

Liquid-liquid phase separation in biology: mechanisms, physiological functions and human diseases

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Cells are compartmentalized by numerous membrane-enclosed organelles and membraneless compartments to ensure that a wide variety of cellular activities occur in a spatially and temporally controlled manner. The molecular mechanisms underlying the dynamics of membrane-bound organelles, such as their fusion and fission, vesicle-mediated trafficking and membrane contact-mediated inter-organelle interactions, have been extensively characterized. However, the molecular details of the assembly and functions of membraneless compartments remain elusive. Mounting evidence has emerged recently that a large number of membraneless compartments, collectively called biomacromolecular condensates, are assembled via liquid-liquid phase separation (LLPS). Phase-separated condensates participate in various biological activities, including higher-order chromatin organization, gene expression, triage of misfolded or unwanted proteins for autophagic degradation, assembly of signaling clusters and actin- and microtubule-based cytoskeletal networks, asymmetric segregations of cell fate determinants and formation of pre- and post-synaptic density signaling assemblies. Biomacromolecular condensates can transition into different material states such as gel-like structures and solid aggregates. The material properties of condensates are crucial for fulfilment of their distinct functions, such as biochemical reaction centers, signaling hubs and supporting architectures. Cells have evolved multiple mechanisms to ensure that biomacromolecular condensates are assembled and disassembled in a tightly controlled manner. Aberrant phase separation and transition are causatively associated with a variety of human diseases such as neurodegenerative diseases and cancers. This review summarizes recent major progress in elucidating the roles of LLPS in various biological pathways and diseases.

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Introduction

Numerous protein interactions and biochemical reactions occur simultaneously within the limited spaces inside eukaryotic cells. Multiple mechanisms have been identified that ensure the spatiotemporal specificity and efficiency of these cellular processes. Organelles delineated by phospholipid membranes provide relatively confined spaces which allow various signaling pathways and biological interactions to proceed efficiently and specifically. The membrane-bound organelles are connected via vesicle-mediated trafficking and distinct membrane contacts, thereby forming elaborate cellular reaction and signaling compartments essential for the well-being of cells.

Biomacromolecules such as proteins and nucleic acids can coacervate into liquid-like membrane-less condensates via liquid-liquid phase separation (LLPS), which provides another means for concentrating and segregating cellular components in a spatiotemporally defined manner for diverse functional processes. The phase-separated condensates are also called aggregates, bodies, granules and membrane-less compartments. There is mounting evidence that protein condensates fulfill a range of distinct physiological functions in living cells. To mention a few examples, phosphorylation-induced phase separation of T-cell receptor (TCR) and its downstream signaling proteins enriches signaling components and expels inhibitory regulators, thus ensuring subsequent signal transduction (Su et al., 2016); gel-like assembly of postsynaptic density (PSD) scaffold proteins may facilitate synaptic signal transduction, synaptic development and plasticity (Zeng et al., 2016; Zeng et al., 2018); phase separation of PGL granules modulated by mTORC1 signaling ensures their efficient degradation by autophagy or their retention as an adaptation to heat stress during development (Zhang et al., 2018a; Wang and Zhang, 2019); the cell fate determinants Numb and Pon form condensates on the basal cortex of the inner surface of the plasma membrane during asymmetric cell division of *Drosophila* neuroblasts (NBs) (Shan et al., 2018). Phase-separated protein condensates also intimately interact with membrane-bound organelles via lipid-binding proteins and membrane-anchored proteins (Banjade and Rosen, 2014; Case et al., 2019a; Liao et al., 2019; Ma and Mayr, 2018; Milovanovic et al., 2018; Yamasaki et al., 2020). The field of biomacromolecular LLPS has just opened up, and many more examples of cellular functions of various biomacromolecular condensates are expected to be discovered.

The physical principles underlying LLPS**Basic principles underlying LLPS**

The LLPS phenomena of polymers have been extensively studied in the fields of polymer chemistry and soft matter physics. Biomacromolecules are polymers and hence the physical basis of biomacromolecular LLPS appears to be the same. The in-depth explanation of the theoretical basis of LLPS, known as the Flory-Huggins theory, has been nicely summarized (Flory, 1953; Michaeli et al., 1957). We can rationalize the condensation process of a biomacromolecular phase separation system using a simple thermodynamics argument. Phase separation systems can contain one or more type(s) of biomacromolecular component(s), but for simplicity, we will use a system comprising one type of biomacromolecule in a solution for further discussion. Biomacromolecules in a solution interact with each other and solvent molecules in a manner that reduces the free energy of the system. Generally, this means that the biomacromolecules will tend to be distributed uniformly throughout the solution volume in monomeric and small-sized complexes to maximize the entropy. If biomacromolecules can engage in more energetically favorable interactions among one another in a condensed solution than in a dilute solution, the extra energy output can compensate for the entropic penalty due to the clustering of biomacromolecules. The higher the biomacromolecule concentration is, the lower the entropic penalty is. The critical concentration of the biomacromolecule solution is defined as the concentration at which the free energy generated from the extra interactions of a molecule in a condensate versus the interactions in dilute solution is equal to the entropy penalty caused by constraining it within a condensate. At concentrations below the critical concentration, the solution is homogeneous. At concentrations above the critical concentration, the solution undergoes phase separation to yield a dilute solution phase and a condensed, biomacromolecule-rich phase. A number of recent reviews have formally dealt with the thermodynamics of phase separation (Banani et al., 2017; Shin and Brangwynne, 2017). Here we introduce recent progress in understanding the mechanisms driving the formation of biomacromolecular condensates.

Multivalent interactions underlying biomacromolecular LLPS

Phase-separated systems normally contain two phases, the

aqueous solution and the biomacromolecular condensates. Phase separation is driven by multivalent, and often weak, interactions (Li et al., 2012), based on electromagnetic forces. Electromagnetic forces can be classified into Coulomb forces and van der Waals forces based on the process by which they are generated (Israelachvili, 2011). For charged particles, such as ions, the inherent positive or negative charges generate permanent electric fields and intermolecular interactions, which are called Coulomb forces or electrostatic forces (Israelachvili, 2011). The ionic bond is the best-known Coulomb force in intermolecular interactions. van der Waals forces exist among all molecules; the precise nature of the force depends on the polarity of the interacting molecules (Israelachvili, 2011). Molecules with larger polarity have stronger van der Waals forces with surrounding molecules, and vice versa. These weak interactions have distinct names in biochemistry, including electrostatic interactions that occur between charged residues, hydrogen bonds, hydrophobic interactions that occur between weakly polar residues, π - π stacking between aromatic residues, and cation- π stacking between positively charged and aromatic residues.

LLPS due to multivalent interactions of IDRs

Emerging evidence indicates that the proteins in phase-separated liquid droplets are mobile and transiently interact with surrounding molecules. Proteins that can undergo phase separation often contain intrinsically disordered regions (IDRs) and low-complexity regions (LCRs). IDRs/LCRs are distinctly different from modular domains in that their amino acid composition and distribution cannot fulfill the requirements for compact folding (Wang and Zhang, 2019; Wright and Dyson, 2015). IDRs lack a stable tertiary structure and often exhibit flexible and versatile conformations. Some IDRs also have highly biased amino acid compositions, and are enriched in a limited subset of residues, such as poly-glycine, poly-glutamine and poly-serine; these domains are also called low complexity regions (LCRs) (Han et al., 2012; Kato et al., 2012; Wang and Zhang, 2019; Wright and Dyson, 2015). The flexible conformations and large numbers of similar residues in IDRs and LCRs perfectly satisfy the requirement for multivalent weak interactions in driving protein LLPS (Posey et al., 2018; Wang et al., 2018). In most cases, protein phase separation is closely linked with the presence of IDRs and LCRs in the phase-separated proteins (Banani et al., 2017; Brangwynne et al., 2015). A number of the above-mentioned weak interactions, including π - π interactions, cation- π interactions, cation-anion interactions, dipole-dipole interactions and reversible amyloid-like interaction (Figure 1A), are often abundant in IDRs/LCRs. These multivalent weak interactions can induce LLPS, resulting in liquid condensate phases.

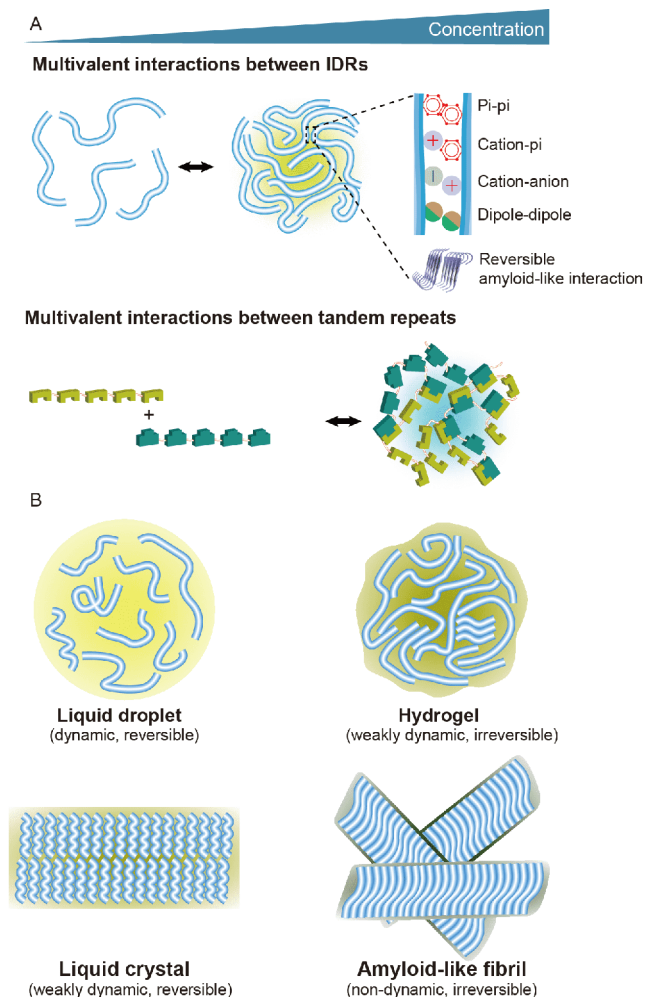


Figure 1 The forces driving phase separation and the material states of condensates. A, Two mechanisms for the formation of phase-separated liquid droplets. Top: interaction of intrinsically disordered regions (IDRs) within one protein species via different kinds of weak contacts (right). Bottom: binding of tandem interacting domains in two different proteins. In both cases, multivalent interactions lead to the formation of phase-separated liquid droplets at higher protein concentrations. B, The different material states of phase-separated condensates. Within the liquid droplets, the protein condensates are highly dynamic and reversibly assembled. The condensates can break up in response to certain changes in the solution conditions, such as protein concentration, temperature and ionic strength. The constituents inside liquid droplets have high mobility and exchange with the surrounding environment. With time, the liquid-like protein condensates may gradually transition into solid-like states, such as hydrogels. Compared to liquid droplets, protein condensates with gel-like structures are less dynamic, and the constituents inside can only undergo very limited exchange with their surroundings. However, the assembly of these hydrogel-like structures can also be partially reversed under certain conditions. In certain scenarios, liquid crystal-like structures can form in cells. The constituents inside liquid crystals are in an ordered arrangement and can realign in response to stimuli. Protein condensates can also transition into amyloid-like fibril structures or other types of aggregates, which are non-dynamic and extremely resistant to changes in solution conditions. The constituents inside amyloid-like fibrils and other aggregates are inert and immobile.

LLPS due to multivalent interactions involving modular protein domains

The multivalent interactions that drive LLPS can also occur

between two or more biomacromolecules which contain multiple interaction domains and/or motifs (Banani et al., 2016; Li et al., 2012; Zeng et al., 2016). The individual interactions between IDRs are often weak and transient. The individual interactions between modular domains are relatively strong and more specific, but are nevertheless on the weaker side of specific biomolecular interactions. A difference between the two phase separation mechanisms is that a single species can undergo IDR-mediated phase separation, while phase separation mediated by multiple interaction domains often involve two or more different protein species (Figure 1A).

Proteins have evolved to be modular, which means that they are often composed of independently folded domains (Pawson, 1995). Some modular proteins are composed of multiple domains of the same type; they are often involved in ligand binding and, by definition, they are multivalent as far as binding is concerned (Banani et al., 2017; Li et al., 2012). Sometimes the binding partners of multivalent proteins are also multivalent. These multivalent interaction pairs have the tendency to undergo LLPS. Cells harness this property and trigger LLPS to execute specific functions at the right time and in the right location. Tandem topology is just one of many ways to achieve multivalence.

It is worth noting that in the field of biomacromolecular LLPS, there is a biased view that IDRs equate to phase separation. As a matter of fact, the presence of an IDR is neither a necessary nor a sufficient condition for phase separation. The majority of IDRs fail to undergo LLPS at physiological conditions, and many physiologically relevant phase separation events do not rely on IDRs. Another major challenge for IDRs-mediated LLPS systems is its intrinsically low specificity. It is hard to rationalize how specific cellular events would only rely on LLPS condensates formed with low specificities. We anticipate that, under physiological conditions, specific molecular interaction together with IDR/LCR-mediated LLPS events act together to fulfill defined cellular processes. In this model, the IDR elements can boost phase separation capacity and the specific molecular interactions function to provide specificity of the condensed assembly.

LLPS due to multivalent interactions of RNA

Biomacromolecular phase separation is not limited to proteins. As a matter of fact, RNAs participate extensively in the formation of RNA/protein-rich membraneless organelles by contributing to phase-separation mechanisms (Banani et al., 2017; Shin and Brangwynne, 2017). In addition, Jain and Vale have demonstrated that repeat-containing RNAs found in a number of diseases can undergo phase separation *in vitro* (Jain and Vale, 2017). These observations are unsurprising as it is the architecture of the molecular interaction, rather than the biochemical identity of the interacting components, that

is the determining factor for phase separation (Li et al., 2012).

Dynamic regulation of biomacromolecular LLPS

Liquid-like condensates assembled via LLPS may undergo changes in their material properties. These changes, known as phase transitions, can affect the mobility inside droplets, viscoelasticity and surface/interfacial tension. Upon phase separation, some liquid condensates are metastable and tend to gradually convert into solid-like states, such as crystals and amyloid-like fibrils (Alberti and Hyman, 2016; Wang and Zhang, 2019). Several intermediate states occur between liquid and solid states, including gel-like structures and liquid crystals (Patel et al., 2015; Rog et al., 2017; Wu and Fuxreiter, 2016; Zhang et al., 2018b) (Figure 1B). Aberrant transition of phase separated condensates is pathological and causatively associated with a variety of human diseases. The physiological functions and pathological features of biomacromolecular condensates are closely related to their material properties.

Protein phase separation and transition are tightly controlled via various mechanisms for proper functions. Physical conditions such as temperature, pH, ionic strength and osmotic pressure, which can change affinities of molecular multivalent, are known to alter phase separation and transition behaviors of biomolecular systems (Banani et al., 2017; Boeynaems et al., 2018; Quiroz et al., 2020). In addition, post-translational modifications (PTMs), including but not limited to phosphorylation (Banjade and Rosen, 2014; Su et al., 2016; Zhang et al., 2018a), methylation (Hofweber et al., 2018; Qamar et al., 2018; Zhang et al., 2018a), acetylation (Gibson et al., 2019), ubiquitination (Sun et al., 2018), and SUMOylation (Banani et al., 2016; Qu et al., 2020), are prevalently involved in modulating phase separation and transition by altering the intermolecular interactions and/or directly altering the valencies of interacting biomacromolecules (Banani et al., 2016).

It has been observed that further solidification of LCR-driven liquid-like condensates is the norm in test tubes. This process is likely detrimental to cells and therefore cells have ATP-dependent and -independent molecular chaperones which maintain the functional form of the liquid state by inhibiting solidification (see section on phase separation and transition in specifying autophagic degradation of protein aggregate for further details and some key examples) (Hondele et al., 2019; Wright et al., 2019).

Aberrant phase separation has been increasingly observed to be linked with malfunctions of cellular functions, which may underlie various human diseases (see section on aberrant protein phase separation in human diseases for more details and key examples) (Shin and Brangwynne, 2017). Extensive effort is required to elucidate the causal link be-

tween aberrant phase separation and diseases, and whether aberrant phase separation can be reversed for therapeutic purposes.

Protein LLPS in the assembly of cytoskeletal networks

Cytoskeletons mainly include microtubules (MTs), filamentous Actin (F-actin), and intermediate filaments. They are constructed into various intracellular structural networks, either alone or in combinations (Dogterom and Koenderink, 2019; Hohmann and Dehghani, 2019). Unlike intermediate filaments, MTs and F-actin are highly dynamic and capable of forming supramolecular networks with diverse architectures, dynamic behaviors, and physiological functions. For instance, MTs are organized into radial arrays in non-polarized cells, thick bundles in neurites, “9+0” or “9+2” configurations in cilia, and spindles in mitotic cells, whereas Actin assembles into cell cortexes, lamellipodia of migrating cells, microvilli of intestinal epithelial cells, sarcomeres of muscle cells, and contractile rings of dividing cells. Accordingly, the MT and Actin cytoskeletons are important for a wide variety of cellular functions, including cell shape maintenance and remodeling, cell division, contraction, movement, and intracellular trafficking (Dogterom and Koenderink, 2019; Hohmann and Dehghani, 2019; Revenu et al., 2004).

The MT and Actin cytoskeletons are often constructed regionally in cells with the help of a large pool of interacting proteins. Among them are various nucleators, cross-linkers, and end-binding, stabilizing, and destabilizing proteins, which enable intricate and elaborate control of the temporal dynamics and spatial architecture of the cytoskeleton (Akhmanova and Steinmetz, 2015, 2019; Bodakuntla et al., 2019; Buracco et al., 2019; Campellone and Welch, 2010; Revenu et al., 2004; Rottner et al., 2017). Recently, emerging lines of evidence suggest that protein phase separation underlies the local assembly of cytoskeletal networks.

LLPS of crosslinked Actin filaments into hydrogels and liquid droplets

Actin filaments underneath the plasma membrane are usually crosslinked into networks with a variety of different geometries and properties (Revenu et al., 2004; Rottner et al., 2017). Crosslinking enhances the rigidity and strength of the Actin meshwork. This renders cells resistant to internal tensions and external mechanical stresses, while also enabling the formation of stable cell-cell junctions for intercellular communication or tissue formation.

Interestingly, *in vitro* studies reveal that crosslinking can also induce phase separation of the networks and can

therefore endow the networks with new physical properties. For instance, it has long been found that Actin-crosslinking proteins such as Filamin can aggregate F-actin into viscoelastic hydrogels *in vitro* (Wang and Singer, 1977), whereas Actin-severing proteins such as Gelsolin can resolve such gels in a calcium-dependent manner (Yin et al., 1981; Yin and Stossel, 1979). These observations provide insights into the mechanical properties and regulation of F-actin networks. Detailed studies reveal that, in the presence of Filamin, short Actin filaments (<1 μm in length) rapidly demix into spindle-like tactoids, whereas long filaments tend to phase-separate into gels (Figure 2A) (Weirich et al., 2017). The cross-link density regulates interfacial tension and viscosity and alters the overall shapes of the tactoids. Interestingly, Actin molecules in the tactoids actively exchange with those in solution, and two tactoids can fuse into one, which indicates that they are dynamic liquid droplets formed by LLPS (Weirich et al., 2017).

LLPS in regional assembly of F-actin networks underneath lipid bilayers

LLPS of signaling proteins has been shown to underlie the formation of stable intercellular junctions. Actin polymerization *in vivo* requires the help of Actin nucleators to form the initial Actin oligomers. Among these nucleators is the Arp2/3 complex, a seven-subunit protein complex that functions in the nucleation of branched F-actin (Campellone and Welch, 2010; Rottner et al., 2017). Hyperphosphorylation of the transmembrane proteins Nephrin in podocytes and LAT in T-cells is known to lead to recruitment of adaptor proteins such as NCK to locally concentrate and activate N-WASP, which in turn activates the Arp2/3 complex to stimulate massive local assemblies of cortical Actin networks at the glomerular podocyte-endothelial cell junctions and T-cell-antigen-presenting cell junctions (Courtney et al., 2018; Dustin and Choudhuri, 2016; Jones et al., 2006; Perico et al., 2016; Rohatgi et al., 2001). Recently, these signaling cascades have been shown to trigger LLPS (Figure 2B) (Case et al., 2019b; Courtney et al., 2018; Li et al., 2012; Su et al., 2016).

The F-actin-abundant processes emanating from glomerular podocytes form a specialized intercellular junction, the slit diaphragm, with glomerular endothelial cells to serve as the major glomerular filtration barrier against macromolecules in the kidney (Pavenstädt et al., 2003; Perico et al., 2016). Podocytes use the Nephrin-NCK-N-WASP-Arp2/3 axis to stimulate assembly of the F-actin meshwork at the slit diaphragm (Jones et al., 2006; Perico et al., 2016; Rohatgi et al., 2001). Incidentally, LLPS of proteins through multivalent interactions was initially conceptualized by mixing synthetic tandem repeats of the proline-rich motifs (PRMs) of N-WASP and their interacting SRC homology 3 (SH3)

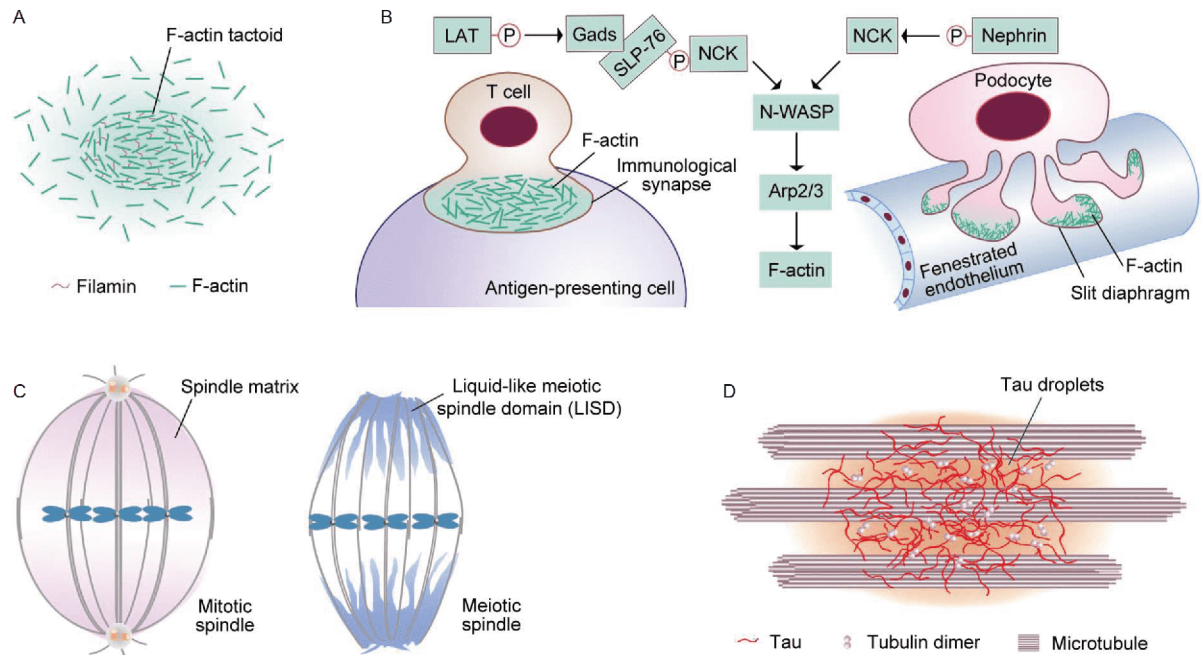


Figure 2 Examples of phase separation of cytoskeleton-related proteins. A, In the presence of Filamin, short actin filaments form tactoids *in vitro* through LLPS. B, LLPS of signaling proteins triggers local F-actin assembly in T-cells at the immunological synapse or in podocytes at the slit diaphragms. C, LLPS of BuGZ and Tacc3 induces the spindle matrix and the liquid-like meiotic spindle domain respectively to facilitate MT polymerization and spindle assembly. D, Liquid droplets of Tau facilitate MT polymerization and bundling *in vitro*.

domains of NCK (Li et al., 2012). Moreover, LLPS of N-WASP and NCK is correlated with increased Arp2/3-induced Actin polymerization *in vitro* (Li et al., 2012). Further detailed reconstitution experiments indicated that LLPS of the phosphorylated Nephrin-NCK-N-WASP complex on lipid bilayers increased the membrane dwelling time of both N-WASP and Arp2/3, resulting in augmented Actin polymerization (Figure 2B) (Case et al., 2019b). In addition, different stoichiometries of Nephrin, NCK, and N-WASP produce LLPS-induced condensates with different membrane dwelling times, which allows elaborate regulation of the Actin polymerization activity (Case et al., 2019b).

The immunological synapse is a transient intercellular junction between a T-cell and an antigen-presenting cell. It is essential for T-cell receptor (TCR) signaling (Courtney et al., 2018; Dustin and Choudhuri, 2016). At the T-cell side, a dense cortical F-actin meshwork ensures the stability of the synapse. Multiple adaptor proteins, including Gads, SLP-76 and NCK, link phosphorylated LAT to N-WASP to activate the Arp2/3 complex and subsequently initiate F-actin network formation (Figure 2B) (Courtney et al., 2018; Dustin and Choudhuri, 2016). Reconstitution experiments indicate that liquid-like clusters form on lipid bilayers through interplay among proteins during TCR activation (Su et al., 2016). The phosphorylation status of LAT correlates with the efficiency and extent of condensate formation. Furthermore, in the presence of Arp2/3, Actin polymerizes massively in the clusters, and the resultant F-actin networks confer a rod-

like morphology on the clusters (Su et al., 2016).

LLPS of MT-binding proteins in spindle assembly and function

The spindle is an MT-enriched organelle essential for segregation of sister chromatids into daughter cells. Mitotic spindles generally use centrosomes as the spindle poles, whereas meiotic spindles are usually acentrosomal (Helmke et al., 2013; Walczak and Heald, 2008). In addition to MTs, spindles also contain the spindle matrix, a membrane-enriched supramolecular network abundant in Lamin B, BuGZ/Znf207, cytoplasmic dynein, Nudel, and possibly many other proteins (Figure 2C) (Jiang et al., 2014; Ma et al., 2009; Tsai et al., 2006; Zheng, 2010). BuGZ contains two zinc finger motifs at the N-terminus that can interact with free Tubulin and MTs. BuGZ undergoes LLPS through intermolecular interactions via its C-terminal disordered region. The BuGZ liquid droplets are capable of concentrating Tubulin dimers, promoting MT polymerization, and bundling MTs. Furthermore, BuGZ phase separation and MT polymerization display a synergistic effect (Jiang et al., 2015). The phase-separation of BuGZ is required for spindle matrix assembly and also enhances the activation of Aurora A, a kinase critical for spindle formation (Huang et al., 2018; Jiang et al., 2015; Tiwary and Zheng, 2019). Accordingly, depletion of BuGZ diminishes spindle size in *Xenopus* egg extracts and spindle MT density in mammalian cells (Jiang et al., 2015;

Tiwary and Zheng, 2019). Therefore, the mitotic spindle is a large non-membrane-bound organelle assembled through protein phase separation.

Meiotic spindles have recently been shown to contain a unique liquid-like meiotic spindle domain (LISD) (Figure 2C) (So et al., 2019). The LISD is a collection of condensates at the spindle pole regions which forms dynamic spherical protrusions both into and beyond the acentrosomal spindle. A microscopy screen of 70 centrosomal and spindle-related proteins identified that 19 of them are concentrated in the LISD. They include proteins that are acentrosomal (Akap450, Cep170, and Kiz), centriolar satellite-localized (Cep72, Pcm1, and Lrrc36), dynein-related (Hook3, NudE, Nudel, and Spdl1), and MT-associated (e.g., Camsap3, Mcak, Myo10, and Tacc3). Moreover, the MT-binding protein Tacc3 is found to phase-separate into liquid droplets through its N-terminal disordered region. LISD formation depends on the LLPS of Tacc3. Depletion of Tacc3 in mammalian oocytes decreases the spindle size, and this defect is not rescued by a mutant lacking the N-terminal disordered region (So et al., 2019). Interestingly, the LISD appears to be structurally independent of the spindle matrix because it does not contain BuGZ (So et al., 2019). Aurora A is required for assembly of the LISD (So et al., 2019); therefore, LISD formation could occur downstream of spindle matrix assembly, considering the activation effect of phase-separated BuGZ on Aurora A (Huang et al., 2018).

LLPS of the MT-binding protein Tau and its neuronal functions

Tau is a well-studied neuronal MT-binding protein that is important for axonal MT growth and stability (Brandt and Lee, 1993; Kadavath et al., 2015), and for axonal transport (Dixit et al., 2008; Ebnet et al., 1998; Stamer et al., 2002). It also bundles F-actin and crosslinks Actin filaments and MTs (Cabrales Fontela et al., 2017). Importantly, it has been strongly linked to multiple neurodegenerative diseases. For instance, the aggregation of hyperphosphorylated Tau in neurofibrillary tangles is tightly correlated with the pathology of Alzheimer's disease and Parkinson's disease dementia, and mutations in *MAPT*, the gene encoding Tau, are frequently identified in familial patients with frontotemporal dementia (Olszewska et al., 2016; Von Bergen et al., 2001; Wu et al., 2017).

MAPT (microtubule-associated protein tau) is expressed as six isoforms in the human brain. The longest isoform, named hTau40 or 2N4R Tau, consists of two hydrophobic N-terminal inserts (N1 and N2), two proline-rich regions (P1 and P2), and a repeat domain (TauRD) containing four hexapeptide repeats (R1-R4). The other five isoforms differ in the N1/N2 and R2 regions (Garcia and Cleveland, 2001; Iqbal et al., 2016). The β -sheet structures in TauRD are im-

portant to facilitate microtubule binding and Tubulin polymerization (Kadavath et al., 2015; Kellogg et al., 2018). The major type of post-translational modification in Tau is phosphorylation, which modulates the interaction of Tau with MTs and F-actin and contributes to tangle formation (Cabrales Fontela et al., 2017; Fischer et al., 2009; Morris et al., 2015; Pavenstädt et al., 2003). Tau is intrinsically disordered in the N1/N2 and P1/P2 regions and undergoes LLPS both *in vitro* and *in vivo* (Ambadipudi et al., 2017; Hernández-Vega et al., 2017; Wegmann et al., 2018; Zhang et al., 2017). Its LLPS is phosphorylation-dependent: the phase-separation time and droplet size vary for Tau with different levels of phosphorylation, and no LLPS is observed when Tau is dephosphorylated through phosphatase treatment, or purified in an unphosphorylated form from *E. coli* (Ambadipudi et al., 2017; Wegmann et al., 2018). Liquid droplets of Tau become markedly enriched in free Tubulin and promote MT polymerization and bundling. The MT bundles in turn induce liquid-like diffusion of the phase-separated Tau (Figure 2D) (Hernández-Vega et al., 2017). Interestingly, both the hyperphosphorylated Tau from human Alzheimer brain and unphosphorylated aggregation-prone mutants of Tau have the ability to undergo LLPS. Furthermore, liquid droplets of Tau, regardless of the source of the protein, become gel-like in minutes and form aggregates in days (Wegmann et al., 2018), consistent with the disease-related behaviors of Tau.

Phase separation in genome organization and gene expression

Eukaryotic chromatin is hierarchically organized and largely composed of euchromatin and heterochromatin (Cohen and Lee, 2002; Grewal and Moazed, 2003). The former is derived from transcriptionally active regions and the latter contains transcriptionally repressed loci. Both euchromatin and heterochromatin contain various types of membraneless organelle-like compartments/condensates (Erdel and Rippe, 2018). Mounting evidence indicates that many factors, including but not limited to, histone modifications (Gibson et al., 2019; Sanulli et al., 2019; Wang et al., 2019b; Zhao et al., 2019), DNA modifications (Wang et al., 2020), RNAs (Huo et al., 2020; Yao et al., 2019), play important roles in regulating chromatin compartments and transcriptional activity, at least in part, via a phase separation mechanism.

The role of phase separation in transcriptional repression

Heterochromatin is a fundamental architectural feature of eukaryotic chromosomes, which compacts particular genomic regions into transcriptionally repressed chromatin domains (Janssen et al., 2018). There are two major types of

heterochromatin, constitutive heterochromatin and facultative heterochromatin, each enriched with characteristic epigenetic marks including histone PTMs and DNA modifications (Cohen and Lee, 2002; Grewal and Moazed, 2003). All these marks can be recognized by specific protein domains known as “reader” domains (Ruthenburg et al., 2007; Taverna et al., 2007). Constitutive heterochromatin encompasses pericentromeric and telomeric regions as well as the enormous clusters of transposable elements that are distributed throughout genomes. Facultative heterochromatin mainly contains repressed tissue-specific genes. Constitutive and facultative heterochromatin are enriched with H3K9me3 and H3K27me3, respectively. H3K9me3 is recognized by the chromodomain motif in heterochromatin protein 1 (HP1) (James and Elgin, 1986). Mammalian genomes encode three HP1 homologs: HP1 α , HP1 β , and HP1 γ . H3K27me3 is recognized by the chromodomains in CBX2, 4, 6, 7 and 8, among other proteins. In *Drosophila* embryos, heterochromatin marked with HP1 α (the homolog of mammalian HP1 α) has been demonstrated to behave like phase-separated liquid-like condensates (Strom et al., 2017). The driving forces responsible for LLPS of heterochromatin organization have been under extensive investigation lately. Phase separation driven by IDR interactions and by multivalent modification-reader interactions is reported to play roles in heterochromatin organization in animals and plants (Larson et al., 2017; Larson and Narlikar, 2018; Sanulli et al., 2019; Strom et al., 2017; Wang et al., 2019b; Wang et al., 2020; Zhao et al., 2019). Nucleosome arrays also have an intrinsic tendency to undergo phase separation, which is reversed by hyper-acetylation on core histones in nucleosomes (Gibson et al., 2019). All these phase separation processes can achieve chromatin compaction and hence transcriptional repression, presumably by blocking the access of DNA to various DNA-binding factors, such as transcription factors (TFs). Hence, these processes likely all play specific roles in heterochromatin regulation in cells.

Phase separation driven by IDRs of a subset of special reader proteins

The H3K9me3 reader protein HP1 contains a chromodomain (CD) (Paro and Hogness, 1991), a less structured hinge region, a chromo shadow domain (CSD) (Aasland and Stewart, 1995), and unstructured N-terminal extension (NTE) and C-terminal extension (CTE) (Canzio et al., 2014; Nishibuchi et al., 2014). This organizational structure of HP1 is conserved from fish to human. The CD is responsible for binding to H3K9me3 via a specialized hydrophobic cage (Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002), while the hinge region can bind nucleic acid, and the CSD can form homodimers by self-association (Cowieson et al., 2000). The unstructured NTE, hinge and CTE are the most divergent in

primary sequence among the three human HP1 homologs.

HP1 α , but not HP1 β and HP1 γ , can undergo LLPS at high protein concentration and low salt concentration (Figure 3A). In addition, HP1 α binds strongly to DNA (Canzio et al., 2014). DNA can interact with the hinge region of HP1 α and may bridge neighboring HP1 α dimers to form higher-order oligomers (Larson et al., 2017). This HP1-DNA-mediated phase separation resembles “coacervate” phase separation (Hancock and Jeon, 2014; Overbeek and Voorn, 1957), which is induced by mixing oppositely charged polymers (Lytle et al., 2016). *Drosophila* HP1 α can undergo phase separation *in vitro* at higher protein concentrations under physiological conditions (Figure 3A), and this mechanism possibly mediates heterochromatin domain formation in early *Drosophila* embryos (Strom et al., 2017). Phase separation of HP1 α is driven by the IDRs in its NTE and hinge region (Larson et al., 2017). Serine phosphorylation in the IDRs of HP1 α promotes its LLPS (Larson et al., 2017). H3K9me3-marked nucleosome arrays drastically elevate the phase separation capacity of HP1 α (Larson et al., 2017). The HP1-mediated formation of constitutive heterochromatin via phase separation is likely to be critical for gene silencing and genome organization (Klosin and Hyman, 2017; Larson et al., 2017; Larson and Narlikar, 2018; Sanulli et al., 2019; Strom et al., 2017; Tatarakis et al., 2017; Wang et al., 2019b; Wang et al., 2020; Zhao et al., 2019).

CBX2, a major reader protein of H3K27me3, the epigenetic mark of facultative heterochromatin, undergoes concentration-dependent phase separation by itself at physiological conditions (Plys et al., 2019; Tatomosian et al., 2019). The CBX2-containing Polycomb repressive complex 1 can also undergo LLPS, driven at least in part by CBX2. Similar to HP1 α , the LLPS potential of CBX2 resides in an IDR fragment, and the force driving LLPS is largely generated by charge-charge interactions (Figure 3A). Endogenous and ectopically expressed CBX2 localize to nuclear condensates, in which H3K27me3 is also enriched (Plys et al., 2019; Tatomosian et al., 2019). In addition, H3K27me3-decorated nucleosome arrays promote LLPS of CBX2. This property of CBX2 may be harnessed by cells to regulate the formation of facultative heterochromatin (Plys et al., 2019).

The intrinsic LLPS potential of nucleosome arrays

Recently Rosen and coworkers showed that unmodified nucleosome arrays (NAs) can undergo LLPS (Figure 3B) (Gibson et al., 2019). A number of physical and chemical factors can regulate LLPS of NAs. Monovalent and divalent cations positively modulate LLPS of NA. The linker histone, H1, also promotes LLPS of NAs. Interestingly, the linker DNA length has a nonlinear effect on LLPS of NAs. For example, NAs with a linker of $10 \times n + 5$ bp are more prone to undergo LLPS than NAs with a linker of $10 \times n$ bp. Im-

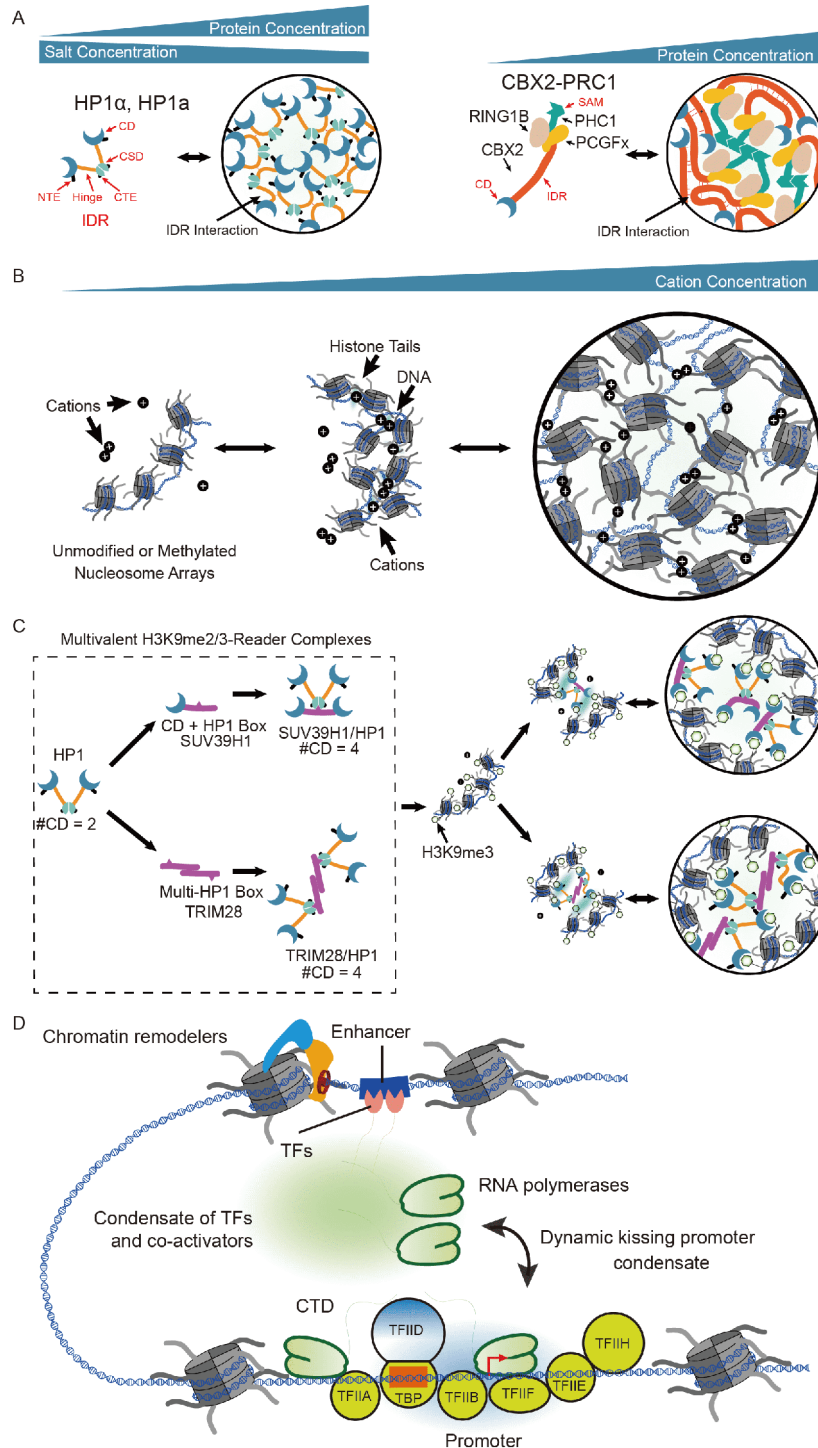


Figure 3 Phase separation in transcription regulation. A, Interactions of the IDRs in two HP1 homologs (human HP1 α and fly HP1a) (left) and CBX2-PRC1 (right) lead to the formation of phase-separated liquid droplets at higher protein concentrations and low salt concentrations *in vitro*. RING1B, PHC1, PCGFx and CBX2 are components of PRC1 complex. IDR, intrinsically disordered region; CD, chromodomain; CSD, chromo shadow domain; NTE, N-terminal extension; CTE, C-terminal extension; SAM, sterile alpha motif domain. B, Nucleosome arrays undergo phase separation under physiological conditions. C, HP1 can form complexes with a plethora of proteins, e.g., SUV39H1 and TRIM28, via their CSD-binding motifs (HP1-boxes). These complexes often contain multiple H3K9me3-recognizing CDs and can undergo phase separation with H3K9me3-marked nucleosome arrays. D, Step-by-step functions of phase separation in transcription complex assembly. (1) Transcription factors (TFs) bind to distal control elements (enhancers or super enhancers) based on their DNA-binding domains and DNA remodelers. (2) TFs interact with cofactors (mediators or chromatin regulators) to form condensates through their IDRs or multivalent domains. These condensates modify chromatin structures to facilitate the recruitment of additional factors. (3) Condensates of TFs and cofactors dynamically assemble at the promoter region to promote a high level of transcription initiation. This involves the recruitment of general transcription factors, and the formation of dynamic transcriptional condensates based on interactions of the C-terminal domain (CTD) of Pol II.

portantly, hyper-acetylation on core histone tails, a modification often correlated with transcriptional activation, efficiently inhibits LLPS of NAs and hence maintains chromatin in an open state (Gibson et al., 2019). The intrinsic LLPS property of unmodified nucleosome arrays might provide another layer of regulation for gene silencing and genome organization.

Phase separation driven by multivalent interactions between epigenetic modifications and readers

Although HP1 α and CBX2 can undergo LLPS by themselves, they may also function in heterochromatin organization via LLPS. The other orthologs of HP1 α , HP1 β and HP1 γ , cannot undergo IDR-driven LLPS (Larson et al., 2017; Wang et al., 2019b). Neither can other orthologs of CBX2, such as CBX7 (Plys et al., 2019; Tatavosian et al., 2019). However, these LLPS-incapable orthologs play similar roles in heterochromatin organization as their LLPS-capable counterparts (Larson et al., 2017; Plys et al., 2019; Tatavosian et al., 2019; Wang et al., 2019b). These observations suggest that IDR-driven LLPS may not be a general mechanism for heterochromatin organization.

Generally, chromatin compartments are enriched with specific histone PTMs as epigenetic marks (Jenuwein and Allis, 2001). Interactions between histone marks and their reader domains are normally weak, and often occur through multivalent recognition (Ruthenburg et al., 2007). Histone PTMs are H3K9me3 and H3K27me3 for constitutive and facultative heterochromatin, respectively. The readers of H3K9me3 are the chromodomains of HP1 proteins and the readers of H3K27me3 are the chromodomains of PRC1 complexes. HP1 dimerizes via its C-terminal chromo shadow domain (CSD), which also acts as a platform to recruit diverse HP1-binding partners (Brasher et al., 2000; Cowieson et al., 2000), and these CSD/partner complexes regulate HP1 to perform diverse functions within heterochromatin (Eskeland et al., 2007; Swenson et al., 2016). HP1-containing complexes tend to contain multiple HP1 dimers and hence multivalent H3K9me3 readers. In addition, some components of PRC1 complexes contain sterile alpha motif (SAM) domains, which form head-to-tail polymers and can multimerize the CD of CBX proteins (Isono et al., 2013; Kundu et al., 2017). It is tempting to hypothesize that H3K9me3 or H3K27me3 modifications regulate chromatin compartmentalization via multivalent modification/reader interaction-driven LLPS.

Indeed, a recent study showed that heterochromatin is likely regulated via LLPS derived from multivalent interactions between H3K9me3 and its reader, CD (Sanulli et al., 2019; Wang et al., 2019b; Zhao et al., 2019). Numerous HP1 binding proteins associate with HP1 dimers by their HP1-box to form multivalent CD containing complexes (Brasher

et al., 2000; Cowieson et al., 2000; Eskeland et al., 2007; Swenson et al., 2016). In the study, two purified complexes, one of HP1 with SUV39H1, an H3K9me3 writer protein, and the other of HP1 with Trim28, an abundant HP1 scaffolding protein, undergo robust LLPS with H3K9me3-decorated NAs but not unmodified NAs (Figure 3C). The resulting condensates in test tubes are reminiscent of heterochromatin in cells as far as their physical properties and their response to mutation and biochemical perturbations (Wang et al., 2019b) are concerned. Similarly, Swi6, the yeast HP1 homolog that oligomerizes, can phase-separate with H3K9me3-marked 12xNA, but not with H3K9me3-marked mononucleosomes (Sanulli et al., 2019; Wang et al., 2019b). Therefore, LLPS driven by multivalent H3K9me3/CD interactions appears to be a critical force underlying heterochromatin formation in eukaryotic cells (Larson et al., 2017; Sanulli et al., 2019; Strom et al., 2017; Wang et al., 2019b; Zhao et al., 2019).

Considering the similarity between constitutive and facultative heterochromatin, it is possible that PRC1, the multivalent H3K27me3 reader complex, also undergoes LLPS with H3K27me3-decorated NAs (Kim and Kingston, 2020). Other repressive histone marks, such as H4K20me2, might also function, at least in part, by driving LLPS with their cognate multivalent readers. The repressive DNA modification, 5-methyl cytosine in CpG islands, also positively regulates LLPS of chromatin with methyl CpG binding protein 2 (Wang et al., 2020). Overall, emerging evidence indicates that epigenetic marks control chromatin compartmentalization in part via multivalence-driven LLPS.

Phase separation in transcription activation

Transcription is mediated by the RNA polymerases I, II, and III. Many aspects of this highly complex process are still a mystery (Roeder and Rutter, 1969). Recent advances in phase separation indicate that activation of transcription, especially by Pol II, may be regulated by the condensation of particular factors in the nucleus. It was subsequently demonstrated that transcription factors (TFs), co-activators and Pol II function as dynamic hubs, clusters or condensates, formed by LLPS through the interactions of intrinsically disordered regions (IDRs) and multivalent domains (Cramer, 2019; Hnisz et al., 2017). A phase separation model has been proposed to explain the regulation of transcription activation (Figure 3D); this model also predicts that not all genes are controlled by such condensates, especially for the lowly transcribed genes (Cramer, 2019).

For gene-specific transcription initiation, active enhancers or promoters are firstly recognized by different TFs. TFs typically consist of one or more DNA-binding domains (DBDs) and trans-activation domains (ADs) (Weirauch et al., 2014). Actually, most eukaryotic TFs contain IDRs

within their ADs, and the degree of disorder in the ADs is significantly higher than that in DBDs (Liu et al., 2006; Staby et al., 2017). Recent studies have shown that phase separation of ADs appears to be a general property of TFs and is associated with gene activation (Boija et al., 2018). The abilities of OCT4 and GCN4 to form phase-separated droplets with Mediator *in vitro*, and to activate genes *in vivo*, both depend on the same amino acid residues in their ADs, and the same is true for estrogen receptor (ER), a ligand-dependent transcription factor which also enhances phase separation with Mediator (Boija et al., 2018). On the other hand, particular properties of enhancers, including the density and affinity of TF binding sites, are critical for driving the formation of condensates containing TFs and co-activators (Boija et al., 2018; Shrinivas et al., 2019). Together, specific structured interactions (TF-DNA) and weak multi-valent interactions (TF-coactivator) drive condensation at particular loci to regulate transcriptional activities in cells (Shrinivas et al., 2019).

Mediator functions as a co-activator that stabilizes pre-initiation complexes (PICs) *in vitro* (Kornberg, 2005). Structural analysis showed that Mediator contains two conserved modules (“head” and “middle”), which contact Pol II and the initiation factors TFIIB and TFIIF (Nozawa et al., 2017; Plaschka et al., 2015; Tsai et al., 2017), whereas the “tail” module binds the ADs of TFs, and the dissociable kinase module is implicated in repression (Jeronimo and Robert, 2017). Live-cell super-resolution and light-sheet imaging showed that Mediator and Pol II each form small transient clusters in murine embryonic stem cells (Cho et al., 2018). They colocalize in phase-separated condensates at super enhancers (SEs), in a manner that is sensitive to transcription inhibitors. Based on this, a dynamic “kissing” model was proposed, in which large clusters of Mediator, recruited by TFs at SEs, dynamically interact with large Pol II clusters in transcriptional condensates to regulate SE-controlled genes (Cho et al., 2018). MED1 and BRD4 also localize in transcriptional condensates at SEs in mESCs (Sabari et al., 2018), and disruption of the condensates by 1,6-hexanediol lead to a decrease of chromatin-bound MED1 and BRD4, as well as a reduction in the level of Pol II at SEs and their regulated genes. *In vitro* assays showed that the IDRs of both MED1 and BRD4 not only form phase-separated droplets, but the MED1-IDR droplets also incorporate BRD4 and Pol II from transcription-competent nuclear extracts. All these results indicate that coactivators form condensates and concentrate the transcription machineries at specific loci through phase separation of IDRs in TFs and coactivators (Sabari et al., 2018).

For PIC formation, TF and co-activator condensates dynamically engage with Pol II and general initiation factors, which are recruited to core promoter regions. The Pol II CTD (C-terminal domain) can also phase-separate by itself in the

presence of crowding agents *in vitro* (Boehning et al., 2018). This suggests that the CTD is likely to be a client of promoter condensates to facilitate the recruitment of Pol II to active genes (Lu et al., 2019). A key function of the PIC is to open the DNA at the transcription start site (TSS). In the Pol I and Pol III systems, DNA is opened spontaneously via binding energy alone (Cramer, 2019), while DNA opening by Pol II generally requires an additional DNA translocase, XPB (Cramer, 2019; Kim et al., 2000). Transcription begins after opening of the double-stranded TSS DNA.

For effective communication, enhancers and promoters must be in close physical proximity. This may require dynamic chromatin interactions, which often involve histone modifications and chromatin remodeling to facilitate the loading of RNA polymerases (Haberle and Stark, 2018; Müller and Tora, 2014). Nucleosome shifts or depletion at enhancers and promoters enables the binding of TFs and Pol II (Core et al., 2014; Neil et al., 2009), which allows transcription to proceed in both directions. A recent study indicated that phase separation is a potential mechanism for forming open chromatin (Gibson et al., 2019). Nucleosomal spacing and acetylation of histone tails are both possible mechanisms for tuning the biophysical properties of chromatin droplets in cells under physiological conditions. Further investigations are required to determine whether the chromatin remodelers trigger LLPS and form condensates to regulate transcription initiation.

After initiation, the transcription activity of many eukaryotic genes is determined by the abundance of poised Pol II at the promoter-proximal regions. Condensates containing the positive transcription elongation factor b complex (P-TEFb), which includes the kinase CDK9 and the cyclin T1, are crucial for transcriptional pause release (Gressel et al., 2019; Lu et al., 2018). The phosphorylated CTD of Pol II can also be incorporated into a condensate formed by elongation factors and splicing factors, which is distinct from promoter condensates (Kwon et al., 2013; Lu et al., 2018).

Phase separation in asymmetric neural stem cell division

As the most complicated organ in human beings, the brain consists of billions of neurons and glial cells, with hundreds of different subtypes. Despite the diversity, neural cells are all highly differentiated and originate from a small pool of neural stem cells called radial glial (RG) cells. In addition to dividing symmetrically to expand the stem cell pool, RG cells and their progenitors can also undergo asymmetric cell division (ACD) to generate diverse neurons and glia (Knoblich, 2008). Essential for multicellular organisms to produce daughter cells with distinct fates, ACD can be induced by an asymmetric environmental niche or asymmetric in-

trinsic cues (Gönczy, 2008; Venkei and Yamashita, 2018). Through unequal segregation of cellular components, including membrane-attached cell fate determinants, organelles or small molecules, the two generated daughter cells will be endowed with different components and functions.

Cell fate determinant-mediated ACD of neural stem cells

During ACD of *Drosophila* neural stem cells neuroblasts (NBs), several evolutionarily conserved proteins are concentrated at restricted regions of the cell cortex during mitosis, followed with their unequal segregation after division (Knoblich, 2008). On the onset of mitosis, the conserved Par complex, which consists of Bazooka (Par3 in mammals), Par6 and atypical protein kinase C (aPKC), forms a condensed crescent on the apical cortex. It is proposed that embryonic NBs inherit the apically localized Par complex from the neurogenic ectoderm, whereas Par proteins in larval NBs are evenly distributed in the cell cortex or cytoplasm in interphase, and accumulate apically during mitosis in a manner that depends on Aurora A activity (Knoblich, 2010; Wirtz-Peitz et al., 2008). Apically localized Par complex then recruits the hub protein Partner of inscuteable (Pins) via the adaptor Inscuteable (Insc) (Schober et al., 1999; Wodarz et al., 1999; Zhu et al., 2011b).

Cell fate determinants Numb, Prospero (Pros), and Brain tumor (Brat) also display a cell cycle-dependent dynamic distribution pattern, and are basally localized in mitosis. Numb and the adaptors Partner of Numb (Pon) and Miranda (Mira) are all membrane-bound proteins and are uniformly cortical in interphase larval NBs. From prophase, these proteins at the apical cortex are phosphorylated by activated aPKC, which disrupts their membrane-binding property and results in their detachment from the apical cortex (Atwood and Prehoda, 2009; Wirtz-Peitz et al., 2008). In addition to this aPKC-mediated apical preclusion, other regulators such as the actomyosin network and *mira* mRNA further promote the basal enrichment of Mira in mitosis (Hannaford et al., 2018; Ramat et al., 2017). Basal localization of Pon requires coordinated phosphorylation by Cdk1 and Polo (Wang et al., 2007; Zhu et al., 2016a). Pros and Brat are then recruited to the basal cortex by interacting directly with the cargo-binding domain of Mira (Ikeshima-Kataoka et al., 1997; Jia et al., 2015; Shen et al., 1997), whereas Numb is basally anchored together with Pon (Lu et al., 1998; Wang et al., 2007). Thus, in mitosis of asymmetrically dividing *Drosophila* NBs, two sets of proteins differentially accumulate at the cell cortex, to set up an apical-basal polarity (Figure 4) (Wen and Zhang, 2018).

To facilitate unequal segregation of the basally localized cell fate determinants into only one of the two daughter cells, the mitotic spindle of a *Drosophila* NB then rotates 90 degrees along the apical-basal axis, which results in the cell

cleavage plane being perpendicular to the cell polarity axis (Figure 4) (Siller and Doe, 2009). The two daughters thus have distinct fates: the apical one receives the Par complex and remains as an NB, while the basal one possesses cell fate determinants and subsequently divides to produce two terminally differentiated neurons. Two redundant pathways both assembled by the apically localized Pins (Pins/Gai/Mud/dynein and Pins/Gai/Dlg/Khc73) have been found to achieve spindle orientation (Izumi et al., 2006; Nipper et al., 2007; Siegrist and Doe, 2005; Siller et al., 2006; Zhu et al., 2011a; Zhu et al., 2011b).

In summary, the cell-fate determinant-mediated ACD of *Drosophila* NBs requires the coordinated accumulation of polarized proteins (polarity proteins and cell fate determinants) at two edges of a cell, accompanied by mitotic spindle rotation along the cell polarity axis, to ensure the inheritance of fate determinants by only one of two daughters (Gönczy, 2008; Knoblich, 2008; Siller and Doe, 2009; Wen and Zhang, 2018). Moreover, a few studies have proposed that the conserved polarity proteins and cell fate determinants may play a similar role in driving ACD of mammalian neural stem cells/progenitors (Schwamborn et al., 2009; Shi et al., 2003; Zhong et al., 1996).

LLPS in driving the local condensation of cortex proteins

An intriguing phenomenon observed during ACD of *Drosophila* NBs is that although the Par complex and cell fate determinants emerge on the apical or basal half of the cell membrane, respectively, at the onset of mitosis, they are gradually concentrated into highly condensed crescents at the two edges of the cell at metaphase (Betschinger et al., 2006; Ikeshima-Kataoka et al., 1997; Knoblich et al., 1995; Schober et al., 1999; Shen et al., 1997; Wodarz et al., 1999). From anaphase, the condensed apical and basal crescents begin to expand throughout the cell cortex, and become uniformly diffuse on the whole plasma membrane of the apical and basal daughter cells, respectively, after cytokinesis. It is largely a mystery how the proteins are recruited into these very limited membrane regions rather than being evenly distributed throughout the apical or basal half of the cell cortex. A couple of studies suggest that Actin cytoskeleton-dependent cortical flows may play a role in condensing and separating the apical Par complex crescent (Oon and Prehoda, 2019; Wang et al., 2017b). However, other studies on the basal proteins Mira and Pon argue against their passive flow in the cortex. FRAP analysis of GFP-Mira or GFP-Pon showed that the bleached GFP signals recovered in a few minutes, with proteins coming from the cytoplasm (which has a low protein concentration) but not the crescent (which has a high protein concentration) (Erben et al., 2008; Lu et al., 1999; Mayer et al., 2005). These experiments reveal the constant exchange of Mira and Pon between the condensed

crescent and the cytoplasm. This interesting phenomenon surely cannot be explained by passive diffusion.

LLPS-mediated basal condensation of the cell fate determinant Numb

A recent study combining both *in vitro* and *in vivo* investigations of the basal proteins Numb and Pon suggested that LLPS could be a potential driving force for polarized condensation of Numb (Figure 4) (Shan et al., 2018), thus bringing the concept of protein phase separation into the regulation of ACD. As described above, during ACD of *Drosophila* NBs, Numb is recruited to the basal cortex and preferentially segregated into the basal daughter cell together with the adaptor protein Pon. It was found that the phosphotyrosine-binding (PTB) domain of Numb specifically interacts with three repeating motifs in the N-terminus of Pon in a non-canonical manner, which may lead to the formation of a heterogeneous, complex Numb/Pon interaction network. *In vitro*, the multivalent Numb/Pon interaction network appears as numerous spherical liquid droplets that quickly fuse into larger ones, a signature phenomenon of LLPS (Figure 4) (Shan et al., 2018). In those droplets, both Numb and Pon are highly enriched, and when co-expressed in living cells, GFP-Pon and mCherry-Numb autonomously form colocalized, highly enriched puncta. Structural studies of the Numb/Pon interaction led to the design of different Pon mutants which are impaired to different degrees in Numb binding and LLPS. In dividing fly NBs expressing these Pon mutants, the efficiency of endogenous Numb basal condensation and the NB over-proliferation phenotype are perfectly correlated with the LLPS ability of the Pon mutant proteins (Shan et al., 2018). This provides evidence for a strong connection between Numb-Pon interaction-induced LLPS and Numb basal localization and asymmetric segregation. An important finding of the study is that there is dynamic exchange of proteins between the Numb/Pon complex in either droplets or puncta and the surroundings, which is reminiscent of

previous findings that these proteins are constantly cycling between the crescent and the cytoplasm during ACD of *Drosophila* NBs (Lu et al., 1999; Mayer et al., 2005). Moreover, LLPS of the Numb/Pon complex has been proved to be a reversible process, and a preformed Numb/Pon liquid phase can be reversed back to the solution state by competitive binding of a Pon peptide that interacts with the Numb PTB (Shan et al., 2018). Interestingly, this “reversing” Pon peptide is one of the three repeating motifs within Pon that is required for LLPS with Numb. The switch from the LLPS-promoting peptide to the reversing peptide seems to be regulated by Cdk1-mediated phosphorylation of Pon (Shan et al., 2018). Cdk1 can phosphorylate Thr63 of Pon *in vitro* (Zhu et al., 2016b), which dramatically enhances the ability of this phospho-Pon peptide to bind Numb. As the multivalent interactions between Numb and the Pon repeating motifs (without modifications) are all weak, the presence of a strong phospho-Pon peptide is expected to disrupt the multivalency that is required for LLPS. However, this experiment was only done *in vitro*, and further investigations are required to determine whether Cdk1 indeed acts as a transition switch to initiate the dispersion of the Numb crescent after anaphase.

LLPS in the localization of polarity proteins

Several other studies have suggested that the apical Par proteins might also exist as a liquid-like state in asymmetrically dividing *Drosophila* NBs. Rapid imaging of larval NBs entering mitosis revealed that aPKC and Bazooka (Baz) form discrete cortical foci, which then fuse with each other to grow into larger patches, which coalesce into an apical cap at metaphase (Oon and Prehoda, 2019). The apical cap then disassembles into spreading cortical patches from anaphase, which finally dissipate at the end of mitosis. The direction of cortical flows during mitosis was suggested to play an important role in regulating the assembly and disassembly of the apical Par cap (Oon and Prehoda, 2019). Another study

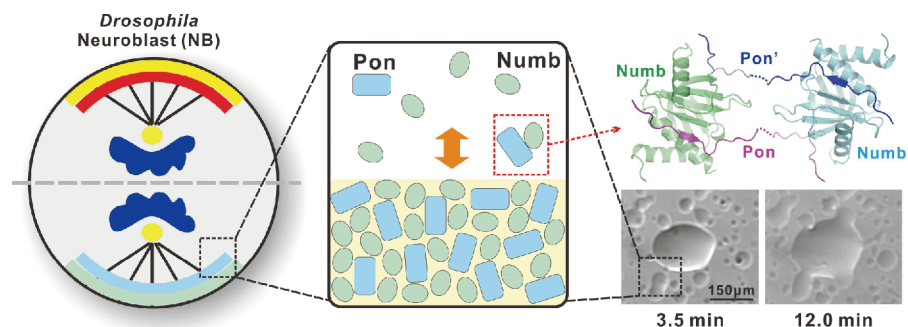


Figure 4 LLPS-mediated basal condensation of the cell fate determinant Numb during ACD of *Drosophila* NBs. In mitotic cells, the Baz/Par6/aPKC complex (yellow) forms a condensed crescent apically with Insc, Pins and Gai (red), whereas the cell fate determinants Numb, Pros and Brat (green) and their adaptors Pon and Mira (cyan) concentrate basally. The specific and multivalent interaction between Numb and Pon leads to LLPS of the Numb-Pon complex, thus driving their basal condensation. The right panel shows the interaction between Numb PTB and one Pon repeating motif (top) and phase-separated droplets formed by Numb PTB and a Pon fragment containing three repeating motifs *in vitro*.

showed that overexpression of Baz induces cortical condensation of the Par complex at interphase in non-polarized *Drosophila* S2 cells (Kono et al., 2019). The cytoplasmic Par proteins first emerge as cortical dots, which then grow into larger patches, and the Par patches undergo continuous fusion and fission. Par complex is also observed as apical patches in *Drosophila* mitotic NBs (Kono et al., 2019). A recent study on *C. elegans* embryonic polarization observed a similar cortical clustering pattern of Par proteins, which occurs by advection as a result of cortical flows (Wang et al., 2017b). The Par clusters exhibit liquid-like behavior in the form of constant fusion and fission. However, no validation has been provided to prove that the Par complex indeed undergoes LLPS *in vitro*.

When considering the mechanisms that generate cell polarity, there are three reasons why the LLPS theory seems to fit better to *in vivo* observations than previous passive diffusion and membrane-anchoring models: (1) the liquid droplets that form autonomously from specific components *in vitro* highly resemble the spherical protein puncta *in vivo*; (2) biomolecular LLPS maintains the dynamic shuttling of apical and basal proteins between the highly enriched crescents (in open contact with cytoplasm) and the cytoplasm without membrane-mediated separation; and (3) LLPS enables rapid assembly/disassembly of the polarized protein condensates during ACD in response to specific cell cycle-dependent cues. In addition to Numb/Pon, most apical and basal proteins are multi-domain proteins that have the potential to build into high-order protein-protein interaction networks. Does such multivalency lead to cell cycle-regulated liquid condensation of those proteins at the cortex? Are the localized concentrations of those proteins high enough to enable autonomous LLPS *in vivo*? How do the protein droplets interact with cortical flows to achieve temporal assembly and disassembly of protein condensates at the two poles of a cell? Further investigations will help us to understand the mechanisms underlying diverse cell polarity-related processes.

Phase separation-mediated formation of pre- and postsynaptic density signaling assemblies

A human brain contains ~85 billion neurons wired together to form an incredibly complex network that can perform remarkably versatile tasks. Neurons take cellular compartmentalization to extremes due to their unusual morphologies. In addition to the membrane-enclosed organelles and membraneless condensates common to other cell types, neurons contain a unique type of compartment that is semi-enclosed in membrane and known as the synapse. Synapses are the sites where two neurons physically connect and communicate with each other, and they are the most basic unit of the

brain network. Each synapse is formed by thousands of proteins and can change its composition and signal processing capacities in response to various stimuli. Thus, synapses are dynamic micro-computational devices. A human brain contains $\sim 10^{15}$ synapses, and each synapse occupies a volume of $\sim 1.0 \mu\text{m}^3$ or less. Underneath the postsynaptic plasma membrane of each synapse resides a condensed protein-rich sub-compartment known as the postsynaptic density (PSD), a structure responsible for receiving, amplifying, and storing signals initiated by presynaptic cells. A very large fraction of neuronal psychiatric disorders is believed to be caused by mutations of genes encoding synaptic proteins (e.g., $\sim 1/3$ of autism-related genes encode synaptic proteins; <https://gene.sfari.org/database/human-gene/>). A major challenge in basic as well as clinical research is to understand how these molecules participate in normal brain functions and why their mutation-induced alterations may cause brain disorders. Interestingly, the assembly of PSDs has key features in common with the formation of membraneless condensates: proteins in PSDs are highly concentrated; PSD condensates can grow or shrink; and components within the condensed PSDs are mobile and can exchange with the corresponding molecules in the dilute cytoplasm of dendritic spines.

A recent study of the interaction between PSD-95 and SynGAP provided the first hint suggesting that PSDs may form via phase separation (Zeng et al., 2016). SynGAP, which catalyzes the conversion of small G proteins such as Ras and RAP from their GTP-bound forms to the GDP-bound forms, serves as an inhibitory factor for synaptic activities (Araki et al., 2015; Chen et al., 1998; Kim et al., 1998; Pena et al., 2008; Vazquez et al., 2004; Zeng et al., 2017). *SynGAP* mutations can cause epilepsy, intellectual disability and autism (Clement et al., 2012; Vazquez et al., 2004). Unlike weak interactions mediated by IDRs/LCRs, the interaction between SynGAP and PSD-95 is mediated by a highly specific and strong interaction between an elongated PDZ binding motif in SynGAP and extended PDZ domain from PSD-95 (Zeng et al., 2016). PSD-95 and SynGAP, either as purified proteins mixed in test tubes or as proteins co-expressed in cells, undergo phase separation (Zeng et al., 2016). It was shown that trimerization of SynGAP via its coiled-coil domain is absolutely required for the PSD-95/SynGAP complex to undergo phase separation (Zeng et al., 2016), consistent with the multivalent interaction-mediated phase separation model for biological systems (Li et al., 2012). Remarkably, a SynGAP mutant, which retains its binding to PSD-95 but is a monomer, is incapable of forming condensates with PSD-95 in test tubes and down-regulating synaptic activities in cultured neurons (Zeng et al., 2016). This observation indicates that a SynGAP mutation leading to defective phase separation of the SynGAP/PSD-95 mixture, but not impairment of the direct interaction between

SynGAP and PSD-95, may be an underlying mechanism for SynGAP mutation caused psychiatric disorders.

PSD assembly can be reconstituted using four purified PSD scaffolding proteins, PSD-95, GKAP, Shank, and Homer (Zeng et al., 2018). When mixed at ratios according to those derived from different synaptic proteome quantification methods, the mixtures all undergo phase separation to form spherical droplets with all four proteins co-condensed. Importantly, phase separation of the mixture containing the four scaffold proteins occurs at individual protein concentration as low as $1 \mu\text{mol L}^{-1}$, which is well below the concentrations of these proteins in synapses (Lowenthal et al., 2015; Sheng and Hoogenraad, 2007; Ting et al., 2012). The multivalent interactions with high specificity and quite strong affinities “cross-link” the PSD scaffold proteins into a large protein network, and such multivalent interactions are critical for the phase separation and co-condensation of the 4× PSD mixture. The condensates formed by the four scaffold proteins are able to recruit and co-condense SynGAP and the tail of multimerized NR2B, the cytoplasmic tail of the the NMDA receptor 2B subunit. Thus, the PSD scaffold condensates can cluster receptors and concentrate enzymes, which are two hallmark features of scaffold proteins in synapses (Feng and Zhang, 2009; Zhu et al., 2016b). The PSD assemblies have also been demonstrated to undergo phase separation beneath the synaptic plasma membranes. The NR2B tail is first tethered to supported lipid bilayers (SLBs) and then the synaptic scaffold proteins and SynGAP are added to the SLBs (Zeng et al., 2018). Interestingly, reconstituted PSD assemblies on SLBs also undergo phase separation. The concentration threshold for the PSD protein mixture to undergo phase separation on 2D SLBs is much lower than in 3D solution. The PSD condensates display a web-like structure on SLBs due to phase separation mediated by a rapid demixing process known as spinodal decomposition. The PSD condensates formed in solution on SLBs can be dispersed by adding increasing amounts of Homer 1a, which is the monomeric splicing isoform of Homer 1c and known to be capable of down-regulating PSD sizes and synapse formation in living neurons. This reveals that the structure of the PSD is regulated by phase separation-mediated formation of protein condensates. Phase separation-mediated formation of the excitatory PSD condensates is highly specific, as the inhibitory PSD master scaffold protein Gephyrin is actively repelled from the excitatory PSD condensates (Zeng et al., 2018). This implies that different membraneless compartments can co-exist in a very small region, such as within a dendritic spine protrusion. The above findings provide quite compelling evidence showing that phase separation is likely to be an effective means for forming highly condensed and very dynamic PSD assemblies in synapses (Figure 5A).

Using the above-mentioned reconstituted PSD system,

Zeng et al. tested whether phase separation-mediated PSD complex formation can modulate synaptic clustering of the AMPA receptor (AMPA) and signal transmission in a more physiologically relevant system. They discovered that transmembrane AMPAR regulatory proteins (TARPs), which are a family of auxiliary subunits of AMPARs critical for the trafficking and transmission of the ion channel in synapses, are clustered in the PSD condensates via phase separation (Zeng et al., 2019). The entire C-terminal tail of each TARP binds to the PDZ12 tandem of PSD-95 with high specificity and a multivalent fashion. The finding answered the long sought-after question on the mechanistic basis for the specific functional interactions between TARPs and membrane associated guanylate kinase family scaffold proteins including PSD-95 in synapses. Using a unique experimental system in which tethered GluA1-TARP γ 8 was expressed in organotypic mouse hippocampal slices with *Gria1-3* knocked out, the authors showed that the multivalent TARP/PSD-95 interaction is essential for clustering of TARPs into the PSD condensates and for AMPAR synaptic transmission *in vivo* (Zeng et al., 2019) (Figure 5B). Mutations of the TARP C-terminal tail that impair TARP and PSD assembly co-phase separation also impair AMPAR synaptic transmission and long-term potentiation of hippocampal neurons. Therefore, phase separation-mediated PSD assembly formation and regulation are linked with the physiological functions of synapses.

Excitatory synapses of central nervous systems are formed by precise juxtaposition of a presynaptic active zone from one neuron with a postsynaptic density from another. Under electron microscope, an active zone contains a layer of electron dense materials beneath the plasma membranes (Couteaux and Pecot-Dechavassine, 1970), and these electron dense materials are believed to be densely packed proteins (Südhof, 2012). The active zones play critical roles in docking and priming readily releasable synaptic vesicles, clustering and positioning of voltage-gated Ca^{2+} channels (VGCCs) at sub-regions of the presynaptic active zone membrane to regulate both the speed and strength of neurotransmitter releases (Südhof, 2012). Analogous to PSDs, presynaptic densities are enriched in scaffold proteins including RIM, RIM-BP, ELKS, Liprin, Munc13, and CASK (Südhof, 2012). Genetic studies have demonstrated that removal of RIM/RIM-BP or RIM/ELKS in mice lead to disappearance of the dense projection structures of active zones and severely impaired neurotransmitter release (Acuna et al., 2016; Wang et al., 2016a). VGCCs are also clustered on the active zone membranes and positioned near the synaptic vesicle (SV) fusion sites to regulate both the speed and strength of neurotransmitter release induced by action potentials (Biederer et al., 2017; Miki et al., 2017; Nakamura et al., 2015; Südhof, 2012; Tang et al., 2016). Using an *in vitro* reconstitution approach, Wu et al. recently demonstrated that

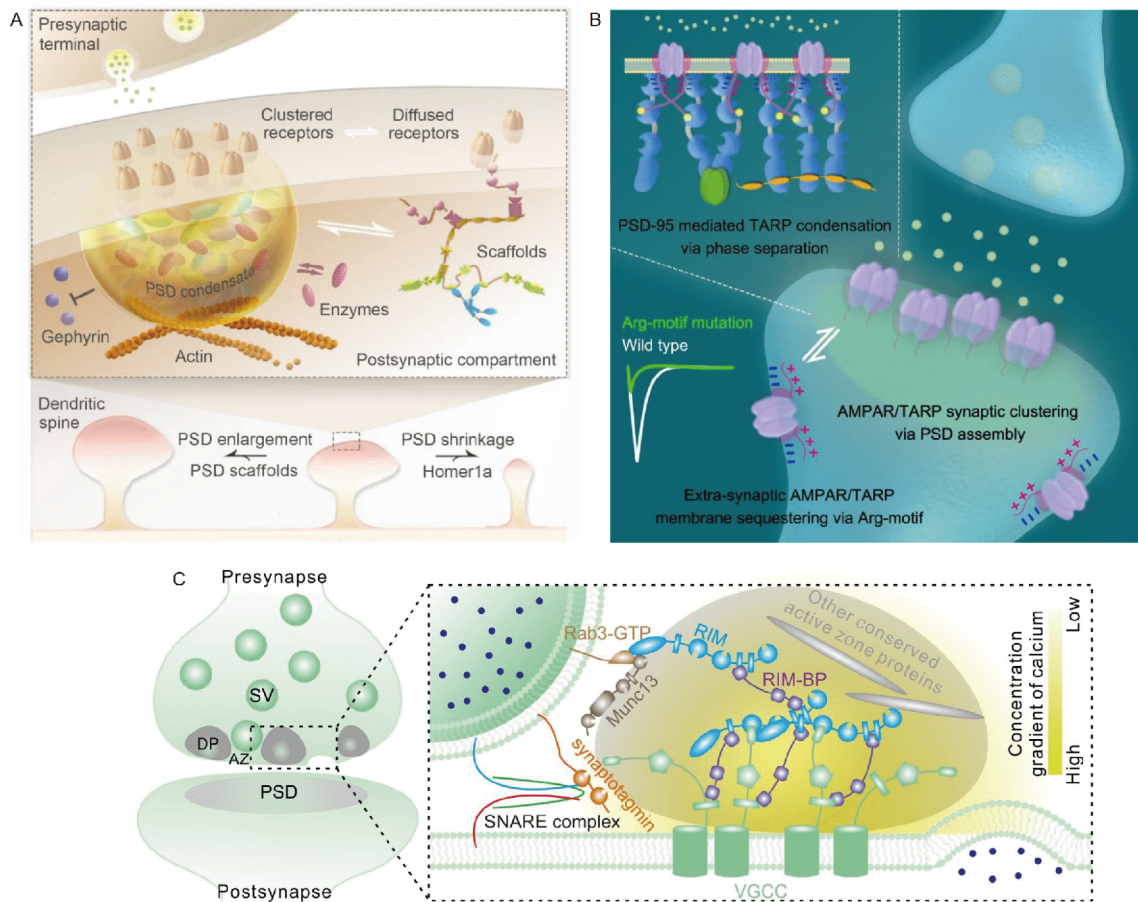


Figure 5 Assembly of pre- and post-synaptic density signaling complexes via liquid-liquid phase separation. A, A diagram showing that the postsynaptic protein complex is likely formed via phase separation-mediated assembly of multiple proteins (adapted from Zeng et al., 2018). B, Role of phase separation-mediated condensation of the TARP/PSD-95 complex in AMPA Receptor (AMPA) synaptic transmission (adapted from Zeng et al., 2019). C, Formation of active pre-synaptic protein condensates via phase separation (adapted from Wu et al., 2019a).

the purified RIM and RIM-BP proteins, when mixed at physiological protein concentrations, underwent phase separation both in 3D solution and on SLBs (Wu et al., 2019a). Like PSD scaffold proteins, the interaction between RIM and RIM-BP is also multivalent, and such multivalent interactions are required for the phase separation of the complex. Interestingly, the cytosolic tail of VGCCs can be recruited to the RIM/RIM-BP condensates via direct binding of the Ca^{2+} channel tail to both RIM and RIM-BP, resulting in a massive enrichment of the channel. The density of VGCCs in the condensed phase on SLBs was quantified by the authors and estimated to be similar to the density of $\text{Ca}_v2.1$ channel in synapses measured by an immuno-EM-based method (Nakamura et al., 2015; Wu et al., 2019a). The finding by Wu et al. is consistent with the concept that fast and accurate neurotransmitter release is supported by both the density of clustered VGCCs on presynaptic plasma membranes and the proximity of the clustered VGCCs to calcium sensors at the SV fusion sites (Eggermann et al., 2011; Südhof, 2013) (Figure 5C). Finally, Milovanovic et al. showed that Synapsin I, a very abundant presynaptic scaffold protein es-

essential for maintaining the vast reserve pool of SVs in synaptic boutons, can co-phase separate with SVs (Milovanovic et al., 2018), thus maintaining the stability of the reserve pool SVs and possibly priming these vesicles for being transported to the release sites upon arrival of action potentials.

Phase separation and transition in specifying autophagic degradation of protein aggregates

The autophagy pathway in multicellular organisms

Autophagy refers to a process involving the sequestration of cytoplasmic contents in a double-membrane autophagosome and its delivery to vacuoles (yeast) or lysosomes (multicellular organisms) for degradation (Feng et al., 2014). In multicellular organisms, autophagy induction triggers the initiation and nucleation of a cup-shaped isolation membrane (IM) in the vicinity of the endoplasmic reticulum (ER). IMs further expand and close to form the autophagosome (Feng et al., 2014; Zhao and Zhang, 2018). Nascent autophagosomes

become gradually acidified by fusing with vesicles originating from endolysosomal compartments to form amphisomes, a process known as autophagosome maturation. The amphisomes eventually fuse with lysosomes to form degradative autolysosomes (Zhao and Zhang, 2019a) (Figure 6A). In response to a variety of stresses such as energy deprivation, autophagy non-selectively removes a portion of cytosol to provide energy and building blocks for cell survival (Levine and Kroemer, 2008). Protein aggregates formed by misfolded or unnecessary proteins can also be selectively removed by autophagy to maintain cellular homeostasis, a process referred to as aggrephagy (Xie and Klionsky, 2007; Stolz et al., 2014). Dysfunction of autophagy has been associated with a variety of human diseases, including neurodegeneration, cancer and immunological disorders (Levine and Kroemer, 2008; Xie and Klionsky, 2007; Zhao and Zhang, 2019b).

A set of autophagy related genes encode ATG proteins that act at different steps of autophagosome formation (Feng et al., 2014; Zhao and Zhang, 2018). In mammalian cells, the initiation of autophagosome formation involves the hierarchical recruitment of ATG proteins, beginning with the FIP200/ULK1 complex, to the ER to generate PI(3)P-enriched subdomains called omegasomes. IMs, whose initial origin remains elusive, are nucleated near omegasomes.

Several ATG proteins are localized on the IM during its expansion, such as the WD40-repeat-containing PI(3)P-binding protein WIPI2 (one of four mammalian Atg18 homologs) and proteins involved in the lipidation of ATG8 homologs (Feng et al., 2014; Zhao and Zhang, 2018). A set of metazoan-specific autophagy genes, known as *Epg* genes, act at steps unique to autophagy in multicellular organisms (Tian et al., 2010; Zhao and Zhang, 2018). For example, EPG-3/VMP1 modulates the dynamics of IM-ER membrane contacts, whose formation is essential for IM expansion (Zhao et al., 2017). EPG-5 acts as a tether protein to determine the fusion specificity between autophagosomes and late endosomes/lysosomes (Wang et al., 2016b).

Phase separation drives the formation of p62-polyubiquitinated protein condensates

Misfolded proteins are constitutively generated by stresses such as hypoxia and hyperthermia, a decline in protein folding capability, mutations in the protein, and protein misfolding (Alberti and Hyman, 2016; Dobson, 2004). Cells have evolved quality control mechanisms to detect misfolded proteins and prevent them from accumulating. Molecular chaperones recognize proteins with aberrant conformations by binding to their ectopically exposed hydrophobic re-

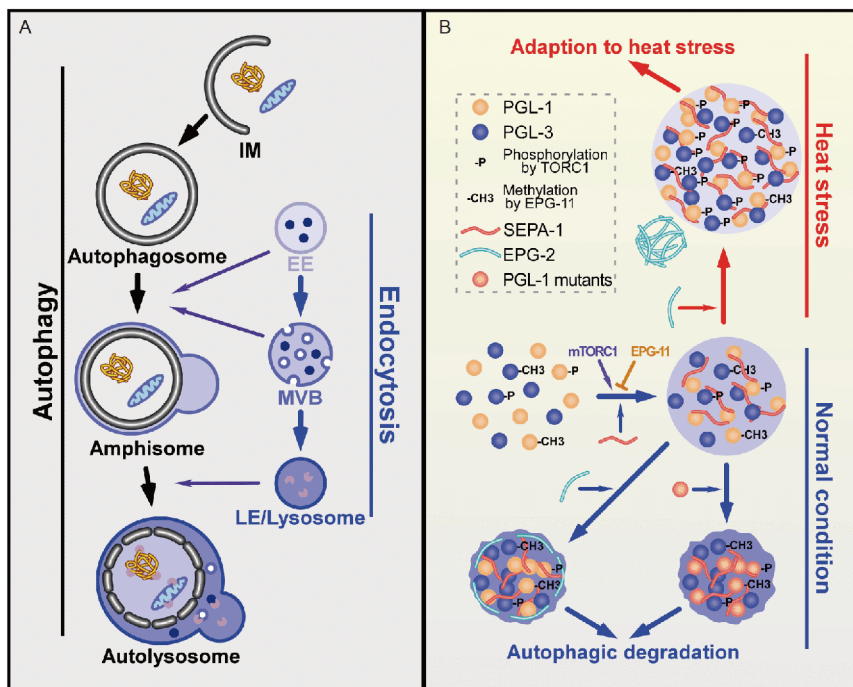


Figure 6 Phase separation and transition specify autophagic degradation of PGL granules. A, The autophagy pathway in multicellular organisms. Nascent autophagosomes fuse with vesicles derived from the endolysosomal compartments to form amphisomes, which further proceed into degradative autolysosomes. IM, isolation membrane; EE, early endosome; MVB, multivesicular body; LE, late endosome. B, LLPS-mediated assembly of PGL granules. PGL-1 and PGL-3 are post-translationally modified by EPG-11 and mTORC1. The receptor protein SEPA-1 mediates aggregation of PGL-1 and PGL-3, which is also modulated by PTMs. The scaffold protein EPG-2 or a gelation mutant of PGL-1 promotes the transition of PGL granules into a gel-like state, which is essential for their autophagic degradation. Under heat stress conditions, assembly of PGL granules is promoted, while levels of EPG-2, which undergoes normal autophagic degradation, are not sufficient to make PGL granules amenable to autophagic degradation.

sidues, and assisting them to refold (Dobson, 2004). The misfolded proteins can also undergo ubiquitination and subsequent degradation by the proteasome pathway (Soto, 2003; Stefani and Dobson, 2003). Misfolded proteins are also prone to oligomerize and further assemble into protein aggregates, which can be selectively recognized by the autophagic machinery (Stolz et al., 2014). Small oligomer forms of misfolded proteins or other states of protein aggregates such as amyloid-like fibrils cause cytotoxicity (Aguzzi and O'Connor, 2010; Soto, 2003; Stefani and Dobson, 2003).

Recognition and clearance of protein aggregates by autophagy require a family of cargo-specific receptor proteins, which bind simultaneously to protein aggregates (cargos) and autophagic machineries, such as ATG8 family members (Stolz et al., 2014). The receptor protein self-oligomerizes and also recruits protein cargos into aggregates (Stolz et al., 2014). p62, a member of the p62/sequestosome 1 (SQSTM1) family, is one of the best characterized receptors mediating autophagic degradation of aggregates formed by misfolded protein (Komatsu et al., 2007). p62 consists of an N-terminal PB1 oligomerization domain and a C-terminal ubiquitin-associating (UBA) domain (Bjørkøy et al., 2005; Pankiv et al., 2007). p62 recognizes the polyubiquitin chains conjugated on cargo proteins, mediating their aggregation (Pankiv et al., 2007). p62 also contains an LC3-interacting region (LIR), which binds to LC3 (mammalian ATG8 homolog) on the inner surface of IMs, therefore linking the cargo to autophagic structures (Bjørkøy et al., 2005; Pankiv et al., 2007). Excess or unnecessary proteins can also be modified by polyubiquitin and undergo p62-mediated autophagic degradation. For example, Dishevelled undergoes polyubiquitin modification and binds to p62 for autophagic degradation, thus controlling the activity of Wnt signaling (Gao et al., 2010).

Recent studies indicate that assembly of p62-polyubiquitinated protein aggregates is mediated by LLPS. Separately, p62 and polyubiquitin chains fail to undergo LLPS, but they form phase-separated condensates when mixed *in vitro* (Herhaus and Dikic, 2018; Sun et al., 2018; Zaffagnini et al., 2018). The p62 condensates are spherical in shape and able to fuse with each other both in an *in vitro* LLPS system and in living cells. LLPS of p62-polyubiquitin chain condensates is driven by multivalent interactions. Deletion of the PB1 domain in p62 or mutation of M404 in the UBA domain, which reduces the affinity of p62 for ubiquitin, abolishes phase separation of p62-polyubiquitinated proteins (Sun et al., 2018). The critical concentration for LLPS decreases when the length of polyubiquitin chain increases (Sun et al., 2018). Fluorescence recovery after photobleaching (FRAP) assays revealed that the mobility of p62 in droplets is low, while polyubiquitin chains exhibit much higher mobility (Sun et al., 2018). LLPS of p62 condensates is modulated by

PTMs. Phosphorylation of S403 and S409 in the UBA domain of p62 by TBK1 or casein kinase 2 (CK2) promotes formation of p62 condensates, as does ubiquitination on K420; in contrast, ubiquitination on K7 in the PB1 domain inhibits the condensate formation (Danieli and Martens, 2018; Sun et al., 2018; Zaffagnini et al., 2018). Disease-related mutations in p62 also affect LLPS of p62 condensates. The M404T and G411S mutations in p62 found in Paget's disease of bone (PDB) inhibit formation of p62 condensates (Sun et al., 2018). NBR1, another member of the p62/SQSTM1 family that interacts with p62, decreases the critical concentration for p62 to undergo LLPS (Zaffagnini et al., 2018). Phase separation-mediated assembly of p62-polyubiquitinated proteins is crucial for the autophagic degradation of misfolded and unnecessary proteins.

Phase separation and transition determining autophagic degradation and stress adaptation of PGL granules

During *C. elegans* embryogenesis, specialized ribonucleoprotein complexes derived from the oocyte, known as P granules, are exclusively localized in germ cell blastomeres during early asymmetric divisions (Strome, 2005). P granules are perinuclearly localized and associate with the nuclear pore complex. P granules serve as the sites for piRNA-mediated surveillance of germline-specific genes and also for the residence of factors essential for transgenerational small RNA inheritance (Almeida et al., 2019). P granules are assembled via liquid-liquid phase separation and possess liquid-like properties (Brangwynne et al., 2009). In the newly fertilized *C. elegans* embryo, P granules are evenly distributed. During the first several rounds of asymmetric divisions separating somatic and germline blastomere cells, P granules that are segregated into the somatic blastomeres become disassembled and degraded (Strome, 2005). The P granule components PGL-1 and PGL-3 are removed by autophagy (Zhang et al., 2009). In autophagy mutant embryos, PGL-1 and PGL-3 accumulate into a large number of aggregates in somatic cells, named PGL granules (Zhang et al., 2009).

The *C. elegans* embryo is enclosed by a hard egg shell and its development is independent of external nutrients. Autophagy activity occurs at a basal level during embryogenesis. The size of autophagosomes is also relatively fixed. The level of diffuse PGL-1 and PGL-3 proteins in somatic cells gradually decreases due to their removal by autophagy. To ensure complete removal of diffuse oocyte-derived PGL-1 and PGL-3 proteins from somatic cells, the assembly of PGL granules amenable for autophagic degradation has to be tightly controlled. Genetic screens revealed that removal of PGL granules in somatic cells requires the activity of the receptor protein SEPA-1, the scaffold protein EPG-2 and multiple PTMs (Li et al., 2013; Tian et al., 2010; Zhang et al.,

2009; Zhang et al., 2018a). The self-oligomerized SEPA-1 protein is required for formation of PGL granules. In *sepa-1* mutant embryos, PGL-1 and PGL-3 fail to be degraded and are diffusely localized in the cytoplasm. SEPA-1 simultaneously binds to PGL-3 and LGG-1, the *C. elegans* ATG8 homolog (Zhang et al., 2009). EPG-2 also self-oligomerizes and binds to SEPA-1 and LGG-1. In *epg-2* mutant embryos, PGL/SEPA-1 granules accumulate and are separated from LGG-1-positive autophagic structures (Tian et al., 2010). Both SEPA-1 and EPG-2 are zygotically synthesized and display a specific temporal expression pattern: expression levels are low at early embryonic stages, become strong at the 100 cell to comma stage, and then decrease in late stage embryos. SEPA-1 and EPG-2 are also degraded by autophagy (Zhang et al., 2009; Tian et al., 2010). Arginine methylation of PGL-1 and PGL-3, which is mediated by the *C. elegans* PRMT1 homolog EPG-11, modulates the association of EPG-2 with PGL/SEPA-1 granules. PGL granules accumulate in *epg-11* mutant embryos, and these granules are separated from EPG-2 aggregates (Li et al., 2013). Interestingly, PGL granules accumulate in *epg-11* mutants independent of SEPA-1 (Li et al., 2013).

Phase separation and transition provide insights into how the concerted actions of SEPA-1, EPG-2 and PTMs ensure efficient clearance of PGL-1 and PGL-3 (Figure 6B). Purified PGL-1 and PGL-3 proteins undergo phase separation in an *in vitro* LLPS system (Zhang et al., 2018a). Co-addition of SEPA-1 promotes LLPS of PGL-1/-3 in a concentration-dependent manner. SEPA-1 increases the size of PGL-1/-3 droplets and lowers the critical concentration of PGL-1/-3 for successful LLPS. SEPA-1 coalesces into and homogeneously disperses into PGL-1/-3 droplets (Zhang et al., 2018a). The SEPA-1/PGL-1/PGL-3 droplets exhibit liquid-like properties. They are spherical in shape, fuse with each other upon encounter, exhibit a wetting phenotype, and have highly mobile interior molecules (Zhang et al., 2018a). Methylation of PGL-1 and PGL-3 decreases their LLPS by increasing the threshold protein concentration for LLPS; the size of droplets formed is also decreased (Zhang et al., 2018a). Addition of EPG-2 reduces the size of PGL/SEPA-1 droplets and the droplet size remains the same over time. The EPG-2/SEPA-1/PGL-1/PGL-3 droplets fail to fuse upon contact, and the mobility of interior molecules is significantly decreased, indicating that EPG-2 promotes liquid-to-gel-like transition of PGL granules (Zhang et al., 2018a). EPG-2 coats the surface of SEPA-1/PGL-1/PGL-3 droplets (Zhang et al., 2018a).

In *C. elegans* embryos, the LLPS-based assembly of PGL granules is modulated by SEPA-1, EPG-2 and EPG-11-mediated modification. Overexpression of PGL-3 induces the formation of PGL granules that exhibit liquid-like properties. They undergo fusion and fission, the interior molecules are highly mobile, and their formation is sensi-

tive to high salts in the culture medium (Zhang et al., 2018a). EPG-2 decorates or coats the surface of PGL granules (Zhang et al., 2018a). The mobility of PGL granules formed in *epg-2* mutants is higher than the mobility of EPG-2-containing granules. The role of EPG-2 in mediating degradation of PGL granules appears to be related to the gelation of PGL granules. Gelation of PGL granules can be greatly facilitated by mutations in PGL-1 in the absence of EPG-2 (Zhang et al., 2018a). Introducing the gelation mutations into PGL-1 renders the degradation of PGL granules independent of EPG-2 (Zhang et al., 2018a). Thus, phase separation and transition of PGL granules is tightly controlled, ensuring autophagic degradation in the somatic cells of *C. elegans* embryos.

C. elegans is grown at 15 to 25°C. Surprisingly, in embryos laid by animals grown under heat stress conditions, such as 26°C, PGL granules accumulate instead of being degraded, which confers a stress adaptation function (Zhang et al., 2018a). Accumulation of PGL granules under heat stress conditions requires the activity of mTORC1. In embryos laid under heat stress conditions, mTORC1-mediated phosphorylation of PGL-1 is significantly elevated (Zhang et al., 2018a). In the presence of phosphorylated PGL-1 and PGL-3, the threshold protein concentration for LLPS is lower, and the droplet size is larger. Therefore, under heat stress conditions, elevated phosphorylation of PGL-1/-3 by mTORC1 may lead to production of PGL granules at a rate that exceeds the gelation capacity of EPG-2, which is normally degraded by autophagy. Overexpression of EPG-2 or PGL-1 gelation mutants dramatically decreases the accumulation of PGL granules under heat stress conditions (Zhang et al., 2018a).

Material properties of stress granules specify their disassembly and autophagic degradation

A variety of stressors such as heat shock, glucose starvation, osmotic stress and oxidative stress trigger the assembly of ribonucleoprotein (RNP) granules in the cytoplasm, called stress granules (SGs) (Protter and Parker, 2016). SGs contain various mRNAs stalled at translation initiation, translation initiation factors, RNA-binding proteins (RBPs), and numerous non-RNA-binding proteins. The composition of SGs depends on the nature and also the degree of the stress, except for the core factors essential for the assembly of the stable substructures within SGs (Protter and Parker, 2016). The dynamics of SGs modulates mRNA localization, translation and degradation and also affects signal transduction via sequestration of signaling factors such as RACK1, TRAF2 and TORC1 (Buchan, 2014). SGs confer on cells the ability to adapt under various stress conditions (Buchan, 2014; Protter and Parker, 2016; Riback et al., 2017; Takahara and Maeda, 2012).

SGs are assembled via phase separation of translation-stalled mRNAs and mRNA-binding proteins, such as the poly(A)-binding protein Pab1 in yeast, and heterogeneous nuclear ribonucleoproteins (hnRNPs), TIA1 and FUS in mammalian cells (Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015; Riback et al., 2017). hnRNPs undergo LLPS *in vitro* and quickly transition into gel-like or amyloid-like fibril structures (Alberti and Hyman, 2016). In living cells, SGs exhibit liquid-like properties (Protter and Parker, 2016). Phase separation and transition of SGs *in vivo* is modulated by multiple factors. RNA and ATP modulate phase separation of hnRNPs in a molar ratio-dependent manner. RNA and ATP inhibit LLPS of hnRNPs at high concentrations, while maintaining the liquid-like properties of hnRNP condensates at low concentrations. RNA and ATP prevent aberrant phase transition of SGs under physiological conditions (Maharana et al., 2018; Patel et al., 2017). Sequestration of misfolded proteins into SGs causes aberrant liquid-to-solid transition of SGs (Mateju et al., 2017). The chaperone surveillance system modulates the composition and material properties of SGs. Molecular chaperones such as HSP27, HSP70 and VCP prevent sequestration of misfolded proteins in SGs and promote disassembly of SGs when the stress diminishes (Kroschwald et al., 2015; Mateju et al., 2017). Transportin-1/Karyopherin- β 2 (TNPO1/Kap β 2), which functions as a nuclear import receptor (NIR) for proteins containing an atypical proline-tyrosine nuclear localization signal (PY-NLS), also acts as a molecular chaperone to inhibit phase separation and gelation of PY-NLS-containing hnRNPs, including FUS, TAF15, EWSR1, hnRNPA1 and hnRNPA2 (Guo et al., 2018; Hofweber et al., 2018; Qamar et al., 2018; Yoshizawa et al., 2018). Overexpression of TNPO1 suppresses accumulation of hnRNPs in SGs (Guo et al., 2018; Hofweber et al., 2018; Qamar et al., 2018; Yoshizawa et al., 2018).

After the stress has passed, the majority of SGs are disassembled and a subset of SGs is removed by autophagy. Dysfunction of autophagy results in accumulation of SGs both in yeast and mammalian cells (Buchan et al., 2013). In mammalian cells, symmetrically methylated arginine residues in the SG components are recognized by the p62-C9ORF72 complex, which tethers SGs to LC3-labeled autophagic structures to mediate their degradation (Chitiprolu et al., 2018). Disease-related mutations in components of SGs such as the D262V mutation in hnRNPA1, the G156E mutation in FUS, and expression of C9ORF72 arginine-rich dipeptide repeats, dramatically promote liquid-to-solid transition of SGs and accelerate formation of amyloid-like fibrils (Boeynaems et al., 2017; Lee et al., 2016; Molliex et al., 2015; Murakami et al., 2015; Patel et al., 2015). Aberrant phase transition of SGs impairs their autophagic degradation, resulting in their accumulation (Boeynaems et al., 2017; Lee et al., 2016; Molliex et al., 2015; Murakami et al., 2015; Patel et al., 2015).

Aberrant phase separation and transition in human diseases

Phase separation and transition of protein condensates are closely scrutinized in cells to ensure that a variety of biological processes occur in a spatially and temporally controlled manner (Hofweber and Dormann, 2019; Liao et al., 2019; Quiroz et al., 2020; Schmidt and Görlich, 2016; Sontag et al., 2017). Factors such as mutations in the genes encoding phase-separated proteins, reduced protein quality control and impaired cellular transportation systems may lead to abnormal protein condensation (Chou et al., 2018; Farhan et al., 2019; Marrone et al., 2019). Dysregulation of protein phase separation and transition has been closely associated with diverse human diseases including neurodegenerative diseases (NDs) (e.g., amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD)) and cancers (Aguzzi and Altmeyer, 2016; Spann et al., 2019).

Abnormal protein phase separation and transition in neurodegenerative diseases

Prior to the explosive and exponential growth in the identification of new proteins that undergo phase separation in different physiological processes (Boeynaems et al., 2018), the main body of the initial studies on protein LLPS came from a series of studies on the abnormal LLPS of several RNA-binding proteins (RBPs), such as FUS, TDP43 and hnRNPA1, and their roles in the pathogenesis of ALS and FTD (Conicella et al., 2016; Kim et al., 2013; Molliex et al., 2015; Murakami et al., 2015). These proteins are referred to as ALS-related RBPs. The discovery of abnormal protein phase separation provides new insights into disease mechanisms as well as new frameworks for therapeutic treatment. So far, several different mechanisms have been demonstrated to explain how aberrant protein phase separation/transition leads to diseases. These include disease-associated mutations and direct modifications of phase-separated proteins, and dysfunction of regulatory proteins, such as molecular chaperones in the protein quality control network and the nucleocytoplasmic transport system (Figure 7).

Aberrant phase separation of RNPs in NDs

Several ALS-related RBPs, such as FUS, TDP-43, hnRNPA1 and TIA1, which shuttle between the nucleus and the cytoplasm, are involved in the formation of stress granule and other nuclear bodies. These proteins undergo LLPS and further mature into a solid phase both *in vitro* and in cells (Lin et al., 2015; Mackenzie et al., 2017; Maharana et al., 2018; Molliex et al., 2015; Patel et al., 2015). The solidification of these RNA-binding proteins into irreversible amyloid aggregates impairs the dynamics of RNP granules

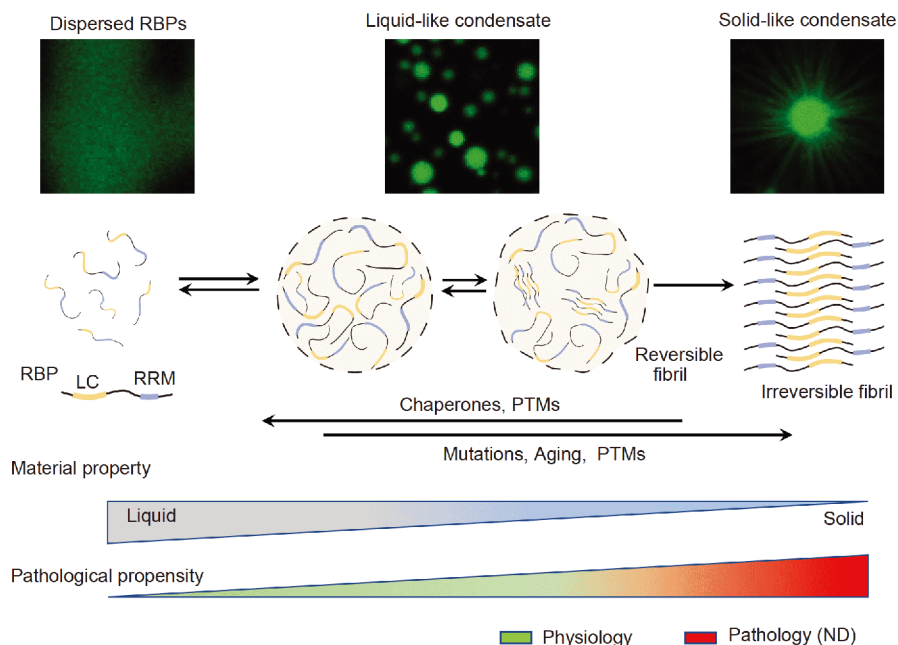


Figure 7 Schematic view of phase transition between different states, and the relationship between aberrant phase separation and neurodegenerative diseases. RNA-binding proteins (RBPs) undergo reversible LLPS to form liquid-like condensates, which can further mature into irreversible aggregates composed of pathological fibrils. This process underpins neurodegenerative diseases. The different states have distinct material properties, with the dynamics and reversibility decreasing as the condensates transition from a liquid-like to a solid-like state. In biological contexts, the LLPS process is precisely regulated by protein quality control systems, protein PTMs and cellular transportation systems. Different chaperones and PTMs may prevent protein phase separation, while disease-associated mutations and certain pathological PTMs may increase the probability that RBPs will form solid-like condensates, thus leading to diseases.

and leads to ALS and FTD (Murakami et al., 2015). These ALS-related RBPs commonly contain LCRs and RNA-recognition motifs (RRMs). The LCR of the ALS-related RBPs exhibits a high LLPS capability, which can drive the phase separation of the full-length protein (Lin et al., 2015). The RRM regulates protein LLPS in the presence of different types of RNAs (Wang et al., 2019a). ALS-associated mutations have been identified within the LCR, which suggests that there is a correlation between the LLPS of RNPs and ALS pathogenesis (Harrison and Shorter, 2017). Two familial ALS-associated mutations, one in the LCR of hnRNPA1 (D262V) and the other in the LCR of hnRNPA2B1 (D290V), dramatically increase the level of pathological cytoplasmic inclusions, leading to neuronal pathology in both cellular and animal models (Kim et al., 2013). Both hnRNPA1 and FUS form highly reversible amyloid fibrils during protein LLPS, which can fine-tune the material properties of the liquid-like state (Gui et al., 2019; Luo et al., 2018). The D262V disease mutation strengthens the amyloid-like interaction, alters the assembly of hnRNPA1 from reversible fibrils to irreversible fibrils, and solidifies the hnRNPA1 condensates (Gui et al., 2019). The ALS-associated mutation G156E in the FUS LCR promotes aberrant liquid-to-solid phase transition of FUS (Patel et al., 2015).

In addition to mutations in the LCR, which directly modulate protein LLPS behavior, mutations in the RRM and

nuclear localization sequence (NLS) were also identified to cause aberrant phase transition of FUS, TDP43 and hnRNPA1 (De Santis et al., 2019; Naruse et al., 2018; Wang et al., 2017a). For instance, an ALS-causing mutation (D169G) within RRM1 of TDP43 abolishes paraspeckle RNA NEAT1-mediated LLPS of TDP43 and the formation of nuclear bodies, while promoting aberrant and irreversible phase transition of stress granules into cytoplasmic inclusions leading to neurotoxicity (Wang et al., 2019a). Moreover, ALS-associated mutations in the NLS of FUS (P525L) and hnRNPA1 (P288A) were found to impair the nuclear localization of these proteins and promote their abnormal aggregation in the cytoplasm (Dormann et al., 2010; Naruse et al., 2018).

PTMs play an important role in modulating protein phase separation and RNP granule dynamics. For example, the level of poly(ADP-ribosylation) (PARylation) is closely associated with the assembly-disassembly dynamics of RNP granules. Both TDP43 and hnRNPA1 can directly bind to PAR by their PAR-binding motifs (PBMs) (Duan et al., 2019; McGurk et al., 2018). PAR can significantly promote hnRNPA1 LLPS and diminish the dynamics of hnRNPA1 condensates both *in vitro* and in cells. Increasing the PARylation level by activating PARP-1 leads to solidification of RNP granules and neurotoxicity, which can be rescued by PARP-1 inhibitors (Duan et al., 2019). Moreover, phosphorylation and methylation can also directly modulate the

LLPS of ALS-related RBPs. Phosphorylation of FUS by DNA-PK reduces the LLPS of FUS and its reversible and irreversible fibrillation, and ameliorates FUS-associated cytotoxicity (Monahan et al., 2017; Luo et al., 2018). Arginine hypomethylation in the FUS RGG region facilitates FUS LLPS and impairs the dynamics of FUS condensates (Qamar et al., 2018). Therefore, different PTMs may act cooperatively to maintain the appropriate phase separation behavior and function of RNP condensates. Aberrant PTMs may also be causative of NDs.

Expansion of the G4C2 repeat in the *C9ORF72* gene was recently identified as the most common cause of ALS and FTD (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Disease-related repeat expansions in the gene result in generation of extra arginine-containing dipeptides. Different disease mechanisms have been proposed including loss of function, or toxic effects of the expanded repeat at the RNA or protein level (Jain and Vale, 2017; Lin et al., 2016). Importantly, the arginine dipeptide repeats can interact with a wide spectrum of LCR-containing RNPs, sequester them in a solidified phase-separated state, and perturb the dynamic assembly and function of different granules (e.g., stress granules, Cajal bodies) (Boeynaems et al., 2017; Lee et al., 2016). Reducing the production of dipeptide repeats or boosting their clearance may provide a means to restore the balance and dynamics of different biomolecular condensates for therapeutic treatment.

Collapse of regulatory networks in NDs

The assembly-disassembly of protein condensates is tightly controlled by different cellular regulatory networks. A wide spectrum of molecular chaperones, including Hsp40, Hsp70, Hsp90, and small heat shock proteins (sHsps), have been identified as protein regulators in RNP granules (Jain et al., 2016; Markmiller et al., 2018). The chaperone network provides the protein quality control system for regulating the formation, dissociation and removal of different RNPs granules. For instance, the HSPB8-BAG3-HSP70 chaperone complex was found to direct the disassembly of aberrant stress granules that contain accumulated pathological ALS-associated RBP inclusions (Ganassi et al., 2016). Hsp70 assists in refolding the misfolded proteins in the nucleolus and maintaining the liquid-like properties of the nucleolar matrix (Frottin et al., 2019). A recent study identified that Hsp27 is incorporated into the aberrant stress granules and is involved in their clearance (Mateju et al., 2017). Yeast Hsp40 proteins (e.g., Sis1 and Ydj1) accumulate in stress granules and may also directly modulate their assembly, dynamics and clearance (Walters et al., 2015).

Recently, the gene *DNAJC7*, which encodes a class III Hsp40 protein, was identified as a novel ALS locus by exome sequencing (Farhan et al., 2019). Mutations in the promoter region of the Hsp27-encoding gene *HSPB1*, which

impair Hsp27 expression in response to stress, have also been identified in sporadic ALS (Dierick et al., 2007). Moreover, mutations of Hsp27 were identified in hereditary motor neuron diseases (Benndorf et al., 2014). These results further strengthen the role of molecular chaperones in regulating RNP granules and their pathological relevance to diseases. Thus, modulating the chaperone networks may provide a new strategy to restore the homeostasis of RNP granules. Indeed, AMX0035, a small molecule that promotes heat shock protein synthesis, exhibits promising activity in preventing neuronal death and degeneration, and is currently in Phase II clinical trial for ALS treatment.

Despite the fact that different molecular chaperones have been identified to regulate the dynamics of RNP granules, the underlying molecular mechanisms and structural aspects remain largely unknown. For instance, how do chaperones differentiate the distinct states of client proteins inside and outside granules? Are chaperones involved in the assembly of granules? How do chaperones efficiently localize within specific granules? Karyopherin- β 2 serves as a noncanonical molecular chaperone in the cytoplasm to prevent and even reverse the LLPS and pathological fibrillation of PY-NLS-bearing RBPs, and reduce their neurotoxicity (Guo et al., 2018; Hofweber et al., 2018; Qamar et al., 2018; Yoshizawa et al., 2018). Intriguingly, Karyopherin- β 2 captures the NLS of FUS with high affinity, while also weakly interfering with multiple regions across full-length FUS to prevent its self-association (Yoshizawa et al., 2018). The ALS-associated mutation P525L in the NLS of FUS impairs FUS/NLR binding (Zhang and Chook, 2012), which highlights the importance of NIRs in chaperoning RNPs away from pathological aggregation under disease conditions.

Abnormal protein phase separation in cancer and other human diseases

Unlike the extensive studies of protein phase separation/transition in NDs, comprehensive cause-consequence studies on the dysregulation of protein phase separation and biomacromolecular condensates in cancers and other human diseases are at a very early stage. Several key signaling transduction proteins (e.g., YAP and ZAP70), which are known to be essential for tumor proliferation and metastasis maintenance, have recently been found to form condensates for signal amplification (Cai et al., 2019; Su et al., 2016). These findings suggest that protein phase separation may serve as a common mechanism for signal transduction and amplification, which is essential for maintaining the highly proliferative state of cancer cells. Moreover, many human cancers (e.g., Ewing's sarcoma and myxoid liposarcoma) are caused by chromosomal translocations which result in the fusion of the LCRs of FET family proteins (e.g., fused in sarcoma (FUS), Ewing's sarcoma (EWS), and TAF15) with

the DNA-binding domain of ETS-related transcription factors (Riggi et al., 2007). Given the generic propensity of LCRs to undergo LLPS, these fusion proteins may trigger abnormal phase separation and transcriptional activation that is causative of the disease. The tumor suppressor SPOP (speckle-type BTB/POZ protein) undergoes phase separation upon binding its substrates (e.g., DAXX) for the delivery of cullin 3 RING ubiquitin ligase (CRL3). Cancer-associated mutations in SPOP disrupt its ability to undergo normal substrate binding and phase separation, which leads to accumulation of pro-oncogenic proteins (Bouchard et al., 2018).

As discussed above (section on phase separation-mediated formation of pre- and postsynaptic density signaling assemblies), protein phase separation plays an essential role in the organization, assembly and function of the postsynaptic density (PSD). Dysregulation of PSDs is directly linked to human psychiatric diseases (Berryer et al., 2013; Hamdan et al., 2009; Parker et al., 2015). It is possible that mutations in genes encoding synaptic proteins involved in PSD condensation may lead to aberrant phase behavior and functional impairment of the PSD. Further work is needed in this area to reveal the direct link between protein phase separation and psychiatric diseases, and to provide new avenues for potential drug development for psychiatric diseases.

Biophysical characterization of biomacromolecular condensates

While the biological significance of LLPS from various biomacromolecular systems is currently under extensive investigation, approaches to uncovering the underlying biophysical mechanisms of the condensed phase droplets in three-dimensional (3D) solution systems or condensed layers in two-dimensional (2D) membrane systems are still in their infancy (Feng et al., 2019). The material properties of the LLPS condensates are important for their distinct biological functions. In many cases, cells utilize the phase-separated condensates as biochemical reaction centers. High diffusivity of the components within the condensates is required to make the reaction more efficient. For condensates serving as structural scaffolds, mechanical properties such as stiffness and elasticity play more important roles. Therefore, characterizing the material properties of the condensates is essential to fully understand the system and its biological functions. Generally, the condensed droplets formed in a 3D solution system can be viewed as a viscoelastic liquid, and thus they display liquid-like behaviors during nucleation, growth, fusion and coarsening/aging. These behaviors usually depend on the diffusivity of the material inside the droplets, the density, viscosity and elasticity of the droplets, and the interfacial tension at the boundary between the

condensed phase (within the droplets) and the dilute phase (the surrounding medium).

Fluorescence recovery after photo-bleaching (FRAP) assays are commonly used to analyze the diffusivity of molecules at different sites within or outside the droplets (Taylor et al., 2019). Specifically, diffusion coefficients obtained by FRAP analysis for molecules deep inside the condensed droplets, in the surrounding dilute phase, and at the boundary between the condensed and dilute phases may reflect various intrinsic kinetic properties of the molecules in the LLPS system (such as the dissociation and association rates). Time-resolved FRAP analysis of droplets can be used to investigate the coarsening/aging process of the droplets (Feric et al., 2016). As FRAP experiments capture the mobility of the molecules and the dynamics of the interactions between the molecules, they can also be used to confirm the existence of LLPS.

The density of LLPS droplets reflects the compactness of the condensed phase. The ratio of the density between the condensed and the dilute phase determines the sedimentation of the droplets under gravity. This in turn limits the location of the droplets within the cells and can enhance their collision probability, which may regulate the biological function of the phase-separated molecules (Feric and Brangwynne, 2013; Feric et al., 2015). It is believed that gravity has little effect on a single biomolecule such as a protein. LLPS reinforces the impact of gravity. Gravity may play a more important role in the behavior of molecules in the condensed phase than in the dilute phase. The density of the LLPS droplets can be measured by the density gradient centrifugation method.

Viscosity is an important parameter for viscoelastic liquids such as LLPS droplets. Microrheology studies have been applied to droplets formed by LAF-1, a DDX3 RNA helicase found in P granules. This approach revealed that the viscosity of the droplets is controlled by the ion and RNA concentrations (Elbaum-Garfinkle et al., 2015), which suggests the importance of droplet viscosity for the biological functions of LLPS in this system. A microfluidic platform has been designed to measure the viscosity of the condensed phase of droplets to improve the throughput of viscosity measurements (Taylor et al., 2016).

Force spectroscopy techniques, such as atomic force microscopy (AFM) (Zemla et al., 2018), biomembrane force probe (BFP) spectroscopy (Chen et al., 2008), and optical tweezers (OT) (Perkins, 2014), can be used to determine the mechanical properties such as stiffness and elastic modulus of LLPS droplets. AFM-based measurements have been used to analyze the mechanical properties of PSD droplets (Zeng et al., 2018). Using an AFM cantilever with a colloidal probe, the elastic modulus of the droplets formed by different LLPS condensates was measured via force-generated deformation of the droplets. The elastic properties of droplets

were found to correlate with their biological functions *in vivo* (Zeng et al., 2018). AFM also has the ability to provide high-resolution scanning images to characterize the morphology of the LLPS droplets (Levenson et al., 2019).

In 3D solution, LLPS droplets adopt a spherical shape as a result of the interfacial tension at the boundary between the condensed and dilute phases. Liquid mechanics indicates that the interfacial tension may be related to the fusion propensity of the droplets (Aarts et al., 2005). The interfacial tension can be fine-tuned by the solution conditions, including ion concentration, pH and temperature. An OT-based method has been developed to measure the interfacial tension of droplets (Jawerth et al., 2018). This technique was used to measure the frequency-dependent rheology and the surface tension of droplets formed by the P-granule protein PGL-3. The results indicated that ion concentration plays important roles in the rheology and dynamics of the LLPS droplets (Jawerth et al., 2018). OT manipulation has also been used to observe the fusion of two droplets, and to determine the fusion time scale (Gui et al., 2019; Alshareedah et al., 2019; Kaur et al., 2019).

BFP spectroscopy is a force spectroscopy technique developed specifically to measure cell mechanics (Chen et al., 2008; Wu et al., 2019b), but which also has great potential for studying the dynamics of LLPS droplets. It utilizes micropipettes with tips with inner diameters ranging from submicrons to several tens of microns to manipulate cells or beads. With this method, an LLPS droplet can be sucked and held by one micropipette, and then pressed repeatedly by a probe bead manipulated by another micropipette. The material properties of the droplet can be calculated by measuring the force profile at the droplet and bead surface. If a second droplet is attached to the probe bead, it is possible to measure the attractive/repulsive forces between the droplets as they do or do not undergo fusion. The behavior of these forces may provide information to link the biophysical properties of the droplets with their biological functions.

Formation of LLPS condensates in a 2D system such as on a cell membrane may facilitate the amplification of signals initiated by various extracellular cues (Case et al., 2019b; Su et al., 2016; Wu et al., 2019a; Zeng et al., 2018). The biophysical properties of these layered LLPS condensates can be observed by various existing methods that have been developed for studying supported lipid bilayers (Zeng et al., 2018; Su et al., 2016; Wu et al., 2019a), including imaging methods such as TIRF and confocal microscopy and super-resolution techniques such as STORM/PALM. These imaging techniques enable FRAP measurements, molecular tracking analyses or quantification of the condensate or condensate components in the condensed and dilute phase (Wu et al., 2019a). Techniques to analyze the material/mechanical properties of 2D condensates remain to be developed, although it is possible to measure the hardness of the condensed layers using AFM.

Another interesting aspect of LLPS condensates is the biophysical mechanisms at the molecular level. The concentrations of molecules and the interactions between individual components in a LLPS system may govern the biological and biophysical behaviors of the condensates (Li et al., 2012; Case et al., 2019b). The interactions are multivalent and may involve the participation of various ions. Thus, quantifying the kinetics (i.e., association and dissociation rates) of the multivalent binding partners as well as the interactions between individual components as they undergo LLPS will provide information to decipher the molecular mechanisms underlying the formation of phase-separated compartments and to understand their functional roles. In addition to the force spectroscopy techniques described above (AFM, BFP and OT), new techniques need to be developed to accomplish these quantitative measurements.

Due to the high heterogeneity of condensates, it appears to be very difficult to obtain structural information about the constituent proteins/RNAs by X-ray crystallography. In contrast, nuclear magnetic resonance (NMR) spectroscopy has emerged as a leading technique to study biomolecular condensates, especially those containing intrinsically disordered regions (Murthy and Fawzi, 2020). The basic solution NMR approaches, including chemical shift perturbation, nuclear Overhauser effect spectroscopy (NOESY) and paramagnetic relaxation enhancement (PRE), have been utilized to provide atomic resolution information about strong or transient intra-/inter-molecular interactions in both dispersed-phase and condensed-phase samples (Conicella et al., 2016; Murthy et al., 2019; Ryan et al., 2018). Solution NMR also has the advantage of being able to monitor protein motion and conformational changes at atomic resolution in the dispersed or condensed phase and even during transition (Conicella et al., 2016; Murthy et al., 2019; Ryan et al., 2018). Solid-state NMR (ssNMR) should be a suitable strategy to characterize the atomic structures of insoluble amyloid fibrils, which are correlated with phase transition and human diseases (Loquet et al., 2018; Murray et al., 2017). Currently, there is still a significant gap between *in vitro* and *in vivo* studies of biomolecular condensates. New biophysical techniques are urgently needed to make the connection, and in-cell NMR might potentially be one such approach.

Conclusions and perspectives

LLPS mediates the assembly of a large body of biomacromolecular condensates that participate in a wide variety of cellular activities, including cell division, signal transduction, higher-order genome organization and gene regulation, formation of pre- and postsynaptic density assemblies and

sorting of proteins for autophagic degradation. LLPS sheds new light on how cellular biomacromolecular condensates fulfill their distinct functions. Our understanding of the dynamic assembly and physiological functions of phase-separated condensates is still in its infancy. Numerous questions remain to be addressed. How do different signaling cascades integrate with the key proteins to trigger phase separation in a spatiotemporally controlled manner? How is the composition of biomacromolecular condensates specified in cells? What mechanisms are responsible for dynamic control of the intermolecular interactions between motifs capable of multivalent interactions and IDRs? Due to the diversity and complexity of intracellular biomacromolecular condensates, it is still difficult to directly visualize phase separation *in vivo*. New tools and techniques are urgently needed to facilitate *in vivo* studies and also to probe the intermolecular interactions in the condensates.

Different biomacromolecular condensates possess distinct material properties that are essential for their distinct physiological function. For example, stress granules and Cajal bodies are dynamic and liquid-like condensates, while amyloid bodies and Balbiani bodies exhibit much less dynamic material properties (Audas et al., 2016; Boke et al., 2016; Woodruff et al., 2017). Gel-like properties of protein condensates appear to be essential for their selective recognition and degradation by autophagy (Zhang et al., 2018a). Therefore, specification of the appropriate material properties of different biomacromolecular condensates (e.g., fluidity, reversibility and mobility) is key under physiological conditions. Very little is known about how the distinct material properties of biomacromolecular condensates are dynamically specified in living cells.

Aberrant phase separation and transition caused by mutations in phase-separated proteins, impairment of quality control systems and alteration of environmental conditions have all been implicated in the pathogenesis of various diseases. Accordingly, identification of molecules that can modulate phase separation and transition provides a novel strategy for drug development and therapeutic treatment in combating human diseases associated with aberrant protein phase separation.

Although we are still at the beginning of our journey to understanding the role of phase separation in the assembly and function of biomacromolecular condensates, we foresee an exciting era in which phase separation will revolutionize our knowledge of diverse biological activities.

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