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Review

Mechanisms and regulation underlying membraneless organelle plasticity control

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Evolution has enabled living cells to adopt their structural and functional complexity by organizing intricate cellular compartments, such as membrane-bound and membraneless organelles (MLOs), for spatiotemporal catalysis of physiochemical reactions essential for cell plasticity control. Emerging evidence and view support the notion that MLOs are built by multivalent interactions of biomolecules via phase separation and transition mechanisms. In healthy cells, dynamic chemical modifications regulate MLO plasticity, and reversible phase separation is essential for cell homeostasis. Emerging evidence revealed that aberrant phase separation results in numerous neurodegenerative disorders, cancer, and other diseases. In this review, we provide molecular underpinnings on (i) mechanistic understanding of phase separation, (ii) unifying structural and mechanistic principles that underlie this phenomenon, (iii) various mechanisms that are used by cells for the regulation of phase separation, and (iv) emerging therapeutic and other applications.

Keywords: liquid–liquid phase separation, membraneless organelles, biomolecular condensates, intrinsically disordered proteins, post-translational modifications

Introduction

The multifaceted cellular biochemistry requires appropriate organization to keep cellular metabolism spatiotemporally regulated. Compartmentalization in eukaryotic cells ensures that specific activities occur in localized places to circumvent the perturbation of dynamic post-translation modifications (PTMs) and proteolysis etc. This compartmentalization is manifested in the form of classical membrane-bound organelles, which are surrounded by lipid bilayer to establish a physical barrier that protects their internal contents from the external surroundings. Emerging evidence indicates that, in addition to membrane-bound organelles, cells do possess membraneless organelles (MLOs) (Liu et al., 2020a). MLO plasticity is defined as the ability of reversible self-assembly and disassembly of those compartments in response to intrinsic and extracellular cues by which PTMs exert the regulatory function on macromolecules such as protein and nucleotide acids. Although the concept of MLOs is not new, as in 1938, Alexander Oparin, a Soviet biochemist, proposed in his book *The Origin of Life* that life came into being as coacervate drops of organic materials (Oparin, 1938). Nonetheless, his theory was not received well because it could not justify the presence of membrane barriers in eukaryotic cells. However, the discovery of MLOs favors Oparin's coacervate theory (Aumiller and Keating, 2016; Feric et al., 2016). There are nuclear MLOs, including the nucleolus, nuclear speckles, Cajal bodies, the Balbiani body, promyelocytic leukemia protein (PML) bodies, and germ granules (Hernandez-Verdun, 2011; Batty et al., 2012), and cytoplasmic MLOs including P-bodies, germ granules, and stress granules (Buchan, 2014). The forces responsible for their formation have long been enigmatic until numerous recent studies revealed that these structures are assembled via the process of phase separation through which a homogenous system demixes into a system that comprises more than one spatially separated, co-existing phases (Banani et al., 2017). When the macromolecule/macromolecule or solute/solute interactions are energetically preferred to the macromolecule/solute interactions and gain-in-free energy is preferred to loss-in-entropic tendency to maintain a homogeneous state, the phenomenon of phase separation takes place (Hyman

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et al., 2014; Lin et al., 2018). Using synthetic biology approaches, Rosen and colleagues reported that protein and RNA-containing bodies can be reconstituted *in vitro*, which supports nucleation of actin polymers (Li et al., 2012). MLOs are also termed as biomolecular condensates due to their material properties (Hernandez-Verdun, 2011). The composition of these MLOs or condensates is very definite with only specific constituents partitioning in the MLOs where the others filtered (Banani et al., 2016; Feric et al., 2016). Given the nature of MLOs, reaction machineries can be assembled and disassembled reversibly and quickly (Feng et al., 2019). Although well-known in polymer chemistry (Flory, 1942), the concept of phase separation in biology specifically as the operational principle mediating the MLO formation for the regulation of biological activities is a fairly recent development. For years, many questions regarding MLOs have remained unanswered that how they are formed, why they exist, and how their physical properties contribute to their biological functions. In this review, we aim to provide the latest progresses toward better understanding of phase separation of biomolecules in cellular processes, emerging mechanisms of action, and regulation of phase separation during cell fate decision.

Phase separation exists in various different forms

A plethora of complementary and comparative *in vivo* and *in vitro* studies have categorized intracellular phase-separated condensates such as liquid–liquid, liquid–gel, or liquid–crystalline according to the spatial ordering and surrounding physiochemical parameters (Alberti, 2017; Boeynaems et al., 2018). There are several criteria to classify MLOs, which include concentration of macromolecules, multivalency of their interactions, kinetics of chemical bonds underlying multivalency, and interfacial tension between the MLO and surroundings (Boeynaems et al., 2018).

The material properties of phase-separated condensates are specified when they undergo further phase transitions, and these distinct material properties contribute to their special functions and pathologies (Kwon et al., 2013; Molliex et al., 2015; Patel et al., 2015; Banani et al., 2017; Franzmann et al., 2018). There are many complex physical and molecular interactions behind these various transitions. Most often, MLOs are regarded as liquids, thus it is worth noting that even liquids are comprised of hard spheres and manifest definite structures whose pair-correlation functions are calculable. According to these functions, liquids display ordered arrangements resembling to crystalline solids, and these ordered arrangements have sizes comparable to a typical molecule (Boeynaems et al., 2018). After assembly, liquid droplet condensate can undergo various physical states. Liquid crystals, on the other hand, are structured fluids in which components have ordered but weak interactions. In gel-like condensates, constituents are bound to each other through powerful interactions, but they are still accessible to other proteins. Solid-like structures are held by very strong interactions and they are almost impermeable to exchange with the external environment (Wang and Zhang, 2019).

Liquid–liquid phase separation (LLPS)

Within living systems, the particularly relevant form of phase separation is LLPS, which is also termed as coacervation sometimes (Shin and Brangwynne, 2017). It is the principle process that governs the formation of MLOs. It is also a physicochemical phenomenon, wherein above a threshold concentration, a homogenous mixture spontaneously demixes into two distinct liquid phases where liquid-like droplets co-exist with their specific liquid surroundings (Shin and Brangwynne, 2017; Feng et al., 2019). The interface around these droplets serves as a boundary between the droplets and the surrounding environment, which permits the selective exchange of molecules thus allowing these droplets to behave as unique compartments (Alberti, 2017). These droplets or condensates are spherical in shape displaying high internal mobility and molecular exchanges (Wang and Zhang, 2019). In short, LLPS is a dynamic process mediated by multivalent weak interactions among folded domains (repeats of the same type of domain or different types of domains) and intrinsically disordered region (IDR)-containing proteins (IDPs) as shown in Figure 1A (Qamar et al., 2018). The challenge ahead is to characterize LLPS-driven activity in live cells and delineate its underlying mechanisms (Alberti et al., 2019).

Mechanistic insights into phase separation

Phase separation is a thermodynamic process in which a system achieves a lowest energy state by the contribution of entropy and enthalpy (Rubinstein and Colby, 2003). Since entropy keeps a system well-mixed, it counteracts the phase separation, and the system uses many other interactions between the constituents interceded by biopolymers especially proteins and nucleic acids to drive their phase separation. These interactions are either homotypic or heterotypic, and entropy-driven mixing of the system is counteracted by strengthening the homotypic interactions over the heterotypic interactions, which results in two-state system having lowest energy (Hyman et al., 2014; Brangwynne et al., 2015). The kinetic barrier that impedes phase separation is overcome by nucleation, a prototypical process that helps in bypassing the free-energy barrier to yield nuclei of new phase within the soluble old phase leading to first-order phase separation. A first-order phase separation delineates the discontinuous changes required for a system to go into a condensed phase from a dispersed phase (or *vice versa*). Nucleation is controlled by the interfacial tension that exists between monomers, dimers, and polymers. Due to fundamental significance of the nucleation in the overall kinetics of phase separation and transition of a wide range of biomolecules, a great deal of efforts have been made to delineate its molecular basis, while many aspects of nucleation remain poorly characterized (Levin et al., 2014). It would be of great interest to design an optic-based kinetic assay similar to the pyrene-actin assembly (Yao et al., 1996), so that the nucleation and LLPS-driven polymerization can be precisely studied in an *in vitro* assay. This would delineate how

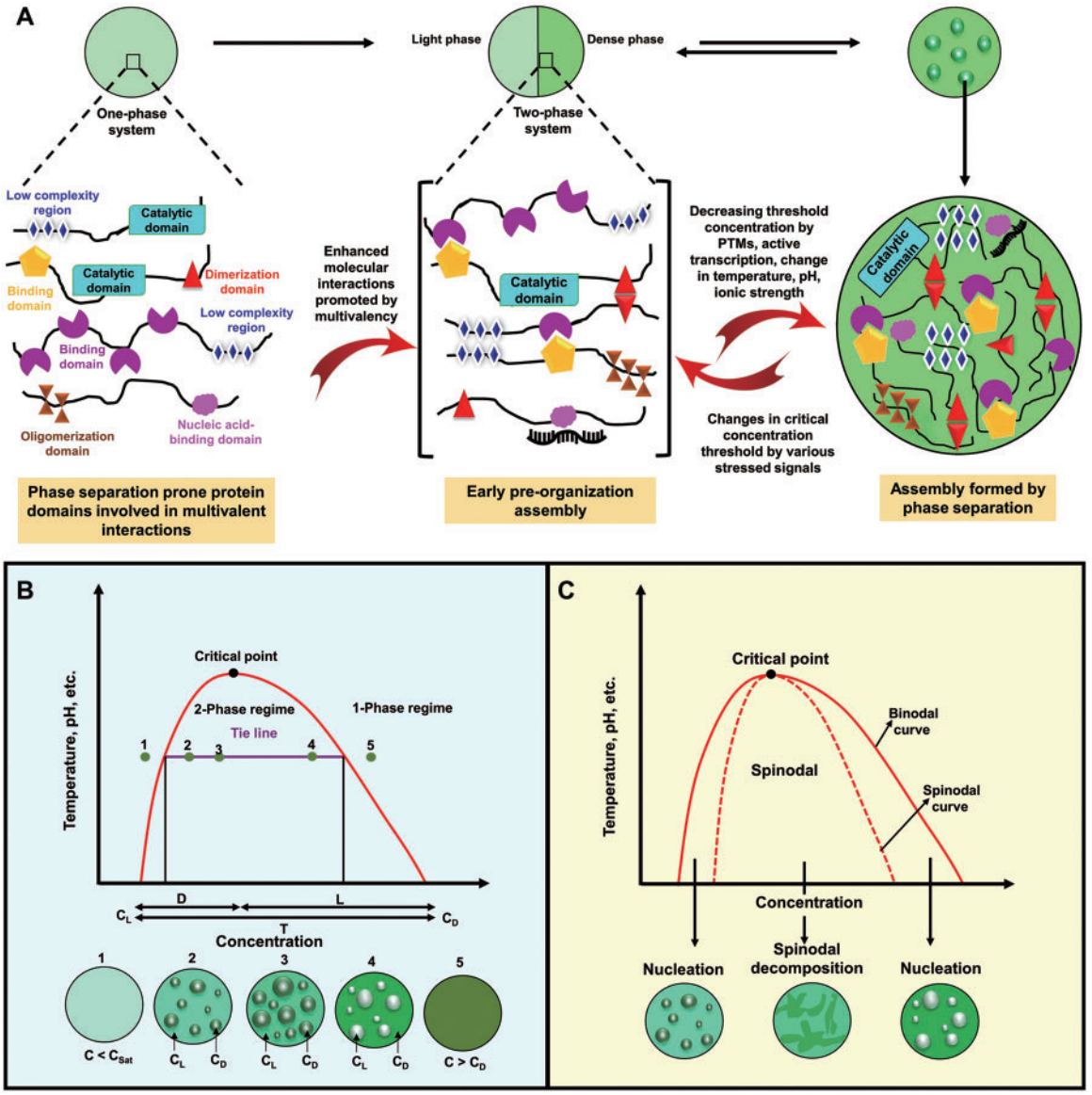

Figure 1 Schematic diagram and molecular basis for phase separation-driven assemblies. (**A**) Proteins that assemble to form MLOs contain different types of domains such as LCRs, oligomerization domains, folded binding domains. They create multivalency and thus offer a framework for establishing multivalent interactions, which bring molecules together and increase their local concentration. When the critical concentration threshold is reached, phase separation takes place. Many factors, such as appropriate PTMs and active transcription of component proteins, favor the nucleation reactions facilitating phase separation and stress signals whereas disfavor the multivalent interactions leading to complete or partial disassembly. (**B**) A phase diagram is constructed by varying protein concentrations and environmental/storage conditions. The solid red line is a function of environmental conditions such as pH, ionic strength, and temperature and differentiates between one-phase and two-phase regimes. A critical concentration threshold must be reached by the system to undergo phase separation. The system is in one mixed phase at $C < C_{sat}$. Within two-phase regime, under any given conditions, the system demixes to form a light phase with $C=C_L$ and a dense phase with $C=C_D$. Tie lines (purple) represent all the conditions that result in two-phase systems, with only fraction volume of two phases, f_L and f_D, fluctuating relative to each other as depicted in examples 2–4. During equilibrium, C_{sat} and C_L are equivalent; however, during nucleation of phase separation, they can vary. (**C**) Solid red line represents the binodal boundary at which molecules reach their solubility limit and demix from the surrounding solution. Dashed red line represents the spinodal curve where the system undergoes spinodal decomposition. In the area between binodal and spinodal curves, nucleation occurs and the system demixes (**B** and **C** were adapted from Alberti et al., 2019).

biological monomers polymerize and phase-transit into functionally distinct polymers, a key step essential for MLO formation.

For each molecular system, a phase diagram can be generated by screening through macromolecular concentration, temperature, pH, or salt concentration to define the set of conditions that will ultimately result in mixed phase and conditions that will lead to phase separation as shown in Figure 1B and C. From the phase diagrams of different proteins, it is obvious that different proteins undergo phase separation at their critical concentration according to the chemical property of individual protein. Nucleation is initiated by the protein having lowest critical concentration, and regulation of their concentration can serve as the rate-limiting step (Alberti, 2017). As shown in Figure 1C, phase boundary defined by the binodal line depicts the boundary at which two distinct phases can coexist stably in the solution. Outside of this binodal curve, molecules exist in the form of a homogenous solution. A metastable region is present between the binodal and spinodal curves where solution demixes through nucleation. In the spinodal zone, which represents a region of instability, spontaneous phase separation takes place when molecules adopt a stable phase bypassing the metastable zone (Feng et al., 2019). By constructing phase diagrams, we obtain important intuitions about the chemical properties and valency of molecules that can control phase separation and whether phase separation can take place in physiologically relevant contexts. However, attention must be paid to the fact that transitions captured by *in vitro* phase separation may not represent the true picture of what happens in cellular environment (Alberti et al., 2019).

Elements associated with phase separation

Multivalency. The key determining factor that underlies LLPS is multivalent interactions, which takes the essence from a classic concept of polymer science that multivalent molecules have higher natural tendency to form polymers or large oligomers (Flory, 1953; Banani et al., 2017). Through multivalency, molecules can establish various inter- and intra-molecular interactions and thus assemble into larger complexes, which possess lowered solubility due to entropy-driven consequences and consequently can easily demix from the solution (Flory, 1953). The concept of multivalency is applicable to various multivalent molecules such as proteins having modular domains and IDRs, DNA, and RNA (Han et al., 2012; King et al., 2012; Li et al., 2012).

(I) Contribution of multivalency in proteins with IDRs. IDPs represent a large class of proteins that have disordered regions referred to as IDRs, and multivalency plays an important role in their phase separation. These IDRs do not form stable secondary and tertiary structures and are conformationally dynamic and heterogeneous. Within IDRs, regions of low sequence complexity (LCRs) are present that have a compositional bias for a limited number of amino acids such as glycine (Gly), glutamine (Gln), serine (Ser), asparagine (Asn), tyrosine (Tyr), and phenylalanine (Phe). In some IDRs, charged residues such as lysine (Lys), arginine (Arg), glutamate (Glu), and aspartate (Asp) are also present. Due to low sequence complexity, these proteins contain blocks of negative or positive charges, poly-Asn and poly-Gln tracts as well as multiple Gly/Ser–Phe/Tyr–Gly/Ser sequences (Gilks et al., 2004; Decker et al., 2007; Reijns et al., 2008; Kato et al., 2012; King et al., 2012; Nott et al., 2015). Hence, they can participate in a variety of homotypic and heterotypic interactions, and these repetitive motifs confer special attributes to IDPs such as *in vivo* and *in vitro* phase-separating behaviors (Jiang et al., 2015; Molliex et al., 2015; Nott et al., 2015), targeting to mitotic spindle (Jiang et al., 2015), and RNA granules (Decker et al., 2007; Reijns et al., 2008; Kato et al., 2012). Aromatic residues have also been shown to significantly contribute to the phase separation of some proteins. In DEAD-box helicase 4 (DDX4), the IDR contains many Phe–Gly (FG) repeats whose aromatic ring establish many inter- and intra-molecular interactions with Arg residues through cation–π interactions (Nott et al., 2015; Figure 2A). The crystallographic studies of LCRs of heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), nuclear pore complex protein 98 (Nup98) and Fused in Sarcoma (FUS) have shown that they are enriched in aromatic residues which stabilize inter- and intra-sheet interactions. These LCRs make kinked β-sheets that allow van der Waals interactions and hydrogen bonding; therefore, they are also referred to as low-complexity aromatic-rich kinked segments (LARKS) (Hughes et al., 2018). IDRs enriched in Ser, Asn, and Gln derive phase separation by mediating dipolar interactions through their side chains (Crick et al., 2013). The blocks of positive and negative charge facilitate phase separation by promoting interaction either within the same protein or among different proteins (Altmeyer et al., 2015; Elbaum-Garfinkle et al., 2015; Nott et al., 2015; Pak et al., 2016). In such systems, the patterning of charged residues plays a significant role as uniformly distributed charge disfavors phase separation while the clustered charged residues promote phase separation (Brangwynne et al., 2015; Nott et al., 2015; Pak et al., 2016). Aromatic, polar or charge-charge interactions confirm the dynamic nature of phase separation because these interactions are short lived and contribute little to the structural stability.

In addition to amino acid side-chains, the secondary structure elements also contribute to the phase separation. In TAR DNA-binding protein 43 (TDP-43) C-terminal domain, an evolutionary conserved, short helical segment forms intermolecular helical interaction necessary for phase separation (Conicella et al., 2016; Figure 2B). LCR of FUS contains a 57-residue segment that participates in the founding of cross-β-sheets which are stabilized by π-stacking interactions and hydrogen bonding (Murray et al., 2017). Human Hdj1 is a class II Hsp40 protein that condenses in ubiquitin-rich nuclear bodies. The high ability of Hdj1 to undergo LLPS is due to its various domains including a dimerization domain (DD), a J domain and an intrinsically disordered G/F-rich region. Hdj1 uses its multiple domains especially its G/F-rich region to chaperone FUS phase separation. This co-phase separation prevents FUS to carry out disease-associated amyloid aggregation. The co-LLPS among Tyr-rich FUS-LC, Arg-rich FUS-RGG, and Arg of N-terminal domain

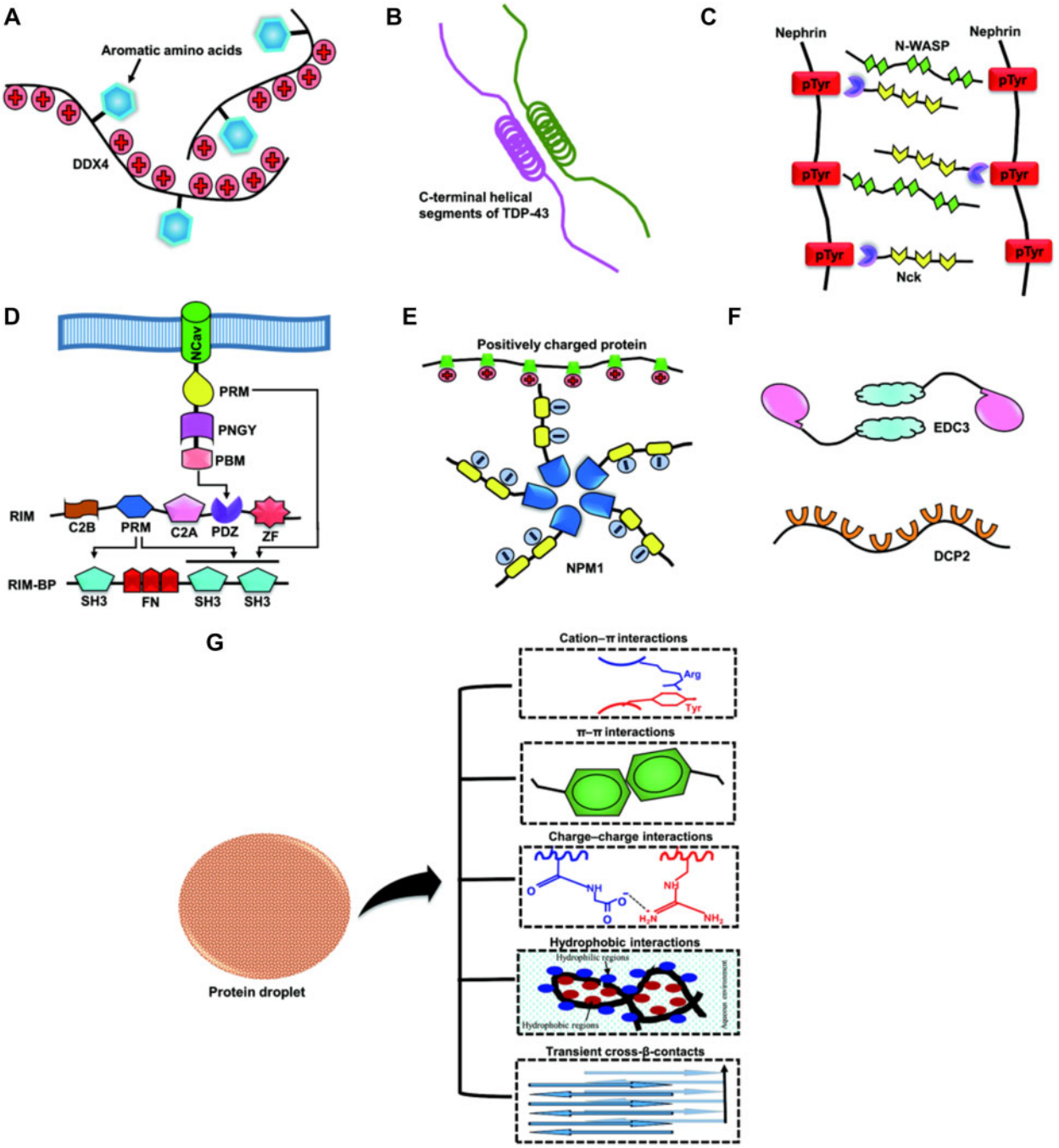

Figure 2 Different modes of multivalent interactions driven by modular domains or IDRs in the system undergoing LLPS and different forces contributing to protein phase separation. (**A**) Proteins containing IDRs such as DDX4 establish cation–π interactions between aromatic amino acids and positively charged basic residues. (**B**) In C-terminal domain of TDP-43, an evolutionary conserved, short helical segment forms intermolecular helical interaction necessary for phase separation. (**C**) Nephrin contains three pTyr motifs (red), which interact with the SH2 domain (purple) of Nck. Nck has three SH3 domains (yellow), which bind to the numerous PRMs (green) in N-WASP. (**D**) Schematic interaction network of presynaptic active-zone proteins RIM and RIM-BP together with the cytoplasmic tail of NCav. Interactions between PRMs of RIM and SH3 domains of RIM-BP drive system's co-clustering that lowers the threshold concentration required to carry out phase separation and also accounts for tight coupling of Ca^{2+} influx and neurotransmitter release in presynaptic zone. (**E**) NPM1 assembles into pentamers via its oligomerizing domain (blue) and binds to proteins that contain positively charged arginine-rich linear motifs (R-motifs) (green) through its negatively charged acidic tracts (yellow). (**F**) EDC3 dimerizes via its YJeF amino-terminal domain (sky blue) and binds to the helical leucine-rich motifs (pink) in DCP2 via its LSm domain (orange). (**G**) Proteins undergoing LLPS utilize various types of interactions including cation–π, π–π, charge–charge, hydrophobic, and transient cross-β-contacts.

of Hdj1 is attributed to cation–π interactions (Gu et al., 2020). Moreover, FUS, TATA-box binding protein associated factor 15 (TAF15), hnRNPA2, Ewing sarcoma (EWS) and cold-inducible RNA-binding protein can be assembled into hydrogels *in vitro* (Kato et al., 2012; Kwon et al., 2013; Elbaum-Garfinkle et al., 2015), nonetheless, some of these proteins have to undergo LLPS first. Analyses through electron microscopy, chemical foot-printing and X-ray diffraction reveal that these hydrogels have long filaments which are speculated to be mediated by interaction between β-strands. These studies indicate that such interactions drive LLPS and yield fibers and hydrogels after a period of a time (Xiang et al., 2015). In short, the composition of amino acids and overall sequence pattern of the IDPs govern the extent to which side chains and backbone interactions contribute to the phase separation. Therefore, computational tools have been developed to facilitate the analyses of regions of IDPs which might undergo LLPS.

The presence of low complexity prion-like domains (PrLDs) and RNA-recognition motifs (RRMs) are another common features of proteins that undergo phase separation. PrLDs is a subset of low complexity domains (LCDs) bearing compositional resemblance to yeast prions and are enriched in uncharged, polar amino acids such as Gln, Asn, Ser, Tyr, and Gly (Alberti et al., 2009; Cushman et al., 2010; March et al., 2016). The prion domains of some yeast proteins such as Rnq1, Ure2, and Sup35 contribute to the formation of infectious prion proteins that propagate by using self-templating amyloid fibrils which are composed of stable cross-β-sheets (Shorter and Lindquist, 2005). These amyloid fibrils are formed via phase separation into solid phase which is difficult to reverse (Chuang et al., 2018). The precise mechanism how these PrLDs contribute to phase separation is currently under active investigation (Alberti et al., 2009; Khan et al., 2018).

In human genome, there are about 240 genes that exhibit biochemical feature of PrLDs in which 72 encode RNA-binding proteins (RBPs) (March et al., 2016). Examples of such proteins include TDP-43, FUS, EWSR1, TAF15, T-cell-restricted intracellular antigen-1 (TIA-1), hnRNPA1, and hnRNPA2, which are present in RNA granules and linked with the pathogenesis of neurodegenerative diseases (King et al., 2012). Some studies have reported that PrLD deletion of key RBPs, e.g. FUS and TIA-1 abrogated the formation of RNP granules completely (Gilks et al., 2004; West et al., 2016). Recently, another type of IDR termed as RGG domains have been shown to interact with PrLDs in order to drive phase separation (Bogaert et al., 2018; Qamar et al., 2018; Yoshizawa et al., 2018). These RGG domains are found in RBPs with PrLDs (Banjade and Rosen, 2014), enriched in Gly and Arg residues (Alberti et al., 2009) and bind RNAs (Cushman et al., 2010; March et al., 2016).

(II) Contribution of multivalency in proteins with modular domains. Ex-periments based on the manipulation of the folded protein valency have established an inverse correlation between the number of binding motifs/domain and the saturation concentration above which a system experiences phase separation. LLPS is significantly influenced by the valency of the interactions and many protein/nucleic acid interaction systems undergo LLPS both *in vivo* and *in vitro* when a specific number of valency is achieved. The threshold concentration or phase boundary for LLPS is dropped by increasing the multivalent interactions (Du and Chen, 2018). Recent studies have reported many examples of natural proteins containing modular domains that undergo phase separation. The first example related to phase separation of modular domains which was described in detail was of the actin-regulatory signaling pathway. This pathway consists of multivalent proteins: neural Wiskott–Aldrich syndrome protein (N-WASP), nephrin and non-catalytic region of tyrosine kinase adaptor protein (Nck). These proteins generate high-order oligomers: Nephrin contains three phosphotyrosine (pTyr) motifs, which interact with the SRC homology 2 (SH2) domain of Nck; Nck has three SH3 domains, which bind to the numerous proline-rich motifs (PRMs) in N-WASP (Figure 2C).

Another such example is T-cell receptor signaling system comprising protein linker for activation of T cells (LAT), growth factor receptor-bound protein 2 (GRB2), GRB2-related adaptor protein 2 (GADS), son of sevenless 1 (SOS1), and SH2 domain-containing leukocyte protein of 76 kDa (SLP76; also known as LCP2) (Su et al., 2016).

In neurons, beneath the post synaptic membranes, an electrodense material, postsynaptic density (PSD) is present. A negative activity regulator of PSD, SynGAP interacts with the major PSD scaffold protein PSD-95 resulting in the droplet formation (Zeng et al., 2016). The high valency provided by the multivalent interactions of PSD constituents enhances the propensity of droplet formation. Furthermore, the presynaptic zone is structured by the co-clustering of cytoplasmic tail of voltage-gated Ca^{2+} channel (NCav), RIM, and RIM-binding protein (RIM-BP). This droplet formation is carried out by the interaction between PRMs of RIM and SH3 domains of RIM-BP (Figure 2D) and lowers the threshold concentration required to carry out LLPS and also accounts for tight coupling of Ca^{2+} influx and neurotransmitter release in presynaptic zone (Wu et al., 2019).

Numerous other examples of phase separation mediated through multivalent interactions of folded domains have been reported which include the nucleolar protein nucleophosmin (NPM1) (Figure 2E), and P-body components mRNA-decapping enzyme subunit 2 (DCP2) and enhancer of mRNA-decapping protein 3 (EDC3) (Figure 2F; Fromm et al., 2014; Mitrea et al., 2016; Zeng et al., 2016).

(III) RNA- and DNA-binding domains. RBPs are another significant class of proteins that exhibit phase separation behavior. RNP granules are consist of RNA and RBPs, and are assembled through LLPS (Uversky, 2017). The IDRs and RRMs in RBPs of RNA granules can establish multiple multivalent interactions in order to exhibit phase behavior (Bogaert et al., 2018; Qamar et al., 2018; Yoshizawa et al., 2018). Whi3, a fungal RBP that regulates cell polarity and nuclear division (Zhang et al., 2015), interacts with RNA to phase separate and encrypt the identity of RNA granule (Langdon et al., 2018). Mutations in RRM of Whi3 block its binding to RNA and also abrogate phase separation (Zhang

et al., 2015). Many RBPs have been shown to undergo LLPS alone under *in vitro* conditions (Elbaum-Garfinkle et al., 2015; Wang et al., 2018a) and IDRs of these proteins are adequate for droplet formation (Conicella et al., 2016; Ryan et al., 2018). Therefore, another property of proteins that can undergo LLPS is their ability to bind to RNA and DNA using zinc fingers, RRMs, or other nucleic acid binding domains (Boeynaems et al., 2018). Additionally, proteins that bind to single-stranded DNA (ssDNA) have also shown propensity to phase separate, for example, in *Escherichia coli*, ssDNA-binding proteins were reported to interact with ssDNA and undergo LLPS to form protein droplets using multifaceted interactions involving various structural domains of the proteins (Harami et al., 2020). Interestingly, many RBPs also have propensity to bind RNA as well as ssDNA making them multi-specific DNA- and RNA-binding proteins (DRBPs) instead of only RBPs (Jankowsky and Harris, 2015; Wang et al., 2015). It has been suggested that nearly 2% of the proteome comprising nearly 400 proteins could be DRBPs (Hudson and Ortlund, 2014). One example of DRBPs is DDX4, that interacts with ssDNA and forms droplets in a concentration dependent manner (Nott et al., 2015).

(IV) Oligomerization domains**.** The presence of oligomerization domains in proteins would enhance their valency and generate a local high concentration of the proteins which promotes their phase separation. Nuclear RBP TDP-43 is such an example which contains oligomer forming N-terminal domain (Afroz et al., 2017; Wang et al., 2018a). The polymerization of N-terminal domain assists the LLPS *in vitro* and mutations in this domain lower the ability of TDP-43 to phase separate (Wang et al., 2018a). Using an optogenetically controlled droplet formation assay, it was shown that oligomerization of Cry2 protein fused with various RBPs was elicited using blue light resulting in the droplet formation of the fusion proteins *in vivo* (Shin et al., 2017). This experiment suggests that context-dependent regulation of oligomerization domains stimulates phase separation.

Forces governing phase separation

Formation of biological condensates requires concentrating numerous molecules into a confined space which can be energetically costly for cells, therefore, nucleic acids and proteins make use of their complex chemistries and develop various weak interactions to counteract the entropic cost for phase separation. These interactions assist in inclusion and exclusion of molecules into phase-separated assemblies thus affect their dynamics and transport properties. Various interaction types have been confirmed to govern phase separation of proteins, and molecular interactions found to be important in phase separation include π–π interactions, cation–π interactions, charge–charge interactions, hydrophobic interactions, and transient cross-β-contacts (Figure 2G). Although these interactions are rather weak, however, their cumulative effect on driving phase separation is likely to be significant. Specific contributions of these interactions determine the selective accumulation of specific proteins within specific MLO. Within MLOs, some amino acids utilize multiple different interactions which may function cooperatively to enhance the phase separating ability of molecules (Murthy et al., 2019; Dignon et al., 2020). Studying the interactions among different components of MLOs is important because they can provide significant information regarding which interactions are most important for a specific assembly, how perturbations in these interactions can affect the assembly and how naturally occurring mutations may have impact on these interactions leading to pathological conditions. However, determining the contribution and relative significance of each interaction mode to phase separation is challenging due to the dynamic nature of MLOs. Different interaction modes which are being used in a phase-separated assembly can be observed using atomic-resolution simulations. However, this approach is still limited and only one study using this approach has been reported (Rauscher and Pomès, 2017).

π–π interactions. In protein structures, π–π interactions play significant roles such as catalysis, RNA binding and structural motifs. The presence of long range π–π interactions identified in a vast number of known phase separating proteins underscores the importance of π–π interactions in LLPS (Vernon et al., 2018). The side chains of amino acids such as tryptophan (Trp), Tyr, Phe, Gln, Arg, Asn, Glu, and Asp carry π-electrons that contribute to the π–π stacking interactions that are critical for phase separation of proteins (Vernon et al., 2018). The π–π interactions between the Phe residues in the FG repeats of nucleoporins are responsible for the gel-like phase of nuclear-pore complex (Schmidt and Görlich, 2015).

The presence of a backbone peptide bond with a partial π-bond in all amino acids suggests that each amino acid may contribute to the planar sp2 interactions throughout the full protein sequence thus facilitating phase separation. It has also been suggested that most phase-separating proteins can establish non-local planar π interactions, which can provide additional assistance in the presence of other dominant forces driving phase separation (Vernon et al., 2018).

Cation–π interactions. Cation–π interactions are another type of interactions that have gained prominence in LLPS of many proteins (Bogaert et al., 2018; Yoshizawa et al., 2018). These interactions take place between positively charged amino acids Arg and Lys, and electron-rich aromatic groups. For example, in DDX4, cation–π interactions between RG and FG regions of the protein drive phase separation *in vivo* and *in vitro* (Nott et al., 2015). Another example is of hnRNPA1 condensate formation, in which cation–π interactions between Arg and Tyr of RNA-binding domain mediate phase separation (Wang et al., 2018b).

The material and transport properties of the condensates are also influenced by cation–π interactions. While being important for proteins, cation–π interactions are also prominent in nucleic acids specially unfolded, ssDNA with exposed aromatic nucleotide bases, therefore, it is often observed that IDP-rich droplets incorporate ssDNA but not dsDNA (Nott et al., 2015; Shakya and King, 2018).

Charge–charge interactions. Next interactions that are important for phase separation are charge–charge interactions. LLPS of IDPs involves interactions between charged amino acid side chains and termini, because IDPs are commonly enriched in charged amino acids (Uversky, 2017). When polymers enriched in one type of charge are brought together, they repel each other thus inhibiting phase separation. However, it has been shown for cationic peptides and RNA that when oppositely charged polymers are brought together, they can neutralize the opposite charge resulting in droplet formation (Boeynaems et al., 2017). The oppositely charged components tend to lower the net charge enhancing the feasibility for condensate formation. The involvement of charge–charge interactions in driving phase separation of proteins *in vivo* and *in vitro* has been reported by many studies (Nott et al., 2015; Pak et al., 2016). For example, the negatively charged nephrin intracellular domain associates with its positively charged partners to yield condensates (Pak et al., 2016). Attractive interactions between oppositely charged stretches of proteins have been proven to be the key driving force for their phase separation. Tau protein is involved in many neurodegenerative diseases including Alzheimer's disease and has strong propensity to exhibit LLPS mediated predominantly by electrostatic interaction between positively charged C-terminal/middle regions and negatively charged N-terminal. In addition, hydrophobic interactions also play a role in its phase separation (Boyko et al., 2019). *Caenorhabditis elegans* protein LAF-1 which is present in P-granules, phase separates via electrostatic interaction between N-terminal RGG-rich domain and Asp/Glu (Elbaum-Garfinkle et al., 2015).

The charge state of amino acids can be altered by many factors such as pH, various PTMs and environment polarity, thus, these factors may also influence the phase separation behavior of target proteins (Isom et al., 2011; Monahan et al., 2017; Saito et al., 2019).

Hydrophobic interactions. The lower fraction of hydrophobic residues in phase separating proteins makes their side-chains remain disordered and their assembly to be liquid-like rather than solid. Moreover, hydrophobic amino acids possibly interact with aromatic amino acids, which are quite prevalent in phase-separating proteins. Hydrophobic interactions also play a major role during protein folding, promoting the formation and stability of various folded domains and oligomers that may additionally facilitate phase separation (Wang et al., 2018a). Hydrophobic interactions are also frequently involved in specific binding of ligands to incorporate them preferentially into the condensed phase (Bah et al., 2015). Hydrophobic interactions are involved in phase separation of many proteins. Speckle-type poxvirus and zinc-finger domain protein (SPOP) participates in ligase substrate recruitment for ubiquitination and subsequent degradation in proteasome. Phase separation of SPOP is essential for condensate formation and is mediated through hydrophobic and polar interactions between multiple meprin and traf homology (MATH) domains in oligomeric SPOP and multiple SPOP binding motifs in its substrate death-domain-associated protein (DAXX) (Bouchard et al., 2018). In *C. elegans*, SPD-5 drives condensate formation through coiled-coil electrostatic, hydrophobic and hydrogen bonding interactions (Woodruff et al., 2015). Furthermore, NMR studies of an *in vitro* constituted MLO also point out the important role of hydrophobic interactions in the formation and stabilization of the condensate (Fromm et al., 2014).

Transient cross-β-contacts. Hydrogen bond donor and acceptor groups are present in most amino acids which suggest the possibility that hydrogen bonding could be frequent in protein condensates. In LCR of FUS, many Gln residues are present and hydrogen bonding is highly prevalent and contributes to phase separation of FUS (Murthy et al., 2019). Many PrLDs that undergo LLPS form fibrils in which amino acids establish transient cross-β-contacts assembling them into cross-β-sheets which bear resemblance to those found in amyloid fibrils. The fibrils of the PrLDs are different from classical amyloid fibrils and contain kinked cross-β-sheets, which are characteristic LARKS. Proteins that have PrLDs enriched in LARKS are found in MLOs assembled through LLPS (Kato and McKnight, 2017; Murray et al., 2017; Hughes et al., 2018). Cross-β-contacts between the PDZ motif of PSD-95 and PDZ binding motif of SynGAP mediates condensate formation in cells and *in vitro* (Zeng et al., 2016).

Regulation of phase separation

Phase separation is regulated by several mechanisms. Changes in the sequence/length, abundance, binding of another molecule or PTM modifications can alter the structure, valency, or binding affinity of the molecules, subsequently, modifying their ability to phase separate.

We next discuss various factors that influence the structure and function of components undergoing phase separation.

Environmental and chemical factors affecting phase separation

Numerous environmental and chemical factors such as chemotoxicity and DNA damage (Louvet et al., 2006; 2014), changes in ionic strength (Elbaum-Garfinkle et al., 2015; Nott et al., 2015), and changes in temperature (Eskiw et al., 2003; Nott et al., 2015) can disrupt phase separation in living cells and under *in vitro* condition.

Based on atomic force microscopic studies, nucleoli exhibited a decrease in their stiffness upon inhibition of RNA polymerase and an increase in stiffness upon inhibition of proteasome (Louvet et al., 2014). Furthermore, the viscoelastic properties and morphology of messenger ribonucleoprotein (mRNP) granules affected due to mutations in their component proteins, for example, mutations in hnRNPA1 and FUS are linked with neurodegenerative disorders (Buchan, 2014; Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015). Environmental and chemical factors can change liquid-like phase into a solid-like state which transform the usual almost spherical droplets into elongated fibril-like structure through a

process referred to as droplet aging (Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015).

Control of concentration of cellular components

Local concentration of the components is an important regulating point during the formation of MLOs. Fundamentally, changes in the expression of proteins, altered localization and degradation change their local concentration which ultimately impact the formation and volume of the condensed phase. It has been demonstrated by experiments that concentrating the basic components at a specific site in the cells, the formation of histone locus bodies, PML bodies, Cajal bodies, nucleoli (natural), and nuclear speckles (artificial) can be induced (Shevtsov and Dundr, 2011; Berry et al., 2015) and by changing the expression level, their sizes are altered. With engineered DDX4 and intracellular domain of the notch protein, it has also been confirmed that their local concentration affects the formation and volume of condensates (Nott et al., 2015; Pak et al., 2016). In *in vitro* model of P-granules involving LAF-1 droplets, by altering the RNA levels of LAF-1 governs the molecular dynamics and viscosity of the liquid-like phase (Elbaum-Garfinkle et al., 2015). Transcriptional control of the RNA levels by RNA polymerase serves as a regulatory mechanism for many MLOs such as paraspeckles and nucleoli in which concentration levels of RNA are essential for their assembly (Fox and Lamond, 2010; Hernandez-Verdun, 2011). Many MLOs act as sensors of stress signals, therefore, the sensitivity of their integrity to RNA and protein concentrations lead to a rapid response to the cellular stresses. Treatment with Actinomycin D to induce the inhibition of RNA Pol I-, II-, and III influences the organization of MLOs in nucleus and cytoplasm (Andersen et al., 2005).

Energy-dependent control of phase separation

Cells need to tune the liquid-like properties and dynamics of the MLOs, therefore, they probably use energy-dependent mechanisms to administer the degree of cross-linking and other processes within MLOs. When condensates are dynamic, their formation is limited; however, it is promoted when they are static. Such dynamic regulation can be the reason for the presence of ATP-dependent disaggregases, molecular motors and chaperones in many RNA granules (Kroschwald et al., 2015; Jain and Vale, 2017). For example, the disassembly of the stress granules upon recovery requires the activity of ATP-dependent chaperones Hsp40/Hsp70, which pile up within stress granules (Walters et al., 2015), depletion of ATP has been linked with enhanced viscosity of nucleoli and stress granules (Jain and Vale, 2017) and numerous ATPases control the persistence of stress granules (Buchan and Parker, 2009; Wu et al., 2019). It can be anticipated that liquid-like physical properties of many MLOs might be controlled through ATP-dependent enzymes such as DEAD-box helicases and kinases that are integrated into them (Louvet et al., 2006; Fromm et al., 2014; Elbaum-Garfinkle et al., 2015; Nott et al., 2015). In addition to the maturation of MLOs, energy-dependent processes can influence many other features of MLOs, for example in *C. elegans* embryos, the spatial distribution and nucleation of the nucleoli is impacted by the transcription of rRNA (Oakes et al., 2007; Berry et al., 2015). The actin cytoskeleton influences the localization of phase-separated LAT clusters at the interface of T-cell antigen presenting cell (Kaizuka et al., 2007; Yi et al., 2012).

Regulation by compositional control of components

The composition of MLOs is complex and their organization is dynamic, i.e. some components are permanently incorporated while others are transiently recruited upon special need (Buchan and Parker, 2009; Groušl et al., 2009). Examples of the components that are necessary for formation of particular MLOs include TIA-1 for stress granules (Kedersha et al., 1999), mRNAs for P-bodies, the NEAT1 non-coding RNA for paraspeckles (Decker and Parker, 2012), and spindle-defective protein 5 (SPD-5) for centrosomes in *C. elegans* (Hamill et al., 2002).

The compositional control of MLOs has been elucidated using simple model systems made up of multivalent scaffolds and their cognate low valency clients as depicted in Figure 3A. Under *in vivo* and *in vitro* conditions, phase-separated droplets generated by polySUMO-polySIM scaffolds employed low valency clients differentially (e.g. GFP-SIM or GFP-SUMO) depending on the relative stoichiometries of the scaffold components. Alteration of SUMO:SIM ratio in the scaffolds changed the composition evidently (Banani et al., 2016).

PTMs as regulators of phase separation

Electrostatic properties of the macromolecules are very crucial for MLO formation, the PTMs altering the charge features of the proteins are a means to control the multivalent interactions of the proteins hence regulating their phase separation behavior (Li et al., 2012; Bhowmick et al., 2015). The frequent occurrence of amino acids such as Ser, Tyr, and Arg also point out the significance of PTMs in regulating the features of MLOs. Since PTMs are very crucial for maintaining the proper structure and function of proteins present in biomolecular condensates, therefore, it is obvious that their aberrant phase separation is involved in many diseases.

Various PTMs have been reported for proteins undergoing phase separation which are discussed here and the regulatory effects of PTMs on phase separation of some proteins are also depicted in Figure 3B and C.

Phosphorylation. Phosphorylation plays a pivotal role in numerous signaling pathways and contributes to the dynamics and structural integrity of MLOs (Wang et al., 2014). Phosphorylation can cast both positive and negative effects on LLPS of proteins as evident from many *in vivo* examples discussed here. The charge of the amino acid side chains is

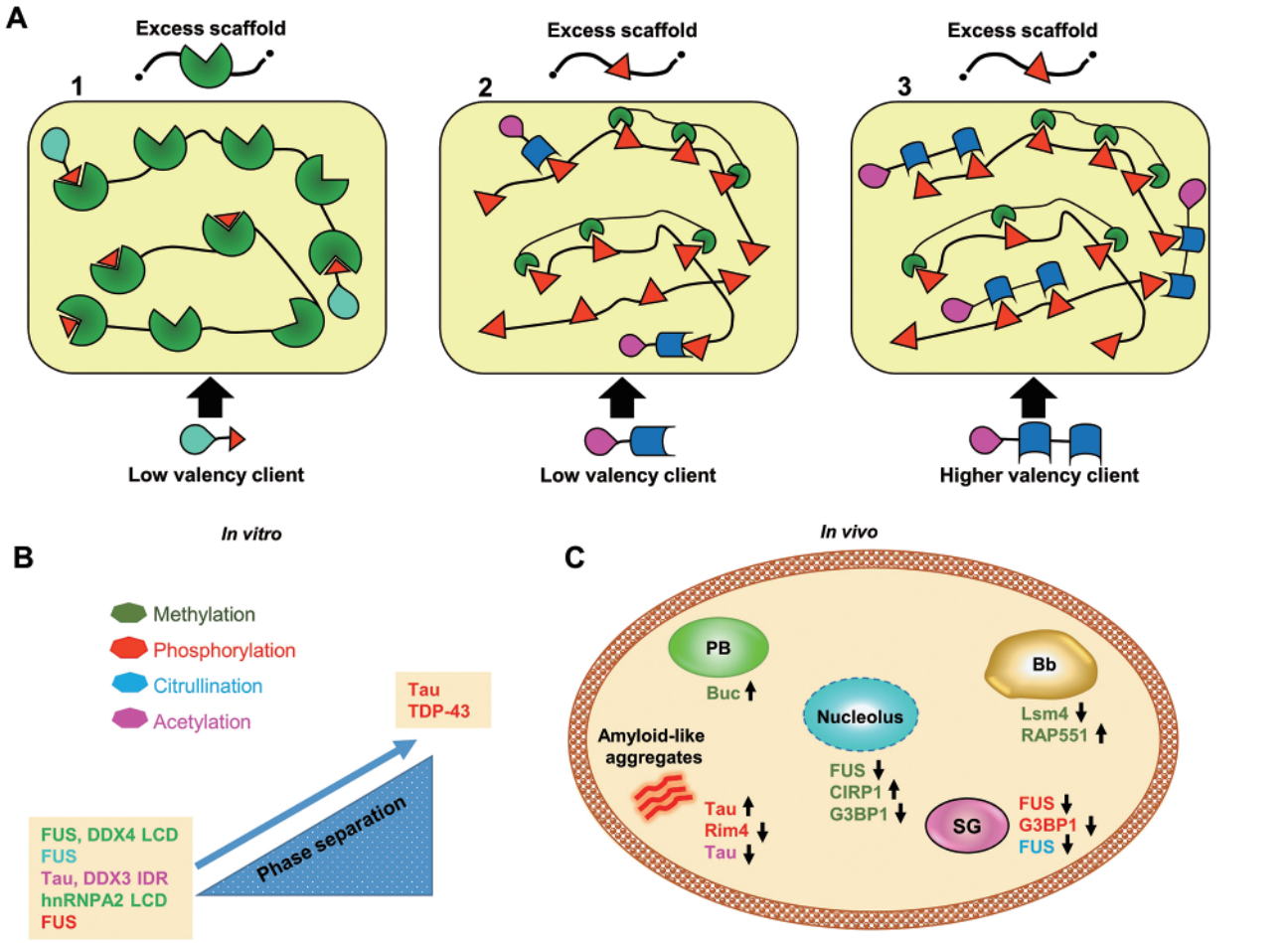

Figure 3 Compositional control model for PTMs on phase separation. (**A**) Multivalent molecules forming the scaffold of the condensates contain complementary modules that facilitate the assembly of the scaffold resulting in phase-separated condensates shown by yellow structures. Client molecules in this example possess interaction domains that are complementary to the scaffold components and are recruited to the condensates by binding to the complementary sites but at a lower valency. (1) Scaffold component having green modules is present in stoichiometric excess yielding free green scaffold sites. Clients having red modules can be recruited to the condensate by binding to the green scaffold sites that are unbound. (2) Scaffold component having red modules is present in stoichiometric excess yielding free red scaffold sites. Clients containing blue modules can be recruited to the condensate by binding to the red scaffold sites that are unbound. (3) Higher valency of the blue client promotes stronger recruitment of this client when the red scaffold module is in stoichiometric excess (Banani et al., 2016). (**B**) Color code represents the respective PTM. Under *in vitro* conditions, various LCDs, RBPs, or IDRs are modified by PTMs that either enhance or suppress their phase-separating behaviors. (**C**) The composition, assembly, or disassembly of various biological condensates such as Balbiani body (Bb), P-body (PB), stress granules (SG), and amyloid-like aggregates is regulated by PTMs *in vivo*. An arrow pointing up depicts promotion of phase separation upon the respective PTM, and an arrow pointing down depicts suppression of phase separation by the respective PTM.

significantly altered upon phosphorylation after receiving two negative charges (PO_4^{-2}), which modify their chemical and steric properties affecting inter- and intra-molecular electrostatic interactions (Bah and Forman-Kay, 2016). For example, phosphorylation of FUS at multiple Thr/Ser residues by DNA-dependent protein kinase (DNA-PK) (Monahan et al., 2017; Rhoads et al., 2018), impedes the phase separation of LCD of FUS (Monahan et al., 2017; Murray et al., 2017), lowers binding to FUS-LCD hydrogels (Han et al., 2012), and annuls phase separation of other interacting proteins which are bound to LCD of FUS (Lin et al., 2017). TDP-43 is linked to the pathogenesis of frontotemporal dementia, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease (AD) (Neumann et al., 2009; Josephs et al., 2014) and it has been shown that a phosphomimetic S48E substitution in TDP-43 N-terminal domain, a site that is constitutively phosphorylated *in vivo* (Hornbeck et al., 2015; Wang et al., 2018a), lowers the LLPS of TDP-43, reduces the formation of nuclear assemblies and diminishes the splicing regulatory activity of mutant TDP-43 as compared to the wild-type TDP-43 (Wang et al., 2018a). Phosphorylation disrupts the phase separation of Rim4 as the dissolution of Rim4 assemblies is proceeded by Ime2 kinase (Carpenter et al., 2018).

Contrary to the negative regulation by phosphorylation, in nephrin/NCK/N-WASP ternary system, Tyr phosphorylation induces phase separation of the system (Li et al., 2012). Active phosphorylation/dephosphorylation cycles maintain the structural integrity of the MLOs. In nucleolus, NPM1 exchange dynamic between nucleolar and nucleoplasmic compartments and structural connectivity between the DFC and the GC regions is governed by the kinase CK2 (Louvet et al., 2006). In *C. elegans*, the assembly and disassembly of P granules during mitosis is controlled by phosphorylation of MEG-3 and MEG-4 proteins by $PP2A^{PPTR-1/2}$ phosphatase and MBK-2/DYRK kinase (Wang et al., 2014). The non-receptor protein tyrosine phosphatase (PTP) SHP2, is essential for RAS-mitogen-activated protein kinase (MAPK) signaling. Loss of function and gain of function (GOF) mutants of SHP2 are thought to acquire capability of undergoing LLPS which promotes its PTP enzymatic activity and activates the RAS-MAPK signaling pathway contributing to many diseases. Thus, LLPS uses a GOF mechanism in the pathogenesis of SHP2-linked human diseases, offering the possibility to therapeutically target LLPS for the treatment of these diseases (Zhu et al., 2020a). In sum, phosphorylation is an important regulatory mechanism governing the assembly and disassembly of MLOs.

Methylation. Methylation on Arg residues results in increased bulkiness, alteration in charge distribution, hydrogen bonding properties and hydrophobicity of the guanidinium head group that influences the intermolecular interactions ultimately tuning the phase separation behavior (Evich et al., 2016).

In hnRNPs and other nuclear RBPs, repetitive RG- or RGG-rich motifs are prevalent (Bedford and Clarke, 2009) and the Arg of these motifs are frequently methylated by the members of arginine methyltransferase (PRMT) family (Tang et al., 2000) and these methylated Arg residues are a key driving force for the phase separation of RBPs (Wang et al., 2018b). For three proteins DDX4, hnRNPA2, and FUS, the negative regulation by Arg-methylation has been confirmed. In these proteins, Arg-methylation reduces the Arg–aromatic (π) interactions which are essential for their LLPS (Nott et al., 2015; Ryan et al., 2018). Loss of Arg-methylation can lead to pathological circumstances, for example in case of FUS, the protein is kept in liquid-like state upon specific Arg-methylation (Hofweber et al., 2018; Qamar et al., 2018), however, loss of this methylation promotes liquid-to-solid-state transition which yields FUS aggregates. Since, it has been reported that the pKa of Arg side chains is nearly unchanged after any type of methylation (Evich et al., 2016), therefore, it can be anticipated that methylation affects protein self-association not only through electrostatic changes but possibly also through changes in hydrophobicity or hydrogen-bonding character.

Eukaryotic chromosomes are compartmentalized for spatiotemporally regulated function by histone modifications. Constitutive heterochromatin is hallmarked by histone methylation such as H3K9me2 and H3K9me3 that are read by heterochromatin protein 1 (HP1). Recent studies show that H3K9me2- and H3K9me3-marked nucleosomal arrays and associated complexes undergo phase separation to form macromolecule-enriched liquid droplets (Strom et al., 2017; Wang et al., 2019b). Chromatin compaction by the *Schizosaccharomyces pombe* HP1 protein Swi6 results in phase-separated liquid condensates. Swi6 substantially increases the accessibility and dynamics of buried histone residues within a nucleosome. Thus, HP1 uses its oligomerization to compact chromatin into phase-separated condensates (Sanulli et al., 2019). Future cross-disciplinary studies will shed light on additional histone methylation in chromatin plasticity control.

Ribonucleoprotein granules are MLOs consisting of RBPs and RNA. RNA granules form through LLPS as multivalent interactions among RBPs and/or RNAs create a dense network of interacting macromolecules and drive the phase separation. Recent work leverages a stickers-and-spacers framework adapted from the field of associative polymers for better understanding of the multivalent protein–RNA interactions driven phase transitions (Choi et al., 2020). Diverse RNAs and RBPs form membraneless granules in cells under stress conditions (Ries et al., 2019). N^6-methyladenosine (m^6A) is the most prevalent modified nucleotide in mRNA (Ries et al., 2019). Recent studies show that m^6A-modified mRNAs are enriched in stress granules, and that m^6A-binding YTHDF proteins are critical for stress granule formation (Fu and Zhuang, 2020; Zaccara and Jaffrey, 2020). Super-resolution imaging suggests that YTHDF proteins are in a super-saturated state and potentially promote stress granule formation by reducing the activation energy barrier and critical size for stress granule condensate formation (Fu and Zhuang, 2020). In sum, RNA methylation promotes YTHDF protein-mediated LLPS *in vitro* and in cells.

Citrullination. Arg residues in RGG/RG motifs can undergo other types of PTMs as well such as citrullination. Members of peptidyl arginine deiminase (PAD) protein family recognize RGG/RG-rich motifs as their consensus sequences and catalyze the conversion of positively charged guanidine group of Arg to a urea group which is neutral, therefore, citrullinated Arg exhibits altered chemical behavior as compare to natural Arg. Recently, it was reported that overexpression of PAD4 competes with Arg-methylation, inhibits it and alleviates the solubility of RBPs such as FUS, hnRNPA1, TAF15, and EWS in cells (Tanikawa et al., 2018). In case of FUS, PAD-mediated citrullination abolishes the LLPS *in vitro* (Qamar et al., 2018), probably by reducing the multivalent Arg–Tyr interactions necessary for LLPS of FUS (Qamar et al., 2018; Wang et al., 2018b). However, it is still ambiguous that what conditions promote PAD4-mediated citrullination and what determines the relative ratios of PAD-mediated citrullination and PRMT-mediated Arg-methylation of RGG/RG motifs. Therefore, future studies are crucial to dissect the interplay between citrullination and Arg-methylation to unravel their roles in phase separation.

SUMOylation. SUMOylation is covalently conjugated a small ubiquitin-like modifier (SUMO) to a Lys residue in proteins. The cytoplasmic polyadenylation element-binding protein 3 (CPEB3) is involved in translational regulation of translation of various mRNAs that are important for long-term synaptic plasticity in the hippocampus. SUMOylation of CPEB3 is essential to drive its phase separation which helps it binding to the

target mRNA. SUMOylation of CPEB3 is therefore, required for its localization to P-bodies *in vivo* and condensate formation *in vitro* (Ford et al., 2019).

Acetylation. Addition of acetyl group to positively charged amino acids can neutralize their charge, e.g. Lys acetylation not only neutralizes its positive charge but also enhances its hydrophobicity (Patel et al., 2011).

In the case of Dead-box RNA helicase 3 (DDX3X), acetylation abrogates its coacervation *in vitro* (Saito et al., 2019). Another example is of Tau protein, whose acetylation by enzymatically-active p300 histone acetyltransferase also disrupts phase separation (Ferreon et al., 2018).

Poly(ADP-ribosyl)ation/PARylation. Poly(ADP-ribosyl)ation or PARylation is a PTM that refers to reversible covalent addition of multiple NAD-derived ADP-ribose (ADPr) molecules to a protein (Alemasova and Lavrik, 2019). PAR polymerases (PARPs) can add ADPr units to Asp, Glu, Lys, Ser, or Arg residues and can be removed by PAR glycohydrolases (PARGs) (Alemasova and Lavrik, 2019). PAR molecules are synthesized by the addition of ADPr units which are now known to alter the phase separation of some IDPs (Altmeyer et al., 2015). In stress granules, PARylation of hnRNPA1 at Lys 298 is necessary for its nucleocytoplasmic shuttling process which is involved in the localization of stress granules and *in vitro* experiments have demonstrated that PARylation enhances phase separation of hnRNPA1 (Duan et al., 2019). TDP-43 also contains a PAR binding motif and co-phase separates with hnRNPA1 in stress granules and *in vitro* (McGurk et al., 2018; Duan et al., 2019).

MARylation. Mono ADP-ribosylation (MARylation), is a PTM that refers to the addition of one ADP-ribose units to target protein. MARylation was shown to be the governing force for a recently identified stress assembly in *Drosophila* cells called Sec body (Aguilera-Gomez et al., 2016).

Ubiquitination. Ubiquitin and ubiquitin-like PTMs on histone proteins can function as signaling molecules by mediating protein-protein interactions. A number of ubiquitin-related molecules have been found to be involved in the regulation of MLOs which arise by LLPS of specific biomolecules. However, it remains unclear whether the proteasome also participates in such regulation. Recent studies show that proteasome-containing nuclear foci form under acute hyperosmotic stress, which are dynamic structures that contain ubiquitylated proteins such as p97 and RAD23B (Cohen-Kaplan et al., 2020; Yasuda et al., 2020). These studies suggest that ubiquitin-chain-dependent phase separation induces the formation of a nuclear proteolytic compartment that promotes proteasomal degradation.

Phase separation: a new road of novel applications in various fields

Cellular regulatory proteins undergo phase separation to construct MLOs, which are critical for cellular regulation and some of these functions are provided in Table 1. Keeping these functions in mind, it is not astonishing that phase separation of proteins can be manipulated to probe applications in *in vitro* settings. Minimalistic models that can recapitulate the functional and structural features of MLOs are represented in the form of all-aqueous emulsions/microfluidics through manipulation of LLPS that offer state-of-art designs for fabricating exquisite biological models whose applications can be harnessed in the field of medicine, tissue engineering, development of artificial liquid organelles, bioreactors formation, biochemical analysis, biomolecules sorting and extraction, and many more (Onghena et al., 2015; Xue et al., 2017). In this section, we discuss some recently reported applications of phase separation in various fields.

Development of circuits and signaling pathways

Development of artificial genetic circuits that can result in formation of networks comprising interacting regulatory molecules for the manipulation of information flow within the cells is one of the focus of synthetic biology. Such genetic circuits can allow user-defined molecular interactions to execute specific biological function. Multivalent protein assemblies can be used to integrate condensates at specific genomic loci to control eukaryotic transcription initiation that enables robust gene regulation (Hnisz et al., 2017; Cho et al., 2018) Recently, a synthetically engineered assembly of multivalent transcriptional factors was reported in yeast that facilitated genetic circuit construction harboring complex signal processing ability (Bashor et al., 2019). Future innovations based on this theme can be expected that can incorporate IDPs in the scaffold designs to enhance the efficiency and specificity and can exploit PTMs to control the assembly and reversal of such synthetic circuits and signaling networks.

Constructing synthetic MLOs

All-aqueous emulsions which are formed due to aqueous-aqueous phase separation bear high similarity to MLOs. Therefore, such emulsions can be regarded as the minimalistic physical model of MLOs. Such synthetic MLOs can be used as signal transduction hubs or factories for synthesizing chemical compounds (Giessen and Silver, 2017). Liposome-coated ATPS droplets are used to generate nucleoid-like artificial MLOs for studying *in vitro* DNA transcription, which contain liposome-coated ATPS droplet-enclosed spermidine and polyuridylic acid, together with components for *in vitro* DNA transcription (Ma et al., 2020). Another important example is encapsulin family of bacterial proteins which can assemble to generate hollow, large nano-compartments which can be loaded with desired cargo proteins (Giessen, 2016).

By employing emerging methods such as optoDroplets, the formation of synthetic MLOs can be controlled spatiotemporally. The properties of OptoDroplets can be varied by using different fusion proteins and light stimulation (Shin et al., 2017).

Table 1 Phase separation plays important roles in a variety of critical cellular processes.

Function	Description	References
Cellular sensing	Various biological condensates can sense the variations in temperature, pH, or other stress signals.	Franzmann et al. (2018); Alberti et al. (2019)
Buffering cellular protein concentrations	Excess protein is stored in MLOs and will enter the dilute phase as required when needed.	Eldar and Elowitz (2010); Alberti et al. (2019)
Immunity	Many signaling components involved in innate immune pathways are capable of oligomerization to form higher-order assemblies.	Wu and Fuxreiter (2016)
Cellular signaling	Condensate formation serves as a conserved signal transduction mechanism in innate immunity and inflammation.	Cai et al. (2014); Dick et al. (2016)
Sequestration	Molecular condensation functions to sequester factors not required for cellular needs and thereby prevents off-target effects.	Protter et al. (2018); Alberti et al. (2019)
Mediating localization of proteins to pre-existing MLOs	Phase separation can mediate targeting of molecules to pre-existing organelles, as has recently been proposed for ubiquilin 2 and SPOP.	Bouchard et al. (2018); Dao et al. (2018)
Force generation	The energy of multivalent molecular interactions that drive phase separation is utilized to alter the macroscopic structural features of other biomolecular assemblies.	Bergeron-Sandoval and Michnick (2018); Forman-Kay et al. (2018)
Formation of physicochemical and mechanical filters	The number and dynamics of the cross-links between the macromolecules that make up the condensate determine the size of pores that serve as filters that allow exchange of specific molecules, e.g. nuclear pores.	Schmidt and Görlich (2016)
Reaction crucible	Phase separation concentrates a specific set of molecules into the condensed state that facilitates efficient cellular reactions between weakly interacting molecules.	Li et al. (2012); Strulson et al. (2012); Banjade and Rosen (2014)
Regulating the specificity of biochemical reactions	Phase-separated compartments could concentrate a protein with a subset of its potential interacting partners while excluding others, imparting specificity to biochemical processes.	Su et al. (2016)
Compartmentalization without physical barriers	Phase separation allows the organization of biomacromolecules spontaneously to form different subcellular compartments without the help of lipid membranes.	Feng et al. (2019)
Direct communications between MLOs and membrane organelles	MLOs can communicate with membrane-bound organelles via direct interactions.	Ma and Mayr (2018); Feng et al. (2019)
Organizational hub	LLPS and the resulting condensates appear to be exploited by cells to organize their internal space.	Jiang et al. (2015); Shin and Brangwynne (2017)
Skin barrier formation	Epidermal structure and functions are driven by phase-separation dynamics.	Quiroz et al. (2020)
Reduction of noise in cells	Compartmentalization of proteins through phase separation has been suggested as a potential mechanism to reduce noise in the cell.	Klosin et al. (2020)
Gene regulation	Many components involved in gene regulation form dynamic protein assemblies that contribute to their regulatory mechanisms.	Hnisz et al. (2017); Strom et al. (2017)
Cell fate decision	MLOs such as nucleoli, centrosomes, heterochromatin, and centromeres confer cellular plasticity and contribute to cell fate decision.	Liu et al. (2020a)
Evolution	Compartmentalization mediated by phase separation reveals how proteins and nucleic acids assemble into condensed bioreactors in the ocean before the emergence of lipid membranes.	Wang et al. (2019a)
Synapse formation and signal transduction	Formation and activity-dependent modulation of PSDs is considered as one of the most basic molecular events governing synaptic plasticity in the nervous system. Phase separation has been reported to play significant roles in the formation of PSDs via condensation of scaffold protein/neurotransmitter receptor complexes.	Zeng et al. (2016, 2018); Bai et al. (2021); Wu et al. (2020)
Establishment of cell polarity	The Par complex exhibits cell cycle-dependent condensation in *Drosophila* neuroblasts, driven by LLPS.	Liu et al. (2020c)
Enzyme or complex-mediated signal transduction	Formation of modular enzyme complex condensates through phase separation can dynamically concentrate enzymes to specific cellular compartments for optimal signaling.	Zhu et al. (2020b)

Both reversible droplets and more stable amyloid-like aggregates can be formed through this method (Chiesa et al., 2020).

Codon reassignment via artificial MLOs

A synthetic MLO was constructed by Reinkemeier et al. (2019) by using spatial targeting and phase separation for protein engineering. They used this system to translate only specific type of mRNA by incorporating RNA-targeting system, ribosomes, stop-codon suppressing machinery, and specific mRNA into the organelle at a specific spatial site of cytoplasm. This setting allows site-specific protein engineering with a customized non-canonical function for a specific codon only in the desired protein (Reinkemeier et al., 2019).

Cellular sensing and signal processing

In cellular environment, biological condensates are associated with sensing of an array of biological and chemical signals and execute appropriate responses to cope with those signals. This ability of protein assembly systems can be used to devise synthetic system that can respond to various stimuli and ligands. Recently, a strategy termed as distributed amphifluoric FRET (DamFRET) was developed which quantifies protein aggregation by utilizing photo-convertible fluorophore to emit FRET signals. DamFRET can be used to study the kinetics of protein aggregation by providing information about the conformation and proximity of protein monomers (Khan et al., 2018). For example, yeast transcriptional reporting of aggregating protein system (yTRAP system) can harness the polymerization states of protein in yeast cells (Newby et al., 2017).

Engineering memory and inheritance

Biological memory is an essential feature to accomplish fundamental biological functions such as cellular differentiation, environmental adaptation and development. Therefore, biological memory can be used to maintain a sustained response to a specific event. Yeast prions represent excellent candidates to generate synthetic memory by exploiting their remarkable features (Inniss and Silver, 2013). Prion alleles with tendency to cure prions were used to design anti-prion drives that can reverse or even eliminate the dominant inheritance of prion (Newby et al., 2017). Recently, a synthetic memory device based on yeast prions was devised which can program the population to remember a transient exposure to increased temperature even after ten generations (Chernova et al., 2017). It would be of great interest to delineate the molecular basis of the cellular plasticity driven by prion inheritance.

Designing protein fibers

Many proteins with abilities to form protein fibers through phase separation can be used as model to design recombinant proteins that can assemble into protein fibers. For example, proteins with folded globular domains at each terminus of a truncated repetitive silk sequence were designed to form fibers driven by LLPS. Such fibers exhibited strong adhesive and self-fusing properties (Mohammadi et al., 2018).

Therapeutic strategies targeting LLPS

Recent discoveries have put LLPS as a compelling culprit in various disease and neurodegenerative disorders, subsequently a new avenue has opened for designing therapeutic strategies (Taylor et al., 2016). A list of various diseases that arise due to aberrant phase separation is shown in Table 2. Several strategies have been proposed to interrogate these diseases, some of which are discussed here.

Antisense oligonucleotides (ASOs). The key players in anomalous phase separation can be knocked down using ASOs, which has been demonstrated successfully in various mouse models (Schoch and Miller, 2017). This strategy was tested in case of TDP-43 protein using ALS models. Ataxin-2 has been demonstrated to recruit TDP-43 to stress granules (Elden et al., 2010) and promote its aggregation associated with ALS. Knockdown of Ataxin-2 in mouse model of ALS lower the TDP-43 aggregates in the spinal cord of the mice and significantly increased the survival (Becker et al., 2017). Another example involves TIA-1 protein of stress granules that interacts with Tau protein and contributes to Tau pathology. TIA-1 knockdown prevented Tau pathology in neuronal culture and rodent models (Apicco et al., 2018). These examples convincingly suggest that LLPS is an attractive target to interrogate pathological protein aggregation.

Modulating quality control machinery. Protein degradation and chaperone machinery tightly control the protein aggregation and phase separation (Ganassi et al., 2016; Mateju et al., 2017), therefore, drugs upregulating these processes and disaggregases antagonizing aberrant phase separation can be potential therapeutic approaches. Many ALS mutations have been reported in members of protein quality control system such as chaperones, members of autophagolysosomal system and ubiquitin/proteasome components (Alberti et al., 2017). Defects in disassembly and dynamics of stress granules can arise due to compromised chaperone function (Ganassi et al., 2016). A potential approach can be focused on the development of specific chaperones that can disassemble aberrant MLOs. For example, Hsp104 variants from yeast were used to design a chaperone that reverted FUS and TDP-43 aggregation and halted their toxicity in yeast (Jackrel et al., 2014; Mateju et al., 2017). Additionally, in mammalian cells, engineered Hsp104 variants also disassembled ALS-associated FUS aggregates (Yasuda et al., 2017).

Autophagy. Autophagy is one of the central events disrupted during ALS pathology, therefore, drugs that can enhance the autophagy have been shown to carry out the clearance and localization of TDP-43 to prevent neurodegeneration (Budini

Table 2 Diseases linked with aberrant phase separation and potential treatment strategies.

Disease	Contribution of phase separation in disease	Potential treatment strategies	References
ALS	Mutations in TDP-43, FUS, hnRNPA1, hnRNPA2, and TIA-1 promote aberrant phase separation and pathological aggregation of the respective protein in stress granules. Abnormal PTMs on RBPs are associated with pathological phase transitions in ALS.	Knockdown of Ataxin-2 in mouse model of ALS through ASOs was shown to lower the TDP-43 aggregates in the spinal cord of ALS mouse models. Engineered Hsp104 variants have been shown to disassemble ALS-associated FUS aggregates in mammalian cells. Enhancing the selective targeting of misfolded proteins to autophagy can enhance their localization and clearance.	Taylor et al. (2016); Rusmini et al. (2017); Yasuda et al. (2017); Hofweber and Dormann (2019)
AD	Disease-linked Tau mutations or aberrant PTMs promote condensed phase and subsequent hardening and aggregation of proteins involved in AD pathology.	TIA-1 knockdown through ASOs prevented Tau pathology in neuronal culture and rodent models.	Apicco et al. (2018); Gan et al. (2018)
Huntington disease (HD)	Poly-glutamine (polyQ) aggregates are formed due to anomalous phase separation in HD. Many of these proteinaceous assemblies can spread from one brain region to another.	Profilin, the product of the PFN1 ALS-associated gene, binds to the monomeric phase of polyQ Huntington protein preferably and suppresses LLPS and pathogenic fibrillar aggregation *in vitro*.	Gan et al. (2018); Posey et al. (2018)
FTD	Dlease-causing mutations of TDP-43 or FUS along with polyadenylate-binding protein 1, eukaryotic translation initiation factor 4 gamma 1, and TIA-1 cause abnormal phase separation. Abnormal PTMs on RBPs are associated with pathological phase transitions in FTD.	Disease-related PTM enzymes that alter client partition into condensates can be potential targets.	Hofweber and Dormann (2019); Wheeler (2020)
Parkinson's disease (PD)	The phase transition of α-Synuclein from a monomeric to oligomeric state and further to fibrils is related to the pathological toxicity of PD.	Use of specific chaperone variants can disassemble aberrant protein aggregates, e.g. use of Hsp104 variant in *C. elegans* PD models.	Jackrel et al. (2014); Han et al. (2020)
Cancer	Cancer mutations promote the formation of anomalous signaling clusters having abnormal composition, which activate downstream oncogenic signaling events.	LLPS inhibitor, 1,6-hexanediol significantly inhibited cell proliferation and induced cell death in all tested pancreatic cancer cells (BxPC-3, PANC-1, AsPC-1, and CFPAC-1). A potential therapeutic strategy for the treatment of tight junction-associated diseases such as tumor invasion and metastasis is the targeting of phase separation by ASOs based on the known actions of miRNAs.	Xu et al. (2016, 2019); Bouchard et al. (2018); Alberti and Dormann (2019); Liu et al. (2020a); Sun and Zhou (2020)
Trinucleotide repeat disorder	Due to repetitive number of nucleotide repeats within the gene, such sequences can engage in multivalent intermolecular interactions and thus form condensates by phase separation.		Alberti and Dormann (2019)
Infectious diseases	Many viruses induce the formation of compartments termed as viral factories, viroplasm, or viral replication centers upon infection of their host cell via LLPS. These compartments promote the production of virus components and allow viruses to evade the immune system, e.g. Measles virus nucleo- and phosphoproteins form phase-separated compartments that promote nucleocapsid assembly.	LLPS is essential for viral assembly for many viruses such as SARS-CoV-2, which hints for developing intervention strategies by disrupting LLPS and viral assembly.	Netherton and Wileman (2011); Schmid et al. (2014); Chen et al. (2020); Guseva et al. (2020)
Primary biliary cirrhosis	Arg-methylation of RNA-associated protein 55A reduces its phase separation in stress granules and P-bodies, which contributes to disease pathology.	Disease-related PTM enzymes that alter client partition into condensates can be potential targets.	Yang et al. (2006); Wheeler (2020)
Fragile X Syndrome	Aberrant Ser/Tyr phosphorylation enhances the phase separation of C-terminal LCD region of fragile X mental retardation protein, which contributes to disease condition.	Disease-related PTM enzymes that alter client partition into condensates can be conventional targets for small-molecule drugs.	Tsang et al. (2019); Wheeler (2020)

et al., 2017). Furthermore, the survival of human-induced pluripotent stem cells (iPSCs)-derived neurons and astrocytes also elevated upon enhanced autophagy (Barmada et al., 2014). A compound colchicine has been reported to increase the expression of HSPB8 and other autophagy players, thus halting TDP-43 accumulation in neurons (Rusmini et al., 2017).

Nuclear-import receptors (NIRs). NIRs represent another entity to be used in novel therapeutic strategies to combat phase separation driven pathologies. Karyopherin-β2 binds with proline–tyrosine nuclear localization signals (PY-NLSs) of RBPs and this binding could revert the fibrillization of TAF15, EWS, FUS, hnRNPA1, and hnRNPA2 (Guo et al., 2018).

Specific kinases. Specialized kinases can modulate the disassembly of specific MLOs, therefore, they can be investigated as promising therapeutic targets. DYRK3 kinase is associated with dissolution of stress granules (Wippich et al., 2013) and exogenously expressed DRYK3 has been shown to disassemble nuclear and cytoplasmic MLOs in a kinase activity-dependent fashion (Rai et al., 2018). Recent studies show that mitotic kinase BubR1 elicits the dynamic assembly of central spindle, a spatiotemporally regulated MLO, by phosphorylation of CENP-E (Huang et al., 2019; Liu et al., 2020b). Thus, it would be of great interest to delineate the phospho-regulation of MLO assembly by identifying the substrates of DYRK3 and BubR1 and visualizing the regulatory function in live cells.

Concluding remarks

Our understanding about the basic biology of cells has revolutionized through the framework of LLPS disclosed by recent studies. Given the complexity of phase separation, we are merely at the starting point of our journey to unravel the mysteries behind the complex protein assembles and must bear in mind that we are still far from the complete comprehension of this dynamic field. There are many outstanding questions that future studies should focus on, such as what are the molecular codes for phase separation? How do cells differentiate the identities of various MLOs and how their regulation is carried out? How do cells respond to different environments to shape their LLPS properties? Although we have basic knowledge about the forces governing LLPS, yet we need information about atomic-level interactions. What factors control the precise composition of each MLO and according to what principle only specific RNAs and proteins are targeted into a particular MLO? Lastly, one of the most important issue is to understand the precise link between phase separation and various disease processes that how do aging, and disease mutations interfere with phase separation? Many viruses have been reported to carry PrLDs (Tetz and Tetz, 2018) and given the huge propensity of such domains to undergo phase separation, it can be speculated that these viruses may exploit LLPS during their infection process, hence a novel strategy for antiviral therapeutics could be based on faulty phase separation blockade. Furthermore, RNA is known to play seeding role in phase separation and protects against aberrant phase separation, therefore, a therapeutic aspect of RNA through expression or delivery of specialized RNA can be explored. Moreover, the role of universal solvent, water, is most often ignored during studying LLPS and generation of MLOs, although water significantly affects the structure–function properties of proteins, thus the properties of water as a solvent must be considered while studying phase separation behavior of proteins.

In summary, we envision that molecular delineation of physiochemical property of MLOs will contribute to better understanding of cell physiology and cell plasticity control which will open new horizons for precision and targeted interrogation of aberrant LLPS-driven pathogeneses.

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