucidated the formation of these MLOs and the relationship between biological functions and associated diseases. The mechanisms underlying the influence of protein concentration and valency on phase separation in cells are also discussed. MLOs undergoing the LLPS process have diverse functions, including stimulation of some adaptive and reversible responses to alter the transcriptional or translational processes, regulation of the concentrations of biomolecules in living cells, and maintenance of cell morphogenesis. Finally, we highlight that the development of this field could pave the way for developing novel therapeutic strategies for the treatment of LLPS-related diseases based on the understanding of phase separation in the coming years.

Keywords: Membraneless organelle (MLO); micro-compartment; liquid-liquid phase separation (LLPS); biological process; cancer

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Introduction

Compartmentalization of cells into distinct, functional rooms in a space- and time-regulated manner is essential in eukaryotic cells, facilitating spatiotemporal regulation of biological reactions. In these separate compartments, there are a multitude of complex biochemical reactions over diverse, active components to carry out various physiological processes (1,2). With the influence of the surrounding environment, cells rapidly regulate the rate and direction of these reactions based on the localization of the reaction components. Specifically, concentrating components within a specific space can significantly increase the reaction kinetics to meet physiological needs, whereas segregating them can remarkably slow or inhibit reactions to protect cells from damaging activities under adverse environmental conditions, such as stress, hypoxia, and low pH (3-5).

Membrane-bound organelles (MOs) are subcellular compartments separated from their surrounding environment by an apparent boundary, orchestrated by

surrounding lipid bilayer membranes, and they include the nucleus, mitochondria, lysosomes, endoplasmic reticulum, and Golgi apparatus (6). In addition to canonical MOs, cells also harbor several novel compartments that have no obvious physical boundaries, which is attributed to the lack of a delimiting membrane. Interestingly, membraneless organelles (MLOs) contain a high concentration of internal components, including proteins, nucleic acids, and other molecules (7-9). Recently, MLOs have attracted considerable interest from researchers due to their potential roles in multiple biological processes. Based on the phase-separation phenomenon, MLOs may endow cells with the ability of self-protection in response to environmental change, including the regulation of gene expression, the stability of protein, and the control of signal transduction (10-14). In this review, we comprehensively elucidated the formation of these MLOs and the relationship between biological functions and associated diseases. The mechanisms underlying the influence of protein concentration and valency on phase separation in cells are also discussed.

MLOs and biomolecular phase separation

In the field of cell biology, MLOs are more generally known as biomolecular condensates, in which the inner molecules are concentrated to a higher concentration relative to their surrounding milieu (15). Interestingly, recent studies have highlighted that the driving force underlying the formation of MLOs is mainly generated by the liquid-liquid phase separation (LLPS) microenvironment (16). Phase transition is a well-known process that spontaneously occurs when the concentration of the components reaches a certain threshold, and is ubiquitous and serves a vital function within living cells (*Figure 1A*). In particular, LLPS within the cells is thought to be the main driving force behind the formation of biomolecular condensates, in which several proteins, RNAs, and other biomolecules undergo phase separation and finally form liquid-like droplets, thereby becoming an important organizing principle for MLOs and further determining the organism responses following the changes in the external environment (17).

Protein structure and LLPS

Generally, internal molecules have several attractive structures that promote the interactions between components to drive the formation of condensed biomolecules (18). There are two main types of structural proteins involved in various biomolecular aggregates, including various proteins carrying different types of multiple modular interaction domains (MIDs) and proteins containing a large number of low complexity domains (LCDs) (*Figure 1B*) (19,20).

There are now several examples of proteins with structural characteristic properties of multiple MIDs that drive phase separation, which is proved to be important for signaling complexes. For example, the actin-regulatory signaling pathway has been described, and mainly contain three types of multivalent proteins: phospho-tyrosine residues of nephrin, Src homology 2 (SH2), and SH3 domains of Nck, and proline-rich motifs (PRMs) of neural Wiskott-Aldrich syndrome protein (N-WASP) (20). When the signaling pathway is activated, these proteins rapidly form phase-separated clusters through multivalent interactions of the internal components, such as between phospho-tyrosine residues and the SH2 domains and between PRMs and the SH3 domains. A recent study by Banani demonstrated that in various condensates, such as promyelocytic leukemia (PML) bodies, proteins comprising multiple repeats of small ubiquitin-related modifier (SUMO) domains could bind to other proteins with SUMO-interacting motif (SIM) ligands, thereby generating liquid-like condensates (21,22).

A second possible structure for condensate formation by proteins contains large LCDs, also named intrinsically disordered regions (IDRs) in most intracellular condensates. The driving force for phase separation of IDR-containing proteins can be provided by the multivalent interactions between proteins carrying with oppositely charged residues or between two molecules harboring repeated sequence elements. Kato showed that phase separation of RNA-binding proteins (RBPs) fused in sarcoma (FUS) was dependent on tyrosine residues enriched in FUS LCDs, and Pak demonstrated that many complementary charged residues were required for phase separation in the disordered intracellular domain of nephrin (23,24). In addition, proteins containing prion-like domains are the second category in aggregated proteins with many LCDs, including α-helical structures (such as TDP43 LCDs) and β-strands (such as hnRNPA2 LCDs), indicating the vital functions of secondary structures of proteins in the formation of liquid-like condensates (25). Conicella *et al.* elucidated that the driving force derived from weak multivalent adhesions of α-helical structures is important in the phase separation of TDP43 (26). Similarly, a recent study indicated that the interactions between β-strands

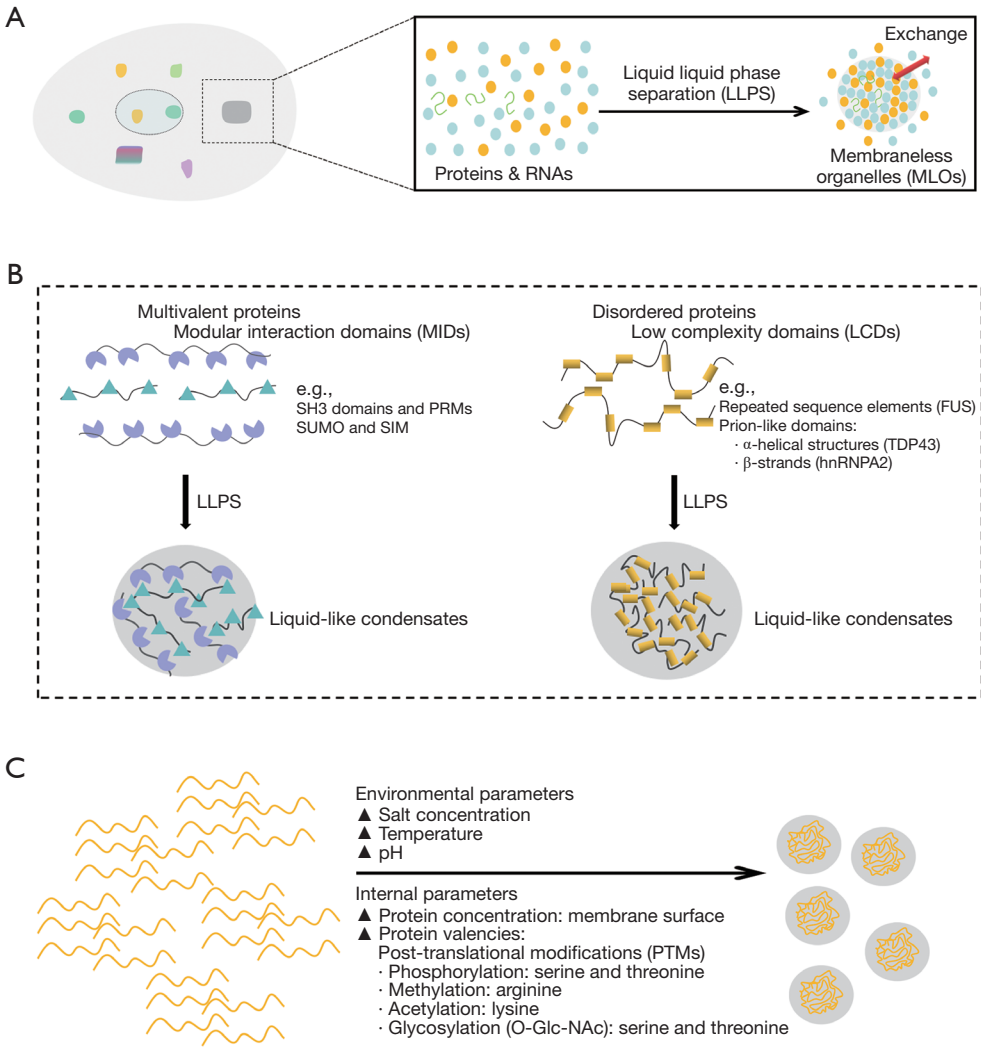

Figure 1 Overview of MLOs in living cells. (A) MLOs formed by LLPS. MLOs are ubiquitous in cells, where inner molecules undergoing liquid-liquid phase separation are concentrated to a higher concentration relative to their surrounding milieu. (B) Molecular characteristics of proteins involved in many liquid-like condensates. Left channel, proteins carrying MIDs are prone to driving phase separation through multivalent interactions of internal components, such as SH2 and SH3 domains of Nck and PRMs of neural Wiskott-Aldrich syndrome protein, as well as multiple repeats of SUMO domains and SIM ligands. Right channel, protein containing a large number of LCDs provides multiple weakly adhesive sequence elements to drive phase separation, including repeated sequence elements (such as FUS and nephrin) and prion-like domains (such as α-helical structures and β-strands). (C) Regulation of phase separation in cellular compartmentalization. In living cells, there are different types of multivalent interactions to facilitate phase separation, which is regulated by several environmental parameters (such as salt concentration, temperature, and pH) and internal parameters (such as protein concentrations and valencies). Specifically, various PTMs of proteins effectively remodel the valency and interaction strength of proteins, thereby tuning the assembly of biomolecular condensates through modulating the process of phase separation. LLPS, liquid-liquid phase separation; MLOs, membraneless organelles; MIDs, modular interaction domains; SH3, Src homology 3; PRMs, proline-rich motifs; SUMO, small ubiquitin-related modifier; SIM, SUMO-interacting motif; LCDs, low complexity domains; FUS, fused in sarcoma; TDP43, TAR DNA-binding protein 43; hnRNPA2, heterogeneous-nuclear ribonucleoprotein group A2; PTMs, post-translational modifications.

could trigger the aggregation of hnRNPA2 proteins undergoing the LLPS process (27). In summary, protein-protein interactions based on repetitive sequences (MIDs or LCDs) are an essential driving force for the formation of MLOs by the LLPS microenvironment.

Regulation of phase separation in cellular compartmentalization

LLPS is an essential process underlying the formation of MLOs by multivalent interactions among proteins and nucleic acids. Accumulating evidence has revealed that there are several different types of multivalent interactions facilitating phase separation, regulated by various environmental parameters (such as salt concentration, temperature, and pH) and phase separation threshold of internal molecules (such as protein concentrations and valencies) (*Figure 1C*) (15,28). Next, we review the mechanisms underlying the influence of protein concentration and valency on phase separation in cells.

Control of cellular concentration

Generally, membrane surface comprising a lipid bilayer can effectively enable regulation over the local concentrations via surface tension compared to the cytosol as well as restrict the diffusion of biomolecules to two dimensions, thereby altering threshold concentrations at which the aggregates are easy to form through phase separation (29). Accumulation of proteins at the membrane surfaces has been confirmed in several signaling cascades, thus facilitating signal transduction. For instance, the transmembrane adaptor protein of T cells, termed linker for the activation of T cells (LAT), is essential for the activation of signaling downstream of the T cell receptor (TCR) (30). Recent studies found that following TCR activation upon engagement of the associated ligands, this protein network showed a trend for triggering phase separates, which was dependent on the formation of membrane-bound clusters, including Grb2 and SOS proteins, involved in intracellular domains of TCR (31).

Control of multivalent interactions

In addition to the concentration, post-translational modifications (PTMs) of proteins, including phosphorylation of serine and threonine, methylation of arginine, acetylation of lysine, and glycosylation of serine and threonine modified with O-linked N-acetylglucosaminylation (O-Glc-NAc), are largely implicated in the modulation of the phase separation process (32). Specifically, phosphorylation of proteins can enhance or suppress phase separation, underlining the specific regulatory effects of PTMs on different condensates, including FUS, CPEB4, and TDP43 proteins (33-35). Arginine methylation has been shown to suppress phase separation via impairing cation-p interactions in several RBPs, including FUS, RNA helicase Ddx4, and hnRNPA2 (36,37). Lysine acetylation has been shown to have differing effects on phase separation in different cases, such as inhibiting phase separation in Tau protein and promoting acetylation of TDP43 (38,39). Glycosylation (O-Glc-NAc) can impair phase separation of associated proteins, decreasing the formation of protein aggregates, such as Tau, hnRNPA1, and α-synuclein (40-42).

In summary, phase separation is an intrinsic feature of several biomolecules at sufficient concentration, including proteins and nucleic acids with various valencies. The cellular mechanisms that regulate phase separation include enrichment of internal molecules and regulation of PTMs, including phosphorylation, methylation, acetylation, and glycosylation. Hence, cells can control the phase separation threshold of internal molecules through regulating membrane surface tension and PTM of proteins, thereby forming several functional condensates at the correct time and location in pathological processes. We present the following article in accordance with the Narrative Review reporting checklist (available at https://dx.doi.org/10.21037/tcr-21-1111).

Several membraneless-organelles and associated functions in cells

MLOs produced by LLPS is mainly distributed in the nucleus, nuclear membrane, cytoplasm, and plasma membrane. Here, we comprehensively discuss the physical principles and functional properties of each phase-separated MLO (*Figure 2*). A review of literature was conducted in PubMed, to identify the latest research on membraneless-organelles and associated functions in cells, and ultimately to generate a narrative review.

Nucleus

Cajal body (CB)

CBs, originally termed nucleolar accessory bodies or coiled bodies, are intranuclear MLOs known as a nuclear center for the assembly of ribonucleoproteins (RNPs), including small nuclear RNPs (snRNPs) and small nucleolar RNPs (snoRNPs) (61,62). Coilin is an essential scaffold protein of

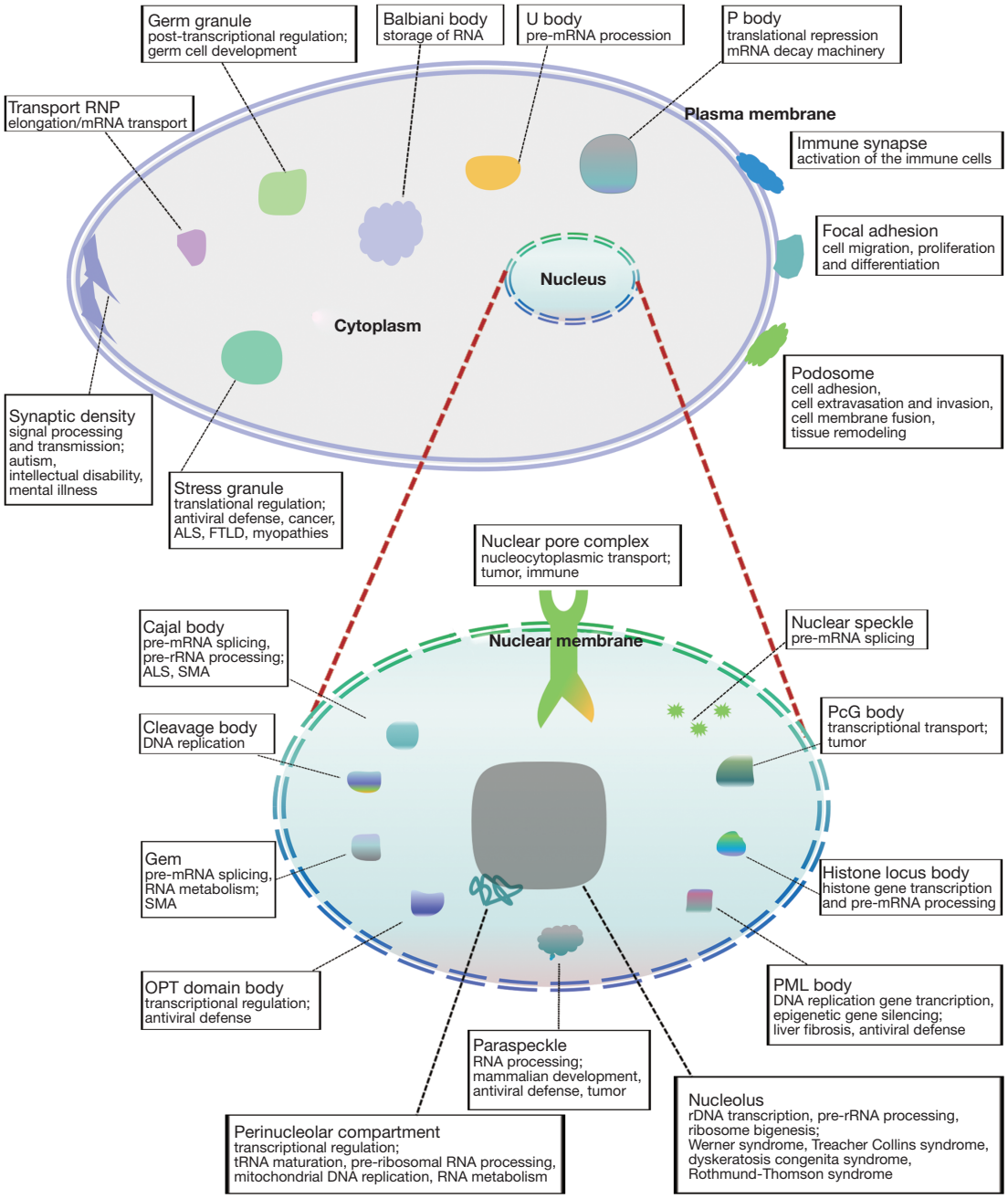

Figure 2 A schematic of various phase separation-organized MLOs in eukaryotic cells. MLOs produced by LLPS are mainly distributed in the nucleus, nuclear membrane, cytoplasm, and plasma membranes of eukaryotic cells. Specifically, several MLOs, including nucleolus, perinucleolar compartment, paraspeckle, Cajal body, cleavage body, Gem, OPT domain body, nuclear speckle, PcG body, histone locus body, and PML bodies, are mainly produced in the nucleus by the LLPS microenvironment, whereas other MLOs are produced in the nuclear membrane (such as nuclear pore complex), cytoplasm (such as stress granule, P body, U body, Balbina body, germ granule, transport RNP, and synaptic density), and plasma membrane (such as immune synapse, focal adhesion, and podosome). MLOs have multiple biological functions related to various diseases, shown in detail in *Table 1*. OPT, Oct1/PTF/transcription; PcG, polycomb group; PML, promyelocytic leukemia; P body, processing body; U body, uridine-rich snRNPs body; ALS, amyotrophic lateral sclerosis; SMA, spinal muscular atrophy; FTLD, frontotemporal lobar degeneration. MLO, membraneless organelle; LLPS, liquid-liquid phase separation.

CBs, determining the structural integrity and function of CBs. The survival motor neuron (SMN) protein complex can form snRNPs in the cytoplasm and accompany them into the nucleus, and then has a tendency to release from the snRNPs for cycling back into the cytoplasm (63). A positive correlation has been reported between transcription rates and CB numbers in HeLa cells (64). Fluorescence recovery after photobleaching (FRAP) assays showed that several CB proteins had a good characteristic of rapid exchange with the nucleoplasm, especially coilin and SMN (65). Therefore, CBs possess dynamic behaviors in various cells, resulting in the variable activity of CBs in response to gene transcription-associated changes.

CBs promote the formation of 'transcriptomes', which is attributed to the assembly of several transcript factors associated with transcription, capping, splicing, polyadenylation, and cleavage of pre-mRNAs. Pellizzoni suggested that the SMN complex facilitated the accumulation of RNA pol II in coilin-containing structures due to the interaction of the C-terminal domain of RNA pol II and Gemins through RNA helicase A (66). According to previous studies, CB dysfunction is associated with multiple neuropathological disorders, such as the mutation of the *SMN1* gene in amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) (43).

Cleavage body

Cleavage bodies are nuclear sub-organelles that contain cleavage stimulation factor CstF-64 and polyadenylation specificity factor CPSF-100 (67). Previous studies showed that cleavage bodies were spatially distributed in various cell cycles, CstF-64-containing cleavage bodies were mainly enriched in the S phase while CPSF-100-containing cleavage bodies were enriched in S and G2 phases. Li suggested that inhibition of DNA replication in cells treated with hydroxyurea could eliminate the majority of CstF-64-containing cleavage bodies, further verifying the function of cleavage bodies in DNA replication rather than RNA transcription (68). The number of cleavage bodies increased while the level of CstF-64 proteins showed no significant change during DNA replication, indicating that the formation of CstF-64-containing cleavage bodies facilitated the redistribution of CstF-64 proteins within the nucleus.

Gem

Gems or Gemini of CBs are liquid-state aggregates of SMN complexes secreted from CBs in the nucleus. Gems are 'storage cabin' for excess nuclear SMN complexes ready for recycling into the cytoplasm and facilitate the formation of snRNPs following their participation in pre-mRNA splicing. Although gems usually overlap with CBs, CB contains both coilin and SMN whereas gem only comprises of SMN. Several studies showed that a high concentration of SMN complexes facilitated the formation of gem organelles; however, gem numbers tended to decrease significantly following the low level of SMN complexes (69). It is well known that SMN is the determining factor for SMA, a genetic condition associated with neuromuscular degenerative disorder (44).

Nuclear speckle (NS)

NSs are one of the most prominent nuclear MLOs containing many pre-mRNA splicing factors [such as snRNPs and serine/arginine-rich (SR) proteins] and are widely distributed in the interchromatin regions of the nucleoplasm (70). Although the process of transcription is not associated with NSs, there are various transcription factors, such as serine-2-phosphorylated RNA pol II, and associated transcription elongation proteins other than splicing factors within NSs, revealing the potential function of NS in transcriptional regulation and processing (71). The phosphorylation-dephosphorylation procedure is essential for pre-mRNA splicing through mediating the formation of the spliceosome complex (72). The reduction of phosphorylated SR proteins involved in NSs inhibits the spliceosome assembly; however, the increase in the level of phosphorylation relieves this block in the spliceosome assembly. The size and number of NSs increased due to the increased accumulation of splicing factors when transcription is halted by inhibitors or heat shock (73). In addition, splicing factors tended to leave NSs when gene transcription was active (74). In conclusion, NSs are essential organelles containing high concentrations of splicing snRNPs and other splicing-related factors and facilitate gene transcription and RNA processing within the nucleus.

Nucleolus

The nucleolus, a distinct subnuclear MLO assembled around chromosomal nucleolus organizer regions (NOR) composed of the repeated ribosomal DNA (rDNA) clusters, is a suitable compartment for rDNA transcription, pre-rRNA processing, and initial assembly and maturation of pre-ribosome (45,75,76). Recent studies have uncovered other

essential functions of the nucleolus, including the regulation of multiple RNP assembly and PTMs of nuclear proteins, such as sumoylation and phosphorylation (77). The nucleolus also plays a special role in regulating multiple aspects of cell cycle progression through inhibiting activities of various specific proteins by sequestering these proteins in a specific compartment. Nucleolus undergoes sequential alternating cycles of disassembly and reorganization during mammalian cell mitosis, which is consistent with the view that the nucleolus is a dynamic structure; the disassembly of the nucleolus compartment turns off when mammalian cells enter mitosis and gene transcription (78). Given that the nucleolus is a ribosome production factory maintaining protein-synthesis levels during cell growth and division, the activity of the nucleolus was tightly regulated by various signaling events in cell-cycle regulation and stress responses (79). The nucleolus exerts its biological functions through regulating the level of tumor-suppressor protein p53, which coordinates cellular response to stress (80). Increasing evidence has also revealed that nucleolar structure or ribosome biogenesis is likely to change to inhibit cancer progression and viral infection (81).

OPT domain body

There is a special subnuclear MLO, termed Oct1/PTF/transcription (OPT) domains, that appear during the G1 stage of the cell division but disappear during the S stage, and contain several transcription factors such as PTF, Oct1, TBF, and Sp1 (82,83). PTF and Oct1 are regarded as useful switches to facilitate the expression of snRNAs and other 'processing RNAs' together with RNA pol II or III. Sp1 is a promoter-specific transcription factor that plays an essential role during cell metastasis and cell-cycle regulation by binding to GC-rich decanucleotide recognition elements. Interestingly, based on various transcription factors involved in OPT domain bodies, there are several important characteristics, including a high dynamic compartment at the transcription sites due to some highly disordered protein of transcription factors (84).

PcG body

Generally, positioning target genes into a special nuclear compartment, termed Polycomb group (PcG) body, induced gene repression due to repressive transcription-complexes, PcG repressive complexes 1 and 2 (PRC1 and PRC2), containing numerous gene-silencing regulators within PcG bodies (46,85). Mechanistically, PcGs often affect chromosome remodeling and compression through PTMs of histones, including methylation of lysine 27 by PRC2 and recruitment of PRC1, to promote chromatin condensation, thereby silencing many important genes. The interaction of PcG protein and cis-regulatory DNA module, termed polycomb response elements (PREs), resulted in remarkable repression of the *Hox* gene (47). PcG bodies have also been identified as sumoylation centers, resulting from the co-collocation of SUMO E2 (Ubc9) and the substrates (CtBP and CTCF) in PcG bodies, thereby serving as SUMO E3 ligases (86).

Perinucleolar compartment (PNC)

The PNC is known to be enriched with RNA processing proteins and RNA pol III transcripts around the nucleolus (87). Based on these proteins, polypyrimidine tract-binding protein often plays essential roles in pre-mRNA splicing, RNA modification, and translation (88). RNase P and RNase mitochondrial RNA processing (MRP), a subset of RNA pol III transcripts belonging to sequence-specific endoribonucleases, catalyze tRNA maturation and pre-ribosomal RNA processing and mitochondrial DNA replication, respectively (89). Based on the significant progress of PNC, it may facilitate RNA processing and assembly of RNA trafficking complexes, therefore regarded as a transitional separation space in which newly synthesized pol III RNAs and the assembly of their final functional complex are performed. Another important feature of PNC is a unique nuclear body that is closely associated with cell metastatic capacity. Frankowski reported that metarrestin, a PNC inhibitor, effectively suppresses metastatic and invasive behaviors of cancer cells (48).

PML body

PML bodies are ubiquitous MLOs in eukaryotic cells, exerting diverse nuclear functions involved in biological processes associated with DNA replication, gene transcription, or epigenetic gene silencing (90). Kurihara found that PML bodies excluded DNA methyltransferase DNMT3A, which maintained the hypo-methylated state at Y-linked gene promoters, thereby providing a novel insight into the functional properties of nuclear structures in gene transcription (91). PML protein is the essential organizer of PML bodies, of which the protein modified with SUMO to form the ubiquitin-like protein plays a critical role in the recruitment of partners. Dai demonstrated that SUMO-modified PML proteins, termed SUMOylation, promoted the development of liver fibrosis (49). A recent study by Stubbe and colleagues demonstrated that PML bodies were

enriched around viral replication centers when viral DNA binding proteins were modified with SUMOs, thereby mediating the replication of the virus (50). In addition, Sehgal revealed that the murine cognate Mx1 could form nuclear condensates overlapping with PML nuclear bodies, further exerting a vital antiviral activity (51).

Histone locus body (HLB)

The HLB is a subnuclear body that frequently appears around the histone-replicating loci to recruit various factors necessary for replication-coupled histone gene transcription and histone pre-mRNAs processing (92). Previous studies demonstrated that HLBs and CBs are not only physically associated but also have the same contents. Coilin, a marker for CBs, was often found in HLBs in high concentrations. It is easy to speculate that the components of HLBs are relatively immobile because they exchange molecules with their environment for a short period, therefore maintaining the stable functioning of metabolically active bodies. Salzler explored the formation mechanism of HLBs using a transgenic assay for ectopic *Drosophila* HLB assembly and demonstrated that HLB assembly was triggered through a special sequence in the H3-H4 bidirectional promoter, resulting in the expression of histone genes (93).

Paraspeckle

Paraspeckles are one of the essential subnuclear MLOs assembled in the interchromatin space of mammalian cells and play a key role in regulating gene expression (94-96). The majority of paraspeckle proteins harboring prion-like domains undergo LLPS through protein-protein interactions. Previous evidence showed that RBM14 and FUS with an intact prion-like domain effectively constructed paraspeckles (97). Several molecular mechanisms are underlying paraspeckle function. Paraspeckles have been shown to effectively regulate certain RNA species in the nucleus based on the nuclear retention mechanism (98). Besides, paraspeckles rapidly sequester their protein components within a spatial space, resulting in a high concentration in local areas compared to their surroundings in the nuclear, exerting accurate and efficient biological functions. Jiang demonstrated that paraspeckle proteins, SFPQ and NONO, coupled with various miRNA processing factors effectively facilitate miRNA formation (99). Furthermore, paraspeckles can regulate gene expression in various developmental and disease scenarios, including mammalian development, response to viral infection, and tumor process (52).

Nuclear membrane

Nuclear pore complex (NPC)

Several biological processes, including gene expression, chromatin organization, and DNA repair, depend on combined regulation of the nucleus and the cytoplasm, especially for the continuous exchange of various molecules (100). NPCs are assembled into the nuclear envelope through the fusion of the outer and inner nuclear membranes, forming a special channel for molecule transport, to facilitate nucleocytoplasmic transport (101). The principal structural element of NPC includes a five-layer architecture from the nucleus to the cytoplasm as follows: the nuclear basket, the nuclear rings, the inner pore ring, the cytoplasmic rings, and the cytoplasmic filaments (102,103). The NPC structure on the cytoplasmic side, the peripheral elements of cytoplasmic rings, associates with the cytoplasmic filaments that have a flexible property due to the components of intrinsically disordered domains.

Recent studies revealed that NPC facilitates nucleocytoplasmic transport in both directions, including free diffusion for small molecules and major active transport routes for various proteins and RNAs (104). Specifically, NPC-mediated import and export of macromolecules contain three cases, including the export of various nuclear RNAs, the import of ribosomal proteins and transcription factors, and the bi-directional shuttling of molecules associated with various signaling pathways. Aberrant scaffold nucleoporins (NUPs) owing to abnormal expression levels, mutations, and gene fusions, especially for human NUP214, NUP188, and NUP358, have been documented in various human cancers (53). NUP-associated cancer could be interpreted as being caused by significant changes in epigenetic chromatin modification resulting from the fallacious interactions of NUPs with chromatin and abnormal transport of cancer-related factors related to the carcinogenic or tumor-suppression process. A recent study of human Nup210 in the adaptive immune system revealed that Nup210-mediated activation of the TCR signaling is an essential prerequisite for naïve $CD4^+$ T cell homeostasis (54).

Cytoplasm

Processing body (PB)

In eukaryotic cells, PBs are one class of MLOs in the cytosol with multiple biological processes related to translational repression and the mRNA decay machinery (105). PBs have been shown to display a wide array of dynamic

behaviors (106). Recent studies revealed the mechanisms underlying the formation of PBs associated with phase transition processes, in which several types of molecular interactions mediating LLPS drive RNAs and proteins to form PBs. Moreover, the PTMs of PB components remarkably affect their formation, including phosphorylation of threonine and serine and methylation of arginine. DCP1A phosphorylated by JNK tends to depart from PBs, while JNK-mediated 4E-T hyperphosphorylation can form the larger PBs (107,108). AKT3-mediated AGO2 phosphorylation significantly enhances the interaction with other proteins, such as TNRC6A/GW182 and DDX6, facilitating PBs formation (109). Matsumoto indicated that RAP55 is important for the assembly of cytoplasmic mRNP granules and PRMT1, a protein arginine methyltransferase, is a prerequisite for RAP55A to localize to PBs (110). One major class of PB compositions is associated with various proteins related to RNA metabolism, further demonstrating that they exert several functions for gene expression. For instance, the XRN1 exonuclease and DCP1/2 decapping factor can control the 5'–3' mRNA decay, while helicase DDX6 and RBP CPEB1 are translational repression factors mediating mRNA translation (111,112). Interestingly, in virus-infected cells, the human interferon (IFN)-inducible 'myxovirus resistance protein A (MxA)' displays antiviral activity with P bodies containing respective viral nucleocapsid proteins (55).

Uridine-rich snRNPs (U snRNPs) body (U body)

U snRNPs play essential roles in pre-mRNA processing in the nucleus. However, recent studies revealed that they are assembled in the cytoplasm of eukaryotic cells (113). Generally, cytoplasmic SMN protein-containing granules, termed U bodies, have a main component associated with U snRNPs, which is coupled with various essential snRNP assembly factors. FRAP assays have indicated that U bodies are spherical, which often has a characteristic on significant dynamics, including fusion or fission of bodies (114). Tsalikis reported that U body formation was associated with the induction of metabolic stresses, such as amino acid starvation, which was triggered by cell membrane damage due to infection with intracellular bacterial pathogens, while U bodies significantly disappeared once the stress was removed, indicating that the process of U body assembly would characterize an adaptive cell-response to metabolic stress (56).

Balbiani body (Bb)

The Bb granule is a non-membrane bound and a dynamic compartment that assembles early during oocyte formation and disappears in late-stage oocytes in mammals (115). Boke showed that Xvelo harboring an N-terminal prion-like domain can form Xenopus Bbs, accompanied by co-recruitment of mitochondria and RNA (116). Escobar-Aguirre showed that microtubule-actin crosslinking factor 1α (macf1α) possesses an essential role in regulating Bb disassembly and oocyte nucleus positioning, which was demonstrated using CRISPR/Cas9 genome editing technology to delete these domains by targeting macf1α endogenous gene (117). Kaufman revealed that the wild-type rbpms2 protein was closely associated with spherical Bb formation, while Bbs disappeared in rbpms2 mutant oocytes, suggesting the crucial role of wild-type rbpms2 protein in Bb assembly (118).

Germ granule

Germ granules are MLOs associated with RNA-rich cytoplasmic bodies of germ cells, carrying many proteins and RNAs related to post-transcriptional regulation specific to germ cells (119,120). A study by Brangwynne revealed that individual components involved in germ granules are highly dynamic (16). Germ granules play significant roles in the development of germ cells. First, germ granule has been implicated in post-transcriptional regulation of mRNAs, such as mRNA localization, stability, and translational activity (121). Seydoux showed that germ granules may be essential to preserve the plasticity of the germline genome due to the control of gene expression in germ cells (57). Second, germ granule determines the germ cell fate because germ granules are used as a part of the germplasm.

Transport RNP

Transport RNPs are prevalent in neurons, and often harbor different mRNAs and associated binding proteins, such as elongation factors and ribosomal proteins or even clusters of ribosomes, possibly serving as subunits of molecular motors that are essential for mRNA transport (122). Monani showed that SMN and Gemin proteins involved in the RNP complex had a beneficial effect on the assembly of spliceosomal RNPs, further playing an essential role in the formation of transport RNPs (123). Additionally, previous studies demonstrated that transport RNPs could facilitate RNA localization and achieve precise translational control.

Synaptic density

In addition to MOs and MLOs, there is a unique type of membrane-semi-enclosed compartments in neuron

cells, termed synapses, that mediate signal processing and transmission involved in the nervous system (124). Postsynaptic densities (PSDs) are typical synapses that contain glutamate receptors and associated signaling and structural molecules. Zeng reported that the multivalent postsynaptic protein-protein interaction between SynGAP and PSD-95 can contribute to the LLPS process, which is possibly a novel mechanism for PSD formation (58). Recent studies indicated that molecular components within PSDs could exert highly dynamic activity, constantly exchanging their components with bulk aqueous cytoplasm in synaptic spines (125). Aberrant development and regulation of PSD often result in an imbalance between excitation and inhibition in neuronal circuits, which causes multiple diseases, such as autism, intellectual disability, and mental illness.

Stress granule (SG)

SG represents assemblies of untranslated messenger RNPs (mRNPs) derived from mRNAs arrested in translation initiation (126,127). Interestingly, SGs have two distinct layers, including a core structure with higher concentrations of proteins and mRNAs and a potentially dynamic shell (128). FRAP assays have shown that most components of SGs are rapidly exchanged, thereby undergoing fusion and fission in the cytosol, suggesting that SGs are dynamic structures (129). A dense network of protein-protein interactions between mRNA-binding proteins significantly contributes to SG formation, which is modulated by various PTMs, including methylation, phosphorylation, and glycosylation. Goulet demonstrated that methylated arginine is necessary for the recruitment of TDRD3 in SGs, facilitating the protein aggregation in SGs (130). Several observations showed that phosphorylated protein impairs granule assembly, suggesting that SG disassembly is promoted by Grb7 phosphorylation and DYRK3 kinase in focal adhesion kinase (FAK) and mTORC1 signaling pathway, respectively (131,132). In addition, Ohn found that O-Glc-NAc glycosylation of proteins enhances SG formation and Duan suggested a crucial role of PARylation in regulating the dynamics of RNP granules, contributing to ALS disease pathogenesis by promoting SG formation (59,60).

SG formation plays an essential role in several biological processes. First, SGs rapidly recruit numerous antiviral proteins at a high local concentration after viral infection, further enhancing the activation of innate immune response and improving viral resistance (133,134). Second, SGs have been proposed to modulate signaling pathways by sequestering components of TOR, RACK1, or TRAF2 signaling pathways (135,136). Importantly, SG formation often seems to be involved in various human diseases, including several degenerative diseases such as ALS, frontotemporal lobar degeneration (FTLD), and various myopathies (137). In addition, SG formation contributes to tumor progression, and chemotherapy drugs effectively disturb SG formation, which might provide a new strategy for cancer treatment.

Plasma membrane

Immune synapse

An immune synapse is often formed on the surface of activated immune cells, such as T cells, B cells, and natural killer (NK) cells (138). Upon encounter with associated antigens, immune cells are activated by the reorganization of its membrane, rapidly forming a specific compartment known as the immune synapse. Benard *et al.* found that LFA-1 and CD28 molecules play a crucial role in enhancing the impact of TCR clustering on cell spreading and actin organization (139,140). In addition, Nowosad and colleagues have demonstrated that germinal center B cells recognize antigens through a specialized immune synapse architecture, thus B cells can selectively recruit antigens by altering individual components in the synaptic architecture (141). For NK cells, recent data have revealed that NKp46 signaling directly regulates the associated immune synapse rearrangement and several immune synapse-related functions (142).

Focal adhesions (FAs)

FA is a special non-membrane structure composed of clustered transmembrane proteins and signaling proteins, such as integrin, talin, paxillin, vinculin, and FAK (143). Recent studies demonstrated that FAs often link to the cellular cytoskeleton, further initiating cell activities, such as cell migration, proliferation, and differentiation (144). Cho reported that the FA molecule, matrix metalloproteinase 1 (Mmp1), effectively regulates astrocyte morphology and glutamate transporters to suppress seizure-like behavior (145). Png showed that transglutaminase-2 influenced phosphorylation of paxillin by JNK, further interacting with matrix proteins and integrins, thereby forming FAs (146). Luo found that ARAP2, an Arf GTPase-activating protein, affects FA dynamics in an Akt activity-dependent manner, further regulating the size and number of FAs (147). Toro-Tapia proposed that Ric-8A-mediated Gα13 signaling is required for proper cell migration by controlling FA

dynamics (148).

Podosome

Podosomes are multimolecular cytoskeletal structures that are particularly formed in various monocytic lineage cells, endothelial cells, and smooth muscle cells (149). Interestingly, podosomes can achieve the cell-matrix linkage between cells and their surroundings, such as integrins (150). Therefore, these dynamic interactions suggest that podosomes may continuously recognize the surrounding information, further reshaping the pericellular environment. Previous studies highlighted the specific functions of podosomes in several primary human monocytic cells, including cell adhesion, cell extravasation and invasion, cell membrane fusion, and tissue remodeling (151).

Conclusions

Given that various biomolecules can undergo the LLPS process under idealized conditions, there is great interest in understanding the specific properties of biological condensates. Many previous studies suggested that biomolecular condensates have pervasive roles in cell biology. However, research in this field tends to progress from the phase behavior of biomolecules to a general platform that explains and predicts bio-functional effects of these biomolecules. Several trials with cellular extracts are essential approaches that may help to explore the functional effects of biomolecular condensates in cellular processes. In this review, we summarize and highlight the diverse biological functions of condensates, providing an important framework for a better understanding of the biological processes associated with the phase behavior of biomolecules (*Table 1*).

First, intracellular MLOs may induce some adaptive and reversible responses that are exquisitely sensitive to changes in physicochemical conditions involved in the extracellular environment, including alteration of transcriptional or translational processes (152,153). In addition, MLOs are essential in controlling endogenous cellular activities through sequestration of molecules; therefore, some components are recruited into a dense phase whereas others distribute in the dilute phase, thereby inhibiting multiple biological processes, such as enzymatic reaction or signaling transduction. Second, MLOs can be used to control biomolecule concentrations in living cells. When the concentration of a biomolecule is in a saturation state, phase-separated MLOs can be locally formed. Interestingly, excess protein stored in organelles with high concentrations generally creates a replenishment pool for biomolecules in cells; intramolecules spontaneously enter the surrounding environment to promote biological processes when the concentration of biomolecules drops. In addition, LLPS driving high concentration of molecules in condensates may activate and accelerate biochemical reactions, promoting various signaling processes. Third, MLOs formed by LLPS may be important for maintaining cell morphogenesis. Specifically, LLPS can mediate the formation of materials with viscoelastic properties, improving cellular structures, including many membrane- and membraneless-containing organelles (154). Schmidt *et al.* demonstrated that LLPS formed by dynamical interactions between the macromolecules facilitates the formation of nuclear pores (155).

In 2019, a novel coronavirus, termed SARS-CoV-2, has rapidly spread around the world, leading to a new public health concern. Interestingly, previous studies revealed that the life cycle of SARS-CoV-2 is closely related to the LLPS phenomenon, including SARS-CoV-2 assembly and replication. For instance, Chen reported that the LLPS of nucleocapsid (N) protein and genome RNA may be essential driving force for SARS-CoV-2 viral assembly, highlighting intervention strategies to combat SARS-CoV-2 infections through the impaired viral assembly attributed to disrupting the LLPS (156). Iserman demonstrated that the nucleocapsid protein (N-protein) may rapidly undergo LLPS. In particular, RNA sequences located at 5' and 3' ends of the genome may promote N-protein condensation while other genomic regions (frameshifting region) exert a special function on facilitating condensate dissolution, presenting a identifying platform for detecting antiviral compounds effective against SARS-CoV-2 based on targeting phase-separation (157). Savastano suggested the RNA genome may effectively induce nucleocapsid protein LLPS, resulting in generation of high-density biomolecule condensates containing viral RNA-dependent RNA polymerase that determines the SARS-CoV-2 replication capacity, and finally providing a theoretical basis for the design of novel therapeutics to combat SARS-CoV-2 through inhibition of the RNA-induced phase separation of N protein (158).

MLOs produced by LLPS in cells may concentrate biomolecules (proteins and nucleic acids) with high concentrations compared to the surrounding environment. This phenomenon has essential functions in multiple cellular processes, such as RNA metabolism, regulation of gene expression, stress adaptation, and transmembrane

Table 1 Basic characteristics of several MLOs

Location	MLO name	Defining components	Functions	Diseases
Nucleus	Cajal body	Coilin, SMN	Pre-mRNA splicing, pre-rRNA processing	ALS, SMA (43)
	Cleavage body	CstF-64, CPSF-100	DNA replication, processing	NA
	Gem	SMN	Pre-mRNA splicing, RNA metabolism	SMA (44)
	Nuclear speckle	snRNPs, SR proteins, Malat1	Gene transcription, RNA processing	NA
	Nucleolus	RNA Pol I machinery	rDNA transcription, pre-rRNA processing, ribosome biogenesis	Werner syndrome, Treacher Collins syndrome, dyskeratosis congenita syndrome, Rothmund-Thomson syndrome (45)
	OPT domain body	PTF, Oct1, TBF, Sp1	Gene transcription, antiviral defense	NA
	PcG body	PRC1, PRC2, PREs	Transcriptional repression, sumoylation centers	Cancer (46,47)
	Perinucleolar compartment	CUGBP, KSRP	tRNA maturation, pre-ribosomal RNA processing, mitochondrial DNA replication	Cancer (48)
	PML body	PML, DNMT3A, Mx1	DNA replication, transcription, epigenetic gene silencing	Liver fibrosis (49), antiviral defense (50,51)
	Histone locus body	NPAT, FLASH	Histone transcription, pre-mRNAs processing	NA
	Paraspeckle	CTN-RNA, PSP1, p54nrb	RNA processing	Mammalian development, antiviral defense, cancer (52)
Nuclear membrane	Nuclear pore complex	FG-NUPs	Nucleocytoplasmic transport	Cancer (53), immune (54)
Cytoplasm	P body	DCP1A, TNRC6A/GW18, DDX6, RAP55, MxA	mRNA metabolism	(55)
	U body	SMN	snRNP metabolism	SMA (56)
	Balbiani Body	macf1α, rbpms2	RNA storage	NA
	Germ granule	NA	Post-transcriptional regulation	Germ cell development (57)
	Transport RNP	Staufen1, Staufen2, FMRP, ZBP1, hnRNPA2, CPEB, Pura, and SMN	mRNA transport	NA
	Synaptic density	SynGAP, PSD-95	Signal processing and transmission	Autism, intellectual disability, mental illness (58)
	Stress granule	RBPs, non-RBPs, TDRD3	Translational regulation, mRNA storage	ALS, FTLD, some myopathies (59,60)

Table 1 (*continued*)

Table 1 (*continued*)

Location	MLO name	Defining components	Functions	Diseases
Plasma membrane	Immune synapse	TCR, NKp46	Immune synapse rearrangement	NA
	Focal adhesions	Integrin, talin, paxillin, vinculin, FAK, Mmp1	Cell migration, proliferation, differentiation	NA
	Podosome	actin-rich proteins, actin-associated proteins	Cell adhesion, migration	NA

MLO, membraneless organelle, NA, not applicable; OPT, Oct1/PTF/transcription; PcG, polycomb group; PML, promyelocytic leukemia; P body, processing body; U body, uridine-rich snRNPs body; ALS, amyotrophic lateral sclerosis; SMA, spinal muscular atrophy; FTLD, frontotemporal lobar degeneration.

signaling cascades. The present review, evaluated the current state of knowledge of MLOs, highlighting their biogenesis, organization, dynamics, and function. Understanding phase separation could pave the way for developing novel therapeutic strategies for the treatment of LLPS-related diseases or tumors in the future.

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