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REVIEW

Biomolecular condensates and disease pathogenesis

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Biomolecular condensates or membraneless organelles (MLOs) formed by liquid-liquid phase separation (LLPS) divide intracellular spaces into discrete compartments for specific functions. Dysregulation of LLPS or aberrant phase transition that disturbs the formation or material states of MLOs is closely correlated with neurodegeneration, tumorigenesis, and many other pathological processes. Herein, we summarize the recent progress in development of methods to monitor phase separation and we discuss the biogenesis and function of MLOs formed through phase separation. We then present emerging proof-of-concept examples regarding the disruption of phase separation homeostasis in a diverse array of clinical conditions including neurodegenerative disorders, hearing loss, cancers, and immunological diseases. Finally, we describe the emerging discovery of chemical modulators of phase separation.

liquid-liquid phase separation | biomolecular condensate | membraneless organelle

INTRODUCTION

Eukaryotic cells exhibit a sophisticated internal architecture that encompasses both membrane-bound organelles and membraneless organelles (MLOs), each fulfilling specialized roles in various biological processes. Canonical membrane-bound organelles compartmentalize biochemical reactions by confining specific biological macromolecules to a certain area enclosed by a lipid bilayer. MLOs lack such membrane structures. MLOs, also called biomolecular condensates, exhibit diverse shapes and typically range from 0.2 to 5 μm in size (Hirose et al, 2022). They are generally formed through liquid-liquid phase separation (LLPS), driven by the multivalent interactions among proteins, nucleic acids (RNA and/or DNA) and other biomolecules (Antifeeva et al., 2022; Wang et al., 2021a). Nucleic acids are inherently capable of multivalent interactions via their charged phosphate backbones. Many proteins in MLOs possess domains facilitating multivalent interactions, which drive the proteins into phase-separated condensates via LLPS under certain conditions. These domains include intrinsically disordered regions (IDRs), which lack folding capacity in physiological environments, and low-complexity domains (LCDs), which are characterized by biased composition of certain amino acids (Alberti and Hyman, 2021; Boeynaems et al., 2018; Protter and Parker, 2016). Some proteins—intrinsically disordered proteins (IDPs)—are conformationally plastic throughout their entire length.

The initial observation of the first MLO, subsequently termed the nucleolus, can be attributed to Felice Fontana in the 1770s. Since then, researchers have identified dozens of distinct MLOs,

distributed within both the cytoplasm and nucleus, that perform a variety of important biological functions (Antifeeva et al., 2022; Hirose et al., 2022; Ripin and Parker, 2023; Guilhas et al., 2020; Ladouceur et al., 2020; Shin and Brangwynne, 2017). This array includes stress granules (SGs), processing bodies (P-bodies), the nucleolus, Cajal bodies, gems, nuclear speckles, paraspeckles, promyelocytic leukemia protein (PML) nuclear bodies, nuclear stress bodies, and others (Figure 1). In this review, we will introduce classical and emerging methods and techniques for detecting and probing phase separation of MLOs both *in vitro* and *in vivo*. We will then summarize recent discoveries about MLOs involved in several physiological and pathological processes, and we will describe the advent of strategies for disease treatment by targeting biomolecular phase separation.

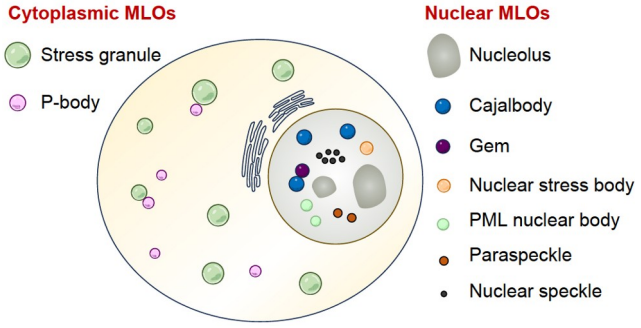

Figure 1. The distribution of various MLOs within the cell.

Detection of phase separation

In vitro detection of phase separation

In contrast to protein aggregates, LLPS condensates are marked by their reversibility and environmental sensitivity (Jin et al., 2021). A variety of methods have been developed to investigate phase separation of purified proteins in test tubes to gain knowledge about the regulation and modulation of such condensates under different conditions (Figure 2). Among them, the most commonly used approach is optical microscopy, since it is easy to perform and can be applied to visualize dynamic processes. Visualization of transparent LLPS condensates is constrained by their low contrast and weak optical absorptivity. Although this can be overcome by staining with dyes or attaching fluorescence tags to the constituent protein, dyes or labels can be phototoxic, conferring the risk of changing the biochemical properties of protein condensates (Hoebe et al., 2007). Differential interference contrast (DIC) is an imaging technique that enables direct imaging of samples with a refractive index gradient in a label-free manner (Koos et al., 2016). Particularly in droplets formed during LLPS, DIC microscopy offers clear imaging of dynamic boundaries between the droplets and the solvent, making it widely used in studies of LLPS *in vitro* (Heydarian et al., 2020; Wang et al., 2023c). Zweckstetter et al. used DIC microscopy to show that the microtubule-binding repeats of Alzheimer's disease (AD)-related Tau protein undergo LLPS and form liquid droplets in solution (Ambadipudi et al., 2017). Under simulated intracellular crowding conditions, formation of droplets was observed at different protein concentrations and pH values. Despite its fine spatial resolution, DIC microscopy is limited to static imaging, which hinders the real-time observation of dynamic processes (Koos et al., 2016). To overcome this, Rosen et al. used fluorescence microscopy, dynamic light scattering (DLS) and small-angle X-ray scattering (SAXS) to complement DIC microscopy (Li et al., 2012). The fluorescence microscopy allowed for visualization of protein droplets, while DLS and SAXS provided information about the size and dynamics of the droplets. Together, these techniques showed that the interactions between multivalent macromolecules produce sharp phase separations and sol-gel transitions in aqueous solution. In the search for higher spatial resolution, confocal microscopy emerges as a powerful tool that enables real-time visualization and dynamic tracking of biological processes (Jonkman et al., 2020; Stadler et al., 2012). Combs et al. used confocal microscopy to investigate the dynamic phase separation of Tau protein *in vitro* in buffers that resemble physiological conditions and found that disease-associated protein modifications significantly enhance droplet formation (Kanaan et al., 2020). Distinct from protein aggregation, phase separation promoted a time-dependent toxic conformation and oligomerization, leading to the formation of non-filamentous pathogenic Tau conformations.

Ultimately, the physical resolution of optical microscopes is limited. Atomic resolution requires the smaller wavelengths provided by electron microscopes (Smith and Chen, 2020). With cryo-transmission electron microscopy (cryo-TEM), Hwang et al. obtained images of condensed droplets with submicron resolution (Kim et al., 2016b). However, due to the stringent conditions required for sample preparation, cryo-TEM can only provide information about the final structures generated by LLPS. As an optimization, Miserez et al. employed *in situ* liquid TEM to observe the dynamic mechanisms of protein self-organization into condensed microdroplets with nanoscale and millisecond resolution (Le Ferrand et al., 2019). They captured the nucleation and initial growth steps of LLPS, which provided insights into the early stages of nucleation and growth associated with MLO formation *in vitro* (Le Ferrand et al., 2019).

Currently, the relationship between the structure and function of phase-separated protein assemblies is challenging to understand clearly, primarily because of their size, dynamics, and heterogeneity (Sahin et al., 2023a). Nuclear magnetic resonance (NMR) and mass spectrometry (MS) have been used as powerful tools to study both the structure and dynamics of LLPS. Rezaei-Ghaleh et al. took advantage of sodium ions which are prevalent in biological samples to report the internal fluidity of condensed phases (Fuentes-Monteverde et al., 2020). They applied ^{23}Na NMR in combination with ^{17}O NMR to study the sodium ion and water mobility within phenylalanine-glycine (FG) peptide hydrogels as well as water-triethylamine (TEA) mixtures. This approach has the potential to unravel properties and regulations of biomolecular condensates under various physical or biochemical conditions. In another report, Kay et al. developed $^{1}H^{\alpha}$ NMR to study the relationship between positions of key residues and phase separation propensity of the disordered region of the RNA-binding protein CAPRIN1 (Wong et al., 2020b). P-bodies are granules, comprised primarily of RNA and RNA-binding proteins, that are responsible for translation repression (see "Processing bodies" section). Sprangers et al. investigated the cellular phase transition process of engineered mRNA degrading

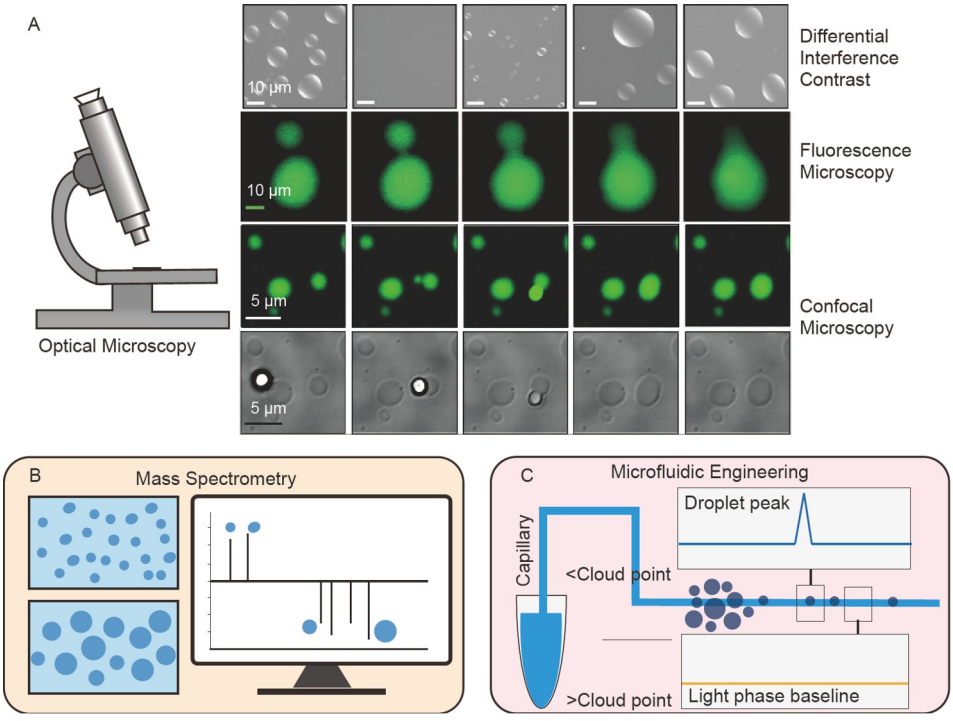

Figure 2. *In vitro* detection of phase separation. A, Application of optical microscopy in *in vitro* LLPS detection (Reproduced from Ambadipudi et al. (2017, open access), Li et al. (2012, with copyright permission from Springer Nature), and Kanaan et al. (2020, open access)). B, Schematic diagram of LLPS detection through mass spectrometry. C, Microfluidic engineering flowchart for LLPS detection.

factors through *in vitro* reconstruction (Fromm et al., 2014). NMR titration revealed that the proteins EDC3 and DCP2 form a network of interactions sufficient to induce phase separation *in vitro*, with the EDC3 LSm domain binding to the helical leucine-rich motifs (HLMs) of DCP2. Moreover, the affinities of these interactions were quantified using isothermal titration calorimetry. Another RNA-binding protein, FUS (fused in sarcoma), which is one of the best-understood heterogeneous ribonucleoproteins (hnRNPs), also undergoes LLPS. Allain et al. employed agarose hydrogels as cytoskeleton mimics *in vitro* to stabilize the droplets of FUS. Using NMR and electron paramagnetic resonance (EPR) techniques, they observed protein signals corresponding to the dispersed and aggregated phases and accurately quantified their respective proportions (Emmanouilidis et al., 2021). However, structural information about the assembly state of hnRNPs is still lacking. Landreh et al. created a pH-responsive LLPS system by combining aggregation-prone hnRNPs with an engineered spider silk domain (Sahin et al., 2023b). They used native MS combined with ion mobility spectroscopy (IM) to identify conformational changes linked to droplet formation (Sahin et al., 2023b).

Recently, microfluidic engineering has been extensively utilized to investigate macromolecular phase transition phenomena (Dolega et al., 2012). Heymann et al. reported a microfluidic device for the determination of saturation concentration changes resulting from phase separation. The microfluidic chip has five cells, each consisting of 20 separate sample chambers. This method quantifies changes in phase behavior at low protein cost, and with high accuracy and statistical efficiency (Bremer et al., 2020). In another report, Buell et al. developed a capillary flow method called Capflex to quantify key parameters of LLPS. Capflex has been successfully applied to characterize the phase separation behavior of three unrelated proteins/peptides—Ddx4n1, RP3 peptide, and α-Synuclein. The versatility and high information content of Capflex make it a powerful tool for characterizing biomolecular LLPS (Stender et al., 2021).

Visualization of LLPS condensates in living cells

The existence of LLPS in living cells was first confirmed by Hyman et al. who observed the dissolution and condensation behavior of membraneless intracellular molecules in *C. elegans* (Brangwynne et al., 2009). So far, a number of methods have been developed for detecting intracellular condensates formed by LLPS (Figure 3). As the main technique for observing phase separation *in vitro*, microscopic imaging also plays an indispensable role in visualizing phase separation in living cells. The detection of live-cell LLPS mainly uses optical microscopy imaging, which combines real-time imaging with fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP). This enables visualization of the size and shape of condensates while also monitoring dynamic changes of biomolecules labeled with fluorescent tags during phase separation in living cells (Che et al., 2023). Furthermore, some light-regulated systems have been reported as advanced techniques for observing LLPS. In addition, AI detection can be used to predict the phase separation of biomolecules (Chen et al., 2022).

Optical microscopy is widely applied in visualizing phase separation in living cells, and it is generally necessary to use dyes or auxiliary labels to stain intracellular biomolecules. Commonly used optical microscopes include the fluorescence microscope (FM), laser scanning confocal microscope (LSCM), and super-resolution microscope (SRM). FM imaging requires fluorescent dyes for protein labelling, and has the advantage of high

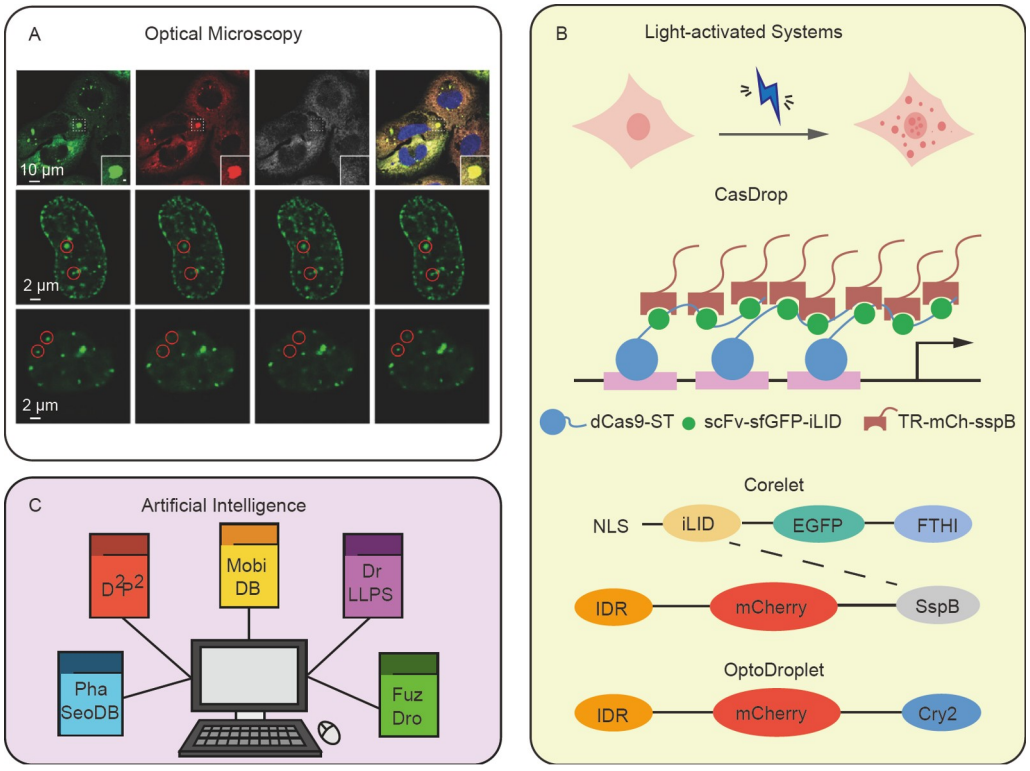

Figure 3. Detection of LLPS condensates in living cells. A, Use of fluorescence imaging to detect LLPS condensates in cells (Reproduced from Lu et al. (2021, open access) and Zhang et al. (2022, open access)). B, Schematic diagram of three light-activated systems: Corelet, OptoDroplet and CasDrop. C, AI-based prediction of LLPS via the D2P2, MobiDB, DrLLPS and FuzDro methods combined with the PhaSepDB database.

sensitivity, high specificity, and the ability to visualize the dynamic behaviors of target biomolecule (Renz, 2013). The gold standards of defining phase separation are that the condensate is spherical, undergoes fusion, and recovers from photobleaching. Therefore, researchers generally took the FRAP phenomenon observed through FM as evidence of phase separation. For example, soluble Tau protein has been reported to undergo LLPS in the cytoplasm, and the droplets formed by LLPS are intermediates of Tau aggregates. The FRAP rate of the full-length GFP-tau (441 aa) is higher in cytosol than when associated with microtubule, suggesting that binding to microtubules reduces the mobility of droplets (Wegmann et al., 2018).

Later evidence suggested that FRAP can be influenced by a variety of factors; thus, more advanced detection methods were developed for reliable identification of the mechanisms of LLPS (Alberti et al., 2019). 1,6-Hexanediol is a small chemical molecule that dissolves phase-separated droplets. A model-free calibrated half-FRAP method was used to distinguish intracellular structures formed through LLPS from other mechanisms by assessing the tolerance of condensates to 1,6-hexanediol as well as the turnover of components in FRAP (Muzzopappa et al., 2022). LSCM and SRM imaging provide more detailed information about biomolecular condensates. LSCM adds a laser scanning device based on FM and can detect intracellular condensates with precision and clarity. Song et al. used confocal imaging to monitor immunostained heparan sulphate (HS) and basic fibroblast growth factor (bFGF) fused with GFP. This revealed that bFGF undergoes LLPS in response to heat shock (Xue et al., 2022). Similarly, the Parkinson's disease (PD)-associated protein α-Synuclein (α-Syn) phase separates under various disease-associated conditions. Single-particle tracking measurements via confocal imaging of FlAsH-stained α-Syn showed that the movement of the liquid-like droplets was directed with the assistance of microtubules (Ray et al., 2020). In addition to the advantages of conventional optical microscopy, SRM has higher resolution and is more friendly to living cells during imaging. FRAP experiments performed using SRM uncovered the phase separation behavior of SARS-CoV-2 N protein. The condensates exhibit a lower viscosity in cells compared to *in vitro*, suggesting that the cellular condensates are dynamically regulated (Lu et al., 2021). Highly inclined and laminated optical sheet (HILO) imaging, which is another specific type of SRM, is also an effective technique to study the separation propensity and fluidity of condensates in living cells (Lee et al., 2022). Additionally, several light-activated systems have emerged as tools to quantify or localize phase separation. The Corelets system, an oligomerizing biomimetic system, can describe the non-equilibrium biophysical characteristics of patterned intracellular phase separation, such as quantifying the concentration of proteins that drive intracellular phase separation (Bracha et al., 2018). The OptoDroplets system employs photoactivation to regulate the formation of liquid droplets in a spatiotemporally definable manner (Shin et al., 2017). Subsequently, Shin et al. (2018) developed a CRISPR-Cas9-based optogenetic system, referred to as the CasDrop system, that enables site-specific formation of LLPS at certain genomic loci. The nuclear condensates produced by phase separation mechanically exclude chromatin and pull target genomic loci closer together as they grow. By driving the rapid aggregation of biomolecules, phase separation enables various essential cellular activities to be carried out efficiently, while abnormal phase separation may lead to many diseases. To achieve a deeper understanding of the

mechanism of phase separation and the relationship between phase separation and human diseases, many researchers have made full use of AI to predict the tendency for phase separation. Many phase separation protein databases have been constructed. For example, D^2P^2 and MobiDB predict the phase separation of proteins based on the analysis of IDR content; DrLLPS is the first comprehensive database that annotates LLPS-related proteins in eukaryotes; and PhaSepDB provides annotation information on the state of phase-separated droplets and the regulation of phase separation (Hou et al., 2023; Ning et al., 2020; Oates et al., 2012; Piovesan et al., 2018). The recently developed FuzDro method calculates the propensity for spontaneous LLPS, thus aiding in identifying proteins that are prone to form condensates. Lim et al. combined FuzDro and PandaOmics, which is a protein target discovery tool, to screen disease targets that may be associated with LLPS (Lim et al., 2023).

Aside from the structure and dynamics of LLPS condensates, the interior composition of MLOs also requires detailed characterization. Taking advantage of the abundance of large nucleoli in late-stage *Xenopus* oocytes, Weeks et al. used mRNAs encoding proteins of interest labelled with a fluorescent protein to investigate the compartmentalization of proteins in the nucleolus (Lavering et al., 2022). Fluorescence microscopy also enables quantification of condensate morphology changes under heat shock or drug treatments. In an alternative approach, Weil et al. developed a fluorescence-activated particle sorting (FAPS) method and identified hundreds of proteins and thousands of mRNAs in purified endogenous P-bodies (Hubstenberger et al., 2017).

Reliable identification of chromatin-associated condensates cannot be achieved in live cell imaging for the small size of them, thus *in situ* biophysical characterization of chromatin-associated condensates remains a challenge. An assay for chromatin-bound condensates by exploratory sequencing (ACC-seq) has been developed by Li et al to grapple with this problem. The accessibility of condensate-mediated DNA regions to the Tn5 transposase under native, fix and 1,6-hex+fix conditions can reflect distinctive DNA accessibility patterns allowing their identification through ATAC-seq. The ACC-seq has been validated through several experiments and hex-released pattern indicates that the differential DNA accessibility to Tn5 transposase belongs to target DNA bound by condensates exclusively. Use of ACC-seq can uncover accessible regions which exhibit traits of condensate occupancy (He et al., 2024).

Techniques to probe interaction networks in MLOs

In the phase separation process, biological macromolecules spontaneously condense and assemble as a result of their own specific biophysical properties (Banani et al., 2017). A large number of MLOs form through phase separation, thereby localizing biological reaction components and regulating biochemical reactions (Banani et al., 2017; Spannl et al., 2019). Phase separation provides a new perspective to understand the mechanisms underlying the spatial and dynamic regulation of protein-protein interactions and biological reactions. However, due to the dynamics of phase-separated condensates and the lack of suitable proteomic tools, the components of MLOs remain largely unknown. Phase separation also plays an important role in various human diseases, including cancers, autoimmune diseases, and neurodegenerative diseases (Du and Chen, 2018; Patel et al., 2015; Song et al., 2020). Explaining the role of phase separation in pathological processes will provide new therapeutic strategies and means for treating diseases.

Enzyme-catalyzed proximity labeling (also known as proximity labeling) is a new method for studying protein-protein interactions and spatial distributions of molecules, such as those in phase-separated condensates in living cells (Gingras et al., 2019; Trinkle-Mulcahy, 2019). Among the proximity labeling methods, BioID technology (proximity-dependent biotin identification) is the most widely used (Roux et al., 2012). Compared with traditional protein-protein interaction techniques, BioID technology can efficiently capture proteins which interact with or are near to a bait protein within cells. The bait protein is tagged with biotin. Since the interaction between biotin and avidin is the strongest known in the biological world (K_d=10^{-15} mol/L) (Green, 1963), BioID can tolerate more stringent lysis and washing conditions, which greatly reduces the interference of contaminating proteins. However, BioID has certain shortcomings. For example, the activity of the biotin ligase BirA is not high, and it usually takes 16–24 h for biotin labeling to produce enough biotinylated proteins for subsequent proteomic analysis (Branon et al., 2020; Roux et al., 2012). In addition, the molecular weight of the BirA enzyme is relatively high (about 36 kD) and the protein includes a DNA-binding domain, which may affect the correct localization of fusion proteins in cells (Kim et al., 2016a). Therefore, various novel biotin ligases with higher activity and less impact on fusion protein localization have been reported in recent years, such as BioID2 (Kim et al., 2016a; Ramanathan et al., 2018) and TurboID (Branon et al., 2020), which enrich the application of BioID.

A novel biotin ligase (PhBPL) in Archaea was recently discovered through bioinformatics analysis. This enzyme has higher activity, a lower molecular weight (~26 kD), and can complete biotin labeling within 5–15 min. It is suitable for detecting transient protein interactions, and is hence named PhastID (PhBPL-assisted biotin identification) (Feng et al., 2024). For example, PhastID has been applied to identify the long-missing regulators of mTORC1 (mechanistic target of rapamycin complex 1). mTORC1 is activated by a small G protein, Rheb, on the lysosome (Livi, 2019; Sabatini, 2017). Rheb mainly senses stimuli from growth factors such as insulin. However, the guanine nucleotide exchange factor (GEF) for Rheb was still controversial (Hsu et al., 2007; Huang and Manning, 2008; Rehmann et al., 2008; Saxton and Sabatini, 2017). Interestingly, a lysosomal membrane protein, ATP6AP1, was found to be significantly enriched in the PhastID proteome at 15 min of insulin stimulation. Combined with other biochemical experiments, it was ultimately discovered that the lysosomal proton pump protein ATP6AP1 acts as the guanylate exchange factor for Rheb to regulate the mTORC1 signaling pathway (Feng et al., 2024). This indicates the power of PhastID in studying dynamic protein-protein interactions.

Telomeres are known to form phase-separated condensates/telomere clusters via telomere-binding proteins such as TRF1 (Jack et al., 2022; Patel et al., 2015; Soranno et al., 2022). TRF1 binds directly to telomeric DNA, where it protects telomeres and regulates their length (Shay and Wright, 2019; Xin et al., 2008). Given that TRF1 exhibits liquid behavior and can drive phase separation together with TRF2, particularly when it is complexed with other Shelterin components and telomeric DNA (Jack et al., 2022), the TRF1 complex could be considered as a nuclear

condensate. The proximal protein network of TRF1 nuclear condensates was also analyzed using PhastID (Feng et al., 2024), and found to contain all six known shelterin/telosome proteins and most other telomere-associated proteins. In addition, PhastID revealed the role of a novel nuclear membrane protein, NUMEN, in regulating telomere end-to-end fusion (Chen et al., 2023). ILF3 was identified as an important regulator of telomere condensates and telomere R-loop structures (Wang et al., 2023a). By PhastID technology, ILF3 was shown to interact with DHX9 to prevent *TERRA* RNA from further invading telomeric double-stranded DNA, thereby stabilizing telomere condensates. PhastID technology has the potential to play a greater role in studying fast and dynamic interaction networks, especially protein-protein interaction networks mediated by LLPS condensates.

In terms of disease treatment, CRISPR/Cas (clustered regularly interspaced short palindromic repeats/Cas)-based transcriptional regulatory tools such as CRISPRa can regulate gene expression without causing permanent DNA sequence changes, thus avoiding potentially harmful mutations (Cheng et al., 2013; Farzadfard et al., 2013; Gilbert et al., 2013; Qi et al., 2013). In clinical practice, CRISPR-related regulatory platforms can compensate for the shortcomings of gene therapy by simultaneously regulating the activity of multiple genes (Cheng et al., 2013; Gilbert et al., 2014). However, gene regulatory platforms represented by CRISPRa often require the fusion of multiple effectors to maximize the transcriptional activation efficiency, which overloads the systems for AAV delivery of components into cells (Polstein and Gersbach, 2015; Zetsche et al., 2015). Therefore, combining the concept of phase separation with CRISPR-specific transcriptional regulatory tools can help in the development of a new generation of disease treatment approaches (Liu et al., 2023; Ma et al., 2023). By fusing the IDRs of a variety of phase-separating proteins to dCas9-VPR (catalytically dead Cas9 joined to three transcription factors), it is possible to incorporate phase-separating proteins into the CRISPRa system (Liu et al., 2023). This system, named dCas9-VPRF, significantly improves the expression and activation efficiency of endogenous genes in mammalian cells (Liu et al., 2023). The dCas9-VPRF system shows a wider gRNA design window (>200 bp upstream of transcription initiation), lower DNA strand targeting preference, and high targeting specificity. The development of efficient transcriptional activation tools utilizing phase separation is expected to be further applied in the treatment of genetic diseases.

Phase separation in RNA-related processes

As described in the Introduction, MLOs can contain a wide array of proteins and nucleic acids. The morphological, structural and functional heterogeneity of MLOs is well illustrated by those involved in synthesis, processing, storage, and recycling of RNAs. In this section, we select two cytoplasmic MLOs (SGs and P-bodies) and three nuclear MLOs (nucleolus, Cajal bodies, and gems) as representatives to introduce the phase-separated MLOs involved in these diverse RNA-related processes.

Stress granules

Stress granules (SGs) are dynamic cytoplasmic structures formed in response to environmental stressors, such as oxidative stress, heat shock, viral infection, or nutrient deprivation. The shape of SGs is often irregular and their diameter ranges between 0.4 and 5.0 μm. Typically, 5 to 30 SGs appear per cell. SGs serve as sites for the storage of untranslated mRNAs and stalled translation initiation complexes, effectively acting as temporary storage sites for these molecules until the stress conditions subside. Therefore, they play a vital role in cellular stress responses by managing and mitigating stress-induced damage (Protter and Parker, 2016).

SGs are characterized by their non-uniform structures, typically featuring a dual-layered organization. This arrangement comprises a densely packed central core and a more dynamic shell. Within the central core of SGs, higher concentrations of specific components, such as the protein G3BP1 and mRNAs, are found. This core is enveloped by a shell that holds the majority of SG components, albeit at lower concentrations (Jain et al., 2016). The formation of SGs is a highly coordinated process initiated by the inhibition of translation initiation. This inhibition is primarily mediated by the phosphorylation of eIF2α or the disassembly of the cap-binding eIF4F complex. This leads to the disassembly of polysomes and the accumulation of untranslated messenger ribonucleoprotein (mRNP) complexes in the cytoplasm. These mRNP complexes consist of mRNAs not currently undergoing translation into proteins, along with the proteins bound to these mRNAs. Subsequently, the mRNP complexes recruit specific RNA-binding proteins (RBPs), such as G3BP and TIA1, to form the core structures of SGs. As these structures mature, they recruit additional protein components, many of which are enriched with IDRs (Protter and Parker, 2016; Yang et al., 2020). The composition of SGs is intricate, encompassing various molecules including RNAs, RBPs, ribosomal subunits, and translation initiation factors involved in the mRNA-to-protein translation process. Additionally, SGs also harbor molecular chaperones and specific signaling proteins, such as kinases, phosphatases, ATPases, GTPases, DNA/RNA helicases, and ubiquitin-modification enzymes. Notably, SGs exhibit substantial variations in composition based on the cellular context. For example, in neuronal cells, SGs possess a sophisticated array of proteins, with an enrichment of chaperones and autophagy factors compared to non-neuronal cells (Markmiller et al., 2018).

When the stressor is eliminated or the cell adapts to the stress, SGs undergo a disassembly process. This critical phase enables the previously sequestered mRNAs to reintegrate into the pool of molecules available for translation, thereby aiding in the recovery and restoration of normal cellular function. The disassembly of SGs is regulated by a spectrum of cellular factors, including molecular chaperones (HSP40/70) that assist proteins in attaining their correct conformation, and DNA/RNA helicases responsible for unwinding DNA and RNA molecules (Chalupníková et al., 2008; Protter and Parker, 2016; Yoo et al., 2022). Additionally, autophagy plays a vital role in clearing SGs, preventing their persistent accumulation in the cell, which could otherwise be deleterious (Buchan et al., 2013). Recent studies emphasize the significance of two post-translational modifications, SUMOylation and ubiquitination, in governing the disassembly of SGs (Keiten-Schmitz et al., 2020). The processes associated with disassembly are typically energy-intensive and are believed to be orchestrated by mechanisms relying on ATP (Hondele et al., 2019). Notably, the duration of stress also influences the disassembly of SGs, with prolonged stress potentially altering the mode of disassembly from autophagy-

independent to autophagy-dependent degradation (Gwon et al., 2021).

As described above, the canonical function of SGs is to selectively sequester mRNAs, preventing their translation into proteins. Despite their inhibitory impact on global protein translation, SGs retain the ability to selectively translate transcripts crucial for the stress response, such as ATF4 (Mateju et al., 2020). This mechanism conserves cellular energy, enabling the prioritization of essential processes during stress conditions. In addition to their role in mRNA storage and translation regulation, SGs contribute to the regulation of stress-related signaling cascades by recruiting specific signaling molecules (Fujikawa et al., 2023; Park et al., 2020). For instance, sequestration of the apoptosis regulatory factor RACK1 into SGs reduces caspase-3 activation, which prevents stress-induced apoptosis (Park et al., 2020). A recent study has unveiled a novel function of SGs associated with endolysosomes. Specialized SGs rapidly form at damaged endomembrane sites, acting as stabilizing plugs and facilitating effective endolysosome membrane repair (Bussi et al., 2023). This discovery suggests that SGs may play diverse roles in cellular homeostasis and stress response beyond their recognized functions. It is crucial to note that the functions of SGs can vary depending on the nature of the stressor. For example, SGs induced by vinca alkaloids adhere to the canonical role by preventing cell death and supporting cell viability (Szaflarski et al., 2016). Conversely, sodium selenite triggers the formation of non-canonical SGs characterized by reduced recruitment of eIF3B and other typical SG components, indicating deficiencies in pro-survival functions (Fujimura et al., 2012).

Processing bodies

P-bodies are consistently present at a basal level in the cytoplasm of numerous cell lines (Hirose et al., 2022). They were initially termed "XRN1 foci" due to the distinct granular subcompartmentalization of the exoribonuclease XRN1 within the cytoplasm. Subsequent research revealed the co-localization of various proteins associated with mRNA turnover in these cytoplasmic regions. Consequently, these structures are commonly referred to as mRNA "processing bodies" (Ivanov et al., 2019). Characteristically, P-bodies exhibit a spherical shape with a diameter ranging between 0.2 and 1 μm when observed in the cytoplasm (Hirose et al., 2022).

P-bodies are primarily composed of mRNAs interacting with a multitude of proteins involved in mRNA decay (such as the 5′–3′ exoribonuclease XRN1, DCP1/DCP2, DDX6, EDC3, and EDC4) and translational repression (including 4E-T and CPEB1). These P-body mRNAs are characterized by low GC content and specific uncommon codons that result in a less efficient translation process (Hubstenberger et al., 2017). The P-body-associated proteins are often enriched with IDRs, which mediate the multivalent interactions between proteins and/or RNAs, facilitating P-body assembly through LLPS (Roy et al., 2022). The compositions of P-bodies are highly dynamic, with frequent flux of both proteins and RNAs in and out, a process influenced by translational activity. The assembly of P-bodies is facilitated by translation inhibition during stress conditions, such as glucose deprivation and heat stress (Roy et al., 2022). Disruptions in mRNA decapping, degradation, or translation initiation enlarge the size and number of P-bodies by increasing the number of mRNPs within these structures. Conversely, preventing translation inhibition decreases P-body size and number by reducing the levels of P-body components (Buchan et al., 2010).

Despite their association with mRNA decay, disruptions in P-body assembly do not interfere with global and local mRNA decay pathways (Ivanov et al., 2019). Subsequent studies revealed that mRNAs sequestered in P-bodies undergo translational repression rather than degradation. This mechanism allows a rapid return of mRNAs into the translational pool as needed, thereby facilitating the local control of mRNA expression (Hubstenberger et al., 2017). Interestingly, P-bodies and SGs are functionally interconnected, playing crucial roles in RNA metabolism. They often spatially interact and exchange contents under stress conditions. Overexpression of proteins such as TTP and BRF1 leads to increased clustering and fusion between these cytoplasmic MLOs (Hirose et al., 2022; Kedersha et al., 2005).

Nucleolus

The nucleolus, recognized as one of the most prominent MLOs, stands as one of the earliest intracellular structures to be thoroughly characterized in eukaryotic cells. Initial descriptions of the nucleolus date back 200 years, prompting extensive research aimed at unraveling its essential role in ribosome biogenesis. This role encompasses the synthesis of pre-ribosomal RNA (pre-rRNA), along with the intricate processes of ribosomal RNA (rRNA) synthesis, processing, and modification, culminating in ribosome assembly (Antifeeva et al., 2022; Hirose et al., 2022). Situated at the actively transcribed rDNA loci, the nucleolus typically adopts a well-defined round shape with a size ranging from 0.5 to 8 μm. Nevertheless, its morphology exhibits considerable variation among different cell types (Lafontaine et al., 2021).

In mammals, the nucleolus exhibits a highly organized structure comprising three distinct subcompartments arranged from the innermost to the outermost as follows: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). These subcompartments play a pivotal role in a series of sequential steps that contribute to ribosome production (Lafontaine et al., 2021; Shan et al., 2023). The FC region serves as the repository for RNA polymerase I (Pol I), nucleolar transcription factor 1 (UBF), and ribosomal DNA (rDNA). At the FC-DFC interface, a platform is established for the transcription of pre-rRNA from rDNA genes, a process driven by Pol I. The DFC, housing fibrillarin and various small nucleolar ribonucleoprotein complexes, is a crucial site where the initial co-transcriptional processing and modification of pre-rRNA take place. Meanwhile, the GC contains nucleophosmin, which acts in the late stages of pre-rRNA processing and subsequent ribosomal assembly (Hirose et al., 2022). An intriguing study by Feric et al. demonstrated that the mixing of fibrillarin and nucleophosmin *in vitro* successfully recapitulated the layered organization observed within nucleoli. This finding underscores the significance of the biophysical properties of nucleolar proteins in shaping the intricate layered structure (Feric et al., 2016). Moreover, the diverse biophysical and viscoelastic properties exhibited by different nucleolar compositions contribute to enhancing the efficiency of rRNA processing reactions and ribosome assembly (Feric et al., 2016). Notably, a recent study reveals that structural variation of the nucleolus may also be linked to the changes in micropolarity among the distinct layers. Specifically,

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the outermost GC layer exhibits a higher micropolarity compared to the inner DFC layer. If their micropolarity is reversed, the DFC layer becomes localized outside of the GC layer (Ye et al., 2023).

Cajal bodies

Cajal bodies are subnuclear organelles situated within the nucleoplasm of eukaryotic cells. These intriguing structures derive their names from the pioneering neurobiologist Ramón y Cajal, who first identified them in neurons over a century ago. Displaying a typically round shape, Cajal bodies vary in size (ranging from 0.1 to 2 μm in diameter) and number (ranging from 0 to 6 per cell). The specific dimensions depend on the cell type and the particular stage of the cell cycle (Hirose et al., 2022).

Cajal bodies exhibit a distinctive coiled-coil structure and encompass several components involved in diverse processes such as transcription, splicing, small nuclear RNA (snRNA) processing, and signaling. These components include p80/Coilin, SMN (survival of motor neuron) protein, small nuclear ribonucleoproteins (snRNPs), and specific small nucleolar RNAs (snoRNAs). It is noteworthy that these factors may undergo assembly or processing within Cajal bodies before being transported to their respective action sites (Sawyer and Dundr, 2018). Coilin, identified as a marker protein for Cajal bodies, plays an essential role in orchestrating their assembly and maintenance (Sawyer and Dundr, 2018). Specifically, the N-terminal domain of Coilin mediates multivalent interactions between Coilin and NOPP140, facilitating biomolecular condensation within the nucleus (Courchaine et al., 2022). It is essential to emphasize that active transcription of gene loci targeted by Cajal bodies is imperative for the assembly process of Cajal bodies, with U snRNAs potentially acting as stabilizers. Additionally, Cajal bodies exhibit a notable abundance of Dyskerin and Fibrillarin. Key regulators influencing Cajal body function include TCAB1/WRAP53, USPL1 (ubiquitin-specific peptidase-like 1), and various proteins from the small nucleolar ribonucleoprotein (snoRNP) family. Moreover, Cajal bodies are enriched with elements facilitating the generation of telomerase ribonucleoproteins (RNPs), which are crucial for the maintenance of telomeres (Machyna et al., 2013).

Cajal bodies exhibit dynamic behaviors in the nucleoplasm: they often fuse to form larger structures or undergo division to give rise to new individual Cajal bodies. Moreover, Cajal bodies engage in the exchange of their molecular components with the surrounding nucleoplasm, with different proteins exhibiting varying rates of exchange (Machyna et al., 2013). Photobleaching experiments suggest a rapid and continuous interchange of proteins between Cajal bodies and the nucleoplasm (Dundr et al., 2004). The regulation of Cajal body formation and dynamics is a nuanced process involving a complex interplay of proteins, notably Coilin, SMN, and various snRNP components. For example, depleting Coilin leads to the absence of Cajal bodies in organisms such as *Drosophila melanogaster*, zebrafish, or mice (Hirose et al., 2022). Conversely, the overexpression of SMN in HeLa cells induces a significant augmentation in both the size and number of Cajal bodies (Hao et al., 2007).

Cajal bodies participate in various facets of RNA-related processes. Primarily, Cajal bodies have a pivotal function in the biogenesis of snRNPs. These snRNPs are indispensable components that contribute to the intricate machinery known as the spliceosome, responsible for gene splicing. Cajal bodies also participate in the assembly of other RNPs, such as telomerase RNPs (Sawyer and Dundr, 2018). Furthermore, the size, number, and/or composition of Cajal bodies undergo alterations in response to various stressors, including UV radiation, heat shock, transcript suppression, osmotic stress, and nutrient deprivation. This implies that Cajal bodies might play a role in cellular responses to stress (Boulon et al., 2010).

Gems

Gemini of coiled bodies, known as gems, are nuclear MLOs with a diameter ranging from 0.1 to 1 μm and a variable quantity per cell (0–6). These structures are commonly found near or associated with Cajal bodies, and their morphologies are often indistinguishable under the microscope (Hirose et al., 2022). While the precise function of gems remains under investigation, their significance has been underscored by their association with a devastating motor neuron disease known as spinal muscular atrophy (SMA).

Despite their morphological similarity, the constituents of gems are significantly different from those found in Cajal bodies. Gems lack snRNPs and are primarily composed of the SMN complex and a protein known as ZPR1. The SMN complex includes the SMN1 protein (also referred to as GEMIN1) and gem-associated proteins 2–8 (GEMIN2–8) (Meister, 2002). The human *SMN* gene, located on human chromosome 5q13, is duplicated, with the *SMN1* gene being the telomeric copy and the *SMN2* gene being the centromeric copy. Deletion of the *SMN1* gene in humans results in SMA, wherein spinal motor neurons degenerate, causing progressive paralysis and muscular atrophy (Lefebvre et al., 1995). The SMN complex plays a crucial role in snRNP biogenesis and is also associated with the assembly of snoRNP particles and the RNA polymerase II transcription/processing machinery (Meister, 2002; Pellizzoni et al., 2001). Notably, a recent study suggests that the Tudor domain of SMN facilitates condensation by binding dimethylarginine (DMA). The distinction between asymmetric and symmetric DMA determines whether gems and Cajal bodies remain separated or docked to one another (Courchaine et al., 2021). While the exact role of gems is not yet fully understood, it is proposed that they may support Cajal bodies in snRNP biogenesis and be responsible for further maturation, storage, or recycling of snRNPs.

Phase separation associated with mitochondria

Mitochondria are the only semi-autonomous organelle, possessing circular mitochondrial DNA (mtDNA) molecules compacted into suborganellar structures called nucleoids in mammalian cells. As the energy factories of the cell, mitochondria have evolved a two-layer membrane structure separated by the interspace, which efficiently compartmentalizes a variety of essential mitochondrial activities. Besides ATP production, mitochondria are involved in many biological processes, including mtDNA transcription, translation, replication, and storage. Many lines of evidence suggest that phase separation enables formation of multiple mitochondrial MLOs, which help to specify the unique structure and function of mitochondria.

Phase separation in mitochondrial nucleoids

Mitochondrial DNA is compacted into nucleoids by mitochon-

drial transcription factor A (TFAM), one of the most abundant proteins in mitochondria, which is considered as the histone-like protein in these organelles (Kukat et al., 2015). TFAM has the ability to phase separate both *in vivo* and *in vitro* (Feric et al., 2021; Long et al., 2021). TFAM is a classical DNA-binding protein, which belongs to the high mobility group (HMG) box family. TFAM contains two HMG domains separated by a disordered linker domain and flanked by an intrinsically disordered C-tail. Nucleoids are self-assembled by phase separation of TFAM-mtDNA, and the linker domain contributes to the phase separation of TFAM. The interaction between TFAM and mtDNA provides the multivalent effect in the formation of nucleoid condensates (Long et al., 2021).

TFAM is not only an organizer of nucleoids, but also a member of the transcription initiation complex. Compared to nuclei, mitochondria have their own specific machineries for DNA transcription (Hillen et al., 2018). Initiated by the phase separation of nucleoids, recruitment of the mitochondrial transcription initiation complex further promotes the formation and stability of nucleoids, which also concentrate substrates such as nucleoside triphosphates (NTPs) for efficient transcription. POLRMT, the RNA polymerase for mtDNA transcription, is unable to undergo phase separation alone. Nevertheless, POLRMT coats the core TFAM-mtDNA structure both *in vitro* and in cells, forming a multilayered phase-separating structure that may play an important role in transcription regulation. The ring structure of POLRMT surrounding the surface of the nucleoid may keep transcription in a standby or low activity status. This can be broken by mitochondrial transcription factor B2 (TFB2M), which melts promoters, or by mitochondrial transcription elongation factor (TEFM), which promotes transcript elongation. Moreover, a similar phenomenon of multilayered phase separation of the mitochondrial transcription termination factor MTERF1 has been observed. Thus, unique architectures based on multilayered phase separation are of importance for the regulation of mitochondrial transcription.

Phase separation orchestrates the roles of TFAM in both mitochondrial nucleoid organization and transcription. TFAM undergoes multiple post-translational modifications (PTMs) (Li et al., 2022a), which may regulate its phase separation.

Phase separation of mitochondrial RNA granules

Human mitochondrial DNA contains 22 tRNA genes, 2 rRNA genes, and 13 genes encoding a subset of the protein subunits of the electron transport chain complexes (Anderson et al., 1981). In the mitochondrial matrix, all kinds of mitochondrial RNAs (mtRNAs), RNA processing proteins and mito-ribosome assembly factors form membraneless structures, termed mitochondrial RNA granules (MRGs) (Xavier and Martinou, 2021). Nucleoids, MRGs, and RNA-protein granules form in the mitochondrial matrix by phase separation (Banani et al., 2017; Rey et al., 2020). Within the MRGs, two MRG-associated proteins, FASTKD2 (Fas-activated serine/threonine kinase domains 2) and GRSF1 (guanine-rich sequence binding factor 1), which are RBPs, show high mobility, revealing that the MRG condensates are in a liquid-like status (Antonicka and Shoubridge, 2015; Jourdain et al., 2013). The morphology of MRGs is dependent on mitochondrial dynamics: inhibition of mitochondrial fission or fusion leads to aberrant accumulation of MRGs into clusters (Quintana-Cabrera and Scorrano, 2023).

Mutations in GRSF1 or FASTKD2 are associated with severe mitochondrial diseases (Ghezzi et al., 2008; Wei et al., 2020; Yoo et al., 2017). Studies uncovering the relationship between the disease-associated mutations of the two MRG markers and phase separation of MRGs may provide important clues for the prevention and treatment of related mitochondrial diseases.

Phase separation in mitochondrial fission

Mitochondria are highly dynamic organelles which undergo continuous fission and fusion to maintain or adapt their morphology, distribution, size, and function. Mitochondrial dynamics are crucial for many cellular processes such as mitophagy, apoptosis, and immunity (Tait and Green, 2012). Mutations or defects in proteins related to mitochondrial fission and fusion are associated with many human diseases (Chan, 2020). Recently, DRP1 (dynamin-related protein 1), a core regulator of mitochondrial fission, has been reported to undergo LLPS driven by its variable domain (VD) (Posey et al., 2023). LLPS enhances membrane binding of DRP1 and can be further promoted by the mitochondrial lipid cardiolipin. The VD is intrinsically disordered and able to undergo phase separation in the presence of crowding agents. LLPS may modulate DRP1 activity and/or other steps of mitochondrial fission and fusion.

Phase separation and neurodegenerative disorders

LLPS in repeat expansion disorders

Expansion of short tandem nucleotide repeats in specific genes causes various monogenic diseases, many of which are neurodegenerative disorders. These diseases are referred to as repeat expansion disorders (REDs). Over 40 REDs have been discovered since the discovery of a CGG repeat expansion in the non-coding region of the *FMR1* gene in 1991 (Verkerk et al., 1991). The pathogenic mechanisms of almost all REDs are unclear, and there has been a lack of effective therapeutic approaches. Several major pathogenic mechanisms have been proposed. For repeat expansions in the coding region, the prevailing view is that they may cause the disease via their encoded repeat-containing polypeptides/proteins, particularly an expanded polyglutamine tract (poly-Q) encoded by the CAG repeat (Feng et al., 2018). In comparison, the pathogenic mechanisms related to repeat expansions in the non-coding region are more controversial. They may cause the disease via repeat-containing polypeptides synthesized by repeat-associated non-AUG (RAN) translation (Cleary and Ranum, 2017); they may also cause the disease via a loss-of-function mechanism due to failed protein synthesis, or via toxicity of the repeat-containing RNA, which may form RNA foci or sequester proteins.

Both the repeat expansion polypeptides and the repeat expansion RNAs may undergo LLPS and phase transition. Phase transition refers to a process following phase separation, during which phase-separated condensates transition from one material state to another, e.g., liquid-to-solid transition. In this section, we will discuss several recent studies in this emerging field, focusing on relevant pathogenic contributions and mechanisms, along with potential therapeutic approaches.

LLPS of repeat expansion polypeptides

One of the most extensively studied repeat expansion polypeptide

is the expanded poly-Q stretch encoded by the expanded CAG repeat, which is the genetic cause of many neurodegenerative disorders including Huntington's disease (HD) and several major types of spinocerebellar ataxias (SCAs). Among the different poly-Q proteins, the mutant HTT protein (mHTT) that causes HD is the best studied one, possibly because HD has the highest prevalence among the poly-Q diseases worldwide and the largest number of animal models. The traditional view is that mHTT may form oligomers and aggregates from monomers in a poly-Q length- and time-dependent manner (Iuchi et al., 2003; Kim et al., 2016c; Legleiter et al., 2010; Olshina et al., 2010; Poirier et al., 2002; Yamada et al., 2008). Recent studies suggest that mHTT may undergo phase separation and phase transition to form aggregates (Aktar et al., 2019; Peskett et al., 2018; Posey et al., 2018). *In vitro* studies using purified HTT N-terminal fragments suggest that they form at least three phases irrespective of the poly-Q length: the M phase (consisting of soluble monomers and oligomers), the S phase (mainly consisting of bigger soluble aggregate spheres sized about 25 nm in diameter), and the F phase (mainly consisting of insoluble fibrillar aggregates) (Posey et al., 2018). *In vitro*, in yeast cells, or in mammalian cells, the exon 1-encoded HTT fragment fused with GFP (HTTex1-GFP) may form dim liquid-like assemblies via LLPS and transform into bright solid-like assemblies via phase transition (Peskett et al., 2018). A similar study in yeast cells also observed phase-separated mutant HTTex1-GFP assemblies, which were not "fully liquid" nor solid but rather gel-like (Aktar et al., 2019). While these studies reported the common findings of LLPS or phase transition of HTT N-terminal fragments, the potential pathological significance of the phenomenon was not demonstrated at all. Further functional investigations may require the discovery of key amino acids required for LLPS of HTT and the development of tools to modulate it.

Another example is the dipeptide repeat proteins (DPRs) expressed by the GGGGCC repeat expansion in the *C9orf72* gene, which is the most prevalent known genetic cause for amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Three of the five DPRs expressed from this mutation are toxic: poly-PR, poly-GR, and poly-GA. Molecular dynamics modeling demonstrates that longer DPRs have a higher propensity to phase separate. The pathogenic threshold (~30) in patients is comparable to the threshold repeat length required for LLPS (25 for poly-PR and 50 for poly-GA), suggesting that the LLPS of DPRs may play an important pathogenic role (Jafarinia et al., 2020). In the cellular context, the highly positively charged repetitive Arg (R)-rich dipeptides (poly-PR and poly-GR) may bind to proteins and nucleic acids at multiple sites via electrostatic forces and cation-π interactions, resulting in LLPS and disturbance of proteins through entrapment in the DPR condensates (Chen et al., 2021). Proteomic analyses revealed that the alternate Arg distribution in poly(PR) is required for the sequestration of proteins with acidic motifs via LLPS. In addition to their own LLPS, DPRs may also influence other MLOs such as nucleoli and stress granules (Boeynaems et al., 2017; Chen et al., 2021).

LLPS of repeat expansion RNAs

In addition to repeat expansion polypeptides, recent research has unveiled the LLPS of repeat expansion RNAs as a pivotal factor in the pathogenesis of several neurodegenerative disorders. In particular, several neurodegenerative disorders—including myotonic dystrophy, fragile X-associated tremor/ataxia syndrome (FXTAS), and *C9orf72*-associated ALS/FTD—are hallmarked by the presence of RNA foci and ribonucleoprotein (RNP) granules within affected cells (Van Treeck and Parker, 2018).

Most LLPS studies are "protein-centric", and RNAs are typically considered as modifiers of protein LLPS by interacting with or influencing the central proteins which assemble the MLOs. Meanwhile, through multivalent intermolecular interactions, including Watson–Crick and non-canonical base pairing, RNA alone can phase separate without the help of proteins. For example, long CUG or CAG or GGGGCC repeat RNAs can form solid-like RNA droplets through phase separation *in vitro* (Jain and Vale, 2017). Similar condensates are also observed in cells, but mostly in the nuclei (Jain and Vale, 2017). These condensates also dissolve when treated with inhibitors of RNA base pairing (Jain and Vale, 2017).

Repeat expansion RNAs may also fold into complex structures, including G-quadruplexes, which aberrantly interact with and sequester RBPs into these RNA condensates (Malik et al., 2021). This mechanism may contribute to disease pathogenesis by depleting RBPs from the nucleoplasm. For instance, in myotonic dystrophy, important alternative splicing factors (MBNL proteins) are sequestered by the expanded CUG repeat RNA in the nuclei, leading to a transcriptome-wide spliceopathy that can be ameliorated by MBNL1 (Kanadia et al., 2006; Miller, 2000; Wang et al., 2019). Besides influencing splicing, RBP sequestration may also influence other RNA maturation reactions such as microRNA biogenesis (Sellier et al., 2013) and alternative polyadenylation (Batra et al., 2014). Finally, the nuclear sequestration of RBPs may also shift RBP localization away from the cytoplasm, impairing their cytoplasmic functions such as modulating mRNA stability, assembling cytoplasmic stress granules, and mediating RNA transport (Malik et al., 2021).

In comparison, LLPS of cytoplasmic RNA has been largely unexplored, possibly due to the absence of cytoplasmic RNA foci in previous studies (Jain and Vale, 2017). More recent studies revealed that cytoplasmic CAG repeat expansion RNAs do undergo LLPS and form gel-like condensates, which are rapidly recognized by the lysosomes for degradation and thus difficult to observe (Pan et al., 2023). Meanwhile, assembly of these cytoplasmic CAG repeat expansion RNA gels may sequester the key translation elongation factor eEF2, leading to suppressed global protein synthesis and possible impairment of neuronal functions (Pan et al., 2023). Noticeably, eEF2 cannot phase separate alone; it only co-separates with the CAG repeat expansion RNA (Pan et al., 2023), confirming a central role of the RNA in this case.

The functional roles of both nuclear and cytoplasmic RNA LLPS and phase transition remain to be further elucidated, and technologies manipulating such processes—such as optogenetic methods—are desired (Li et al., 2022b; Pan et al., 2023).

Phase separation and amyotrophic lateral sclerosis/ frontotemporal dementia

The pathogenesis of neurodegenerative diseases such as ALS and FTD involves the abnormal aggregation and accumulation of proteins, especially RBPs, within neural cells (Purice and Taylor, 2018; Feldman et al., 2022; Grossman et al., 2023). Recent studies have shed light on the emerging role of LLPS of RBPs in the formation and dynamics of biomolecular condensates, which

have implications for the development and progression of both ALS and FTD (Naskar et al., 2023; Song, 2023). In this section, we will discuss the current understanding of the relationship between LLPS, RBPs, and ALS/FTD, highlighting their potential implications for disease pathogenesis.

Overview of ALS and FTD

ALS is a progressive neurodegenerative disease. It mainly affects motor neurons, the nerve cells responsible for controlling voluntary muscle movement, which gradually degenerate and die. This results in a progressive loss of muscle control and, eventually, the inability to move, speak, swallow, and breathe. The symptoms of ALS may vary from person to person, but typically include muscle weakness, muscle atrophy, muscle cramps, difficulty in speaking and swallowing, and finally paralysis. The disease primarily affects the voluntary movements, but leaves other bodily functions, such as thinking and memory, intact. The cause of ALS is not fully understood, although a combination of genetic and environmental factors contribute to the development of the disease. Some ALS cases are inherited, while the majority of cases occur spontaneously without any known family history (van Es et al., 2017; Feldman et al., 2022).

FTD, also referred to as frontotemporal lobar degeneration (FTLD), is a progressive neurodegenerative disorder that primarily affects neurons in the frontal and temporal lobes of the human brain. It is one of the most common forms of dementia, second only to AD in individuals under the age of 65. Unlike AD, which primarily affects memory, FTD often presents with changes in personality, social behavior, language and other executive functions, while memory initially remains intact. These changes can manifest as emotional instability, apathy, social withdrawal, language problems, and impairments of planning, decision-making, and problem-solving. Like ALS, the exact cause of FTLD is unclear, although both genetic and environmental factors play a role in the disease pathogenesis. In some cases, FTD can be inherited in an autosomal dominant manner, with mutations in specific genes associated with the disease (Grossman et al., 2023).

There is currently no definitive cure for ALS or FTD. Some medications, physical therapy and assistive devices are available, with the aim of relieving symptoms, providing supportive care, aiding in mobility and communication, slowing down the progression of the disease, and improving quality of life.

The link between RBPs and ALS/FTD

RBPs play crucial roles in the regulation of RNA metabolism, including transcription, splicing, transport, and translation. Upon cellular stress, clusters of RBPs undergo LLPS to form biomolecular condensates, including SGs (see "Stress granules" section), in the cytoplasm (Baradaran-Heravi et al., 2020; Fakim and Vande Velde, 2024) and TDP-43 nuclear bodies (NBs) in the nucleus (Wang et al., 2020; Gu et al., 2021; Yu et al., 2021a).

Both genetic and pathological evidence has provided significant insights into the link between RBPs and ALS/FTD. Genetic studies have uncovered missense mutations in the genes encoding several key RBPs in patients with familial or sporadic cases of ALD or FTD. These RBPs include TDP-43 (transactive response DNA binding protein of 43 kD), FUS, hnRNP A1, TIA-1 (T cell intracellular antigen-1), EWSR1 (EWS RNA-binding protein 1), etc. (Chen-Plotkin et al., 2011; Ling et al., 2013; Renton et al., 2014; Taylor et al., 2016; Mathis et al., 2019). In addition, the most common genetic cause of familial ALS and FTD is an expansion of a hexanucleotide repeat (GGGGCC) in the non-coding region of the *C9orf72* gene (see "LLPS of repeat expansion polypeptides" section). Although the physiological function of *C9orf72* is not yet clear, it is believed to play a role in RNA metabolism, nucleocytoplasmic transport, vesicle trafficking, and autophagy. As discussed in "LLPS of repeat expansion polypeptides" section, the repeat expansion leads to the formation of abnormal aggregates containing RNAs and dipeptide repeat proteins, which disrupts various cellular processes and underlies the development of ALS and FTD (Peters et al., 2015; Zhang et al., 2015; Aoki et al., 2017; Shi et al., 2018; Zhu et al., 2020b).

More importantly, the ALS/FTD-associated RBPs are known to become insoluble and form protein inclusions. For example, in normal cells, TDP-43 is predominantly nuclear and regulates RNA processing; however, in the disease environment, it becomes abnormally aggregated and forms cytoplasmic inclusions, which are a hallmark pathological feature of ALS, FTD and several other neurodegenerative diseases (Mackenzie et al., 2010; Ratti and Buratti, 2016; Neumann and Mackenzie, 2019). It is believed that the RBPs undergo LLPS and abnormal liquid-to-solid phase transition, which gives rise to the pathological protein inclusions (Purice and Taylor, 2018; Naskar et al., 2023; Song, 2023). These RBP aggregates disrupt RNA homeostasis and impair the pathways required for cellular functions, resulting in degeneration and loss of neurons in the affected brain regions (the motor cortex and spinal motor neurons in ALS and the frontal and temporal lobes in FTD), which leads to a progressive decline in motor or cognitive functions (Neumann and Mackenzie, 2019; Feldman et al., 2022; Grossman et al., 2023).

Factors regulating protein condensation of ALS/FTD-associated RBPs

Almost all of the disease-associated RBPs contain intrinsically disordered and/or low-complexity domains, which drive the LLPS of RBPs. A combination of factors can regulate the LLPS of RBPs and the properties of the biomolecular condensates. A few such key factors are discussed below.

(1) Concentration and stoichiometry: Similar to other IDPs, the concentration of RBPs is a critical factor in LLPS. Increasing the protein concentration can promote the formation of RPB condensates (Kato et al., 2012; Lin et al., 2015; Molliex et al., 2015; Pak et al., 2016; Babinchak et al., 2019; Tsoi et al., 2021). Meanwhile, the stoichiometry of RBPs and their RNA-binding partners can also influence LLPS (Burke et al., 2015; Farag et al., 2023). Therefore, alterations in concentration or stoichiometry can modulate the assembly and dynamics of the RBP condensates.

(2) Subcellular micro-environments: Cellular conditions such as osmolarity (Babinchak et al., 2019; Hans et al., 2020; Tsoi et al., 2021; Gao et al., 2022a), temperature (McDonald et al., 2011; Udan-Johns et al., 2014; Kroschwald et al., 2018; Babinchak et al., 2019;), pH (Babinchak et al., 2019; Tsoi et al., 2021; Pintado-Grima et al., 2022), and the presence of specific ions such as Ca^{2+} and metabolites such as ATP (Jain et al., 2016; Kroschwald et al., 2018; Kang et al., 2019; Nahm et al., 2020; Dang et al., 2022; 2023) all impact on condensate formation. Changes in these environmental factors can alter the driving forces for phase separation, affecting the stability and properties of the RBP condensates.

(3) Post-translational modifications: Various PTMs, including phosphorylation, ubiquitination, methylation, acetylation, PAR-

ylation, and glutathionylation, can regulate the phase separation behavior and aggregation of ALS/FTD-associated RBPs (Table 1). PTMs can alter the charge, hydrophobicity, and other biophysical properties of the RBPs, affecting the formation of liquid droplets, protein condensates, and pathological aggregates of RBPs.

(4) Protein-protein interaction and competition: RBPs can interact with other proteins, cofactors, and cellular components, affecting LLPS. Partner proteins such as nuclear importer receptors and protein chaperones modulate the LLPS behavior of RBPs and disaggregate wild-type RBPs and their disease-associated mutants (Guo et al., 2018; Hofweber et al., 2018; Qamar et al., 2018; Gu et al., 2020; Liu et al., 2020; Gu et al., 2021; Yu et al., 2021a; Lu et al., 2022), while RNA helicases and ATP-dependent RNA chaperones limit the condensation of RNA and reduce the formation of SGs (Jain et al., 2016; Tauber et al., 2020). Meanwhile, RBPs can compete for the binding sites and the interaction with other RBPs or binding partners in the condensates (Wang et al., 2018b; Yang et al., 2020; Gu et al., 2021; Boeynaems et al., 2023). This cooperation or competition influences the interplay between RBPs and their interaction partners, thereby modulating the LLPS behaviors and regulating the composition and function of the RBP condensates.

(5) Protein-RNA binding: RBPs can bind to RNA molecules, which regulate the LLPS capability of the RBPs (Mann and Donnelly, 2021). RNA binding can promote LLPS by acting as a scaffold molecule or coacervating agent, bringing RBPs together (Schwartz et al., 2013; Bounedjah et al., 2014; Van Treeck and Parker, 2018; Guillén-Boixet et al., 2020; Wang et al., 2020; Tsoi et al., 2021). The type, sequence, length, and secondary structure of RNA can impact LLPS, with specific RNAs enhancing or inhibiting RBP condensate formation (Lee et al., 2013; Conlon et al., 2016; Fay et al., 2017; Maharana et al., 2018; Mann et al., 2019; Wang et al., 2020; Koehler et al., 2022). In addition, modifications of RNA *per se* can impact on protein condensates and their cellular functions (Liu et al., 2017; Anders et al., 2018; Ries et al., 2019; Fu and Zhuang, 2020; Sun et al., 2023; Zhao et al., 2023).

(6) Proteostasis regulation: The protein degradation machineries, such as the ubiquitin-proteasome system (UPS) and the autophagy pathway, mediate the turnover and clearance of biomolecular condensates including SGs (Ryu et al., 2014; Chitiprolu et al., 2018; Turakhiya et al., 2018; Zhang et al., 2019; Gwon et al., 2021). Malfunction of the UPS or autophagy can lead to aggregation of RBP condensates, a pathological hallmark observable in ALS and FTD patients. The related topics have been reviewed elsewhere (Hu et al., 2022; Tolay and Buchberger, 2022) and are not discussed in detail here.

It is important to note that the LLPS of RBPs is a complex and dynamic process, and the interplay of these factors can vary depending on specific RBPs, cellular contexts, and disease conditions. Further research is needed to gain a deeper understanding of these regulatory factors and their impact on physiological and pathological processes.

Protein condensation associated with Alzheimer's disease

AD is characterized by the presence of amyloid β (Aβ) plaques and Tau neurofibrillary tangles (Goedert and Spillantini, 2006; Hardy and Selkoe, 2002; Long and Holtzman, 2019). These amyloid fibrillar structures, comprising Tau and Aβ, are central to AD pathology, leading to issues including imbalanced proteostasis, microtubule instability, cell membrane damage, and neuroinflammation (Knopman et al., 2021; Li and Liu, 2022). Accumulation of these insoluble fibrillar inclusions in the brain is the key pathological hallmark and primary pathology of AD. Intriguingly, mounting evidence reveals that Tau and Aβ can undergo a process of LLPS (Figure 4A), forming dynamic condensates that change over time, a process known as condensate aging and maturation (Figure 4A) (Louros et al., 2023; Michaels et al., 2023). These condensates can eventually solidify into amyloid fibrils, which suggests that LLPS of Tau and Aβ is a possible step in the progression of AD (Nedelsky and Taylor, 2019; Shin and Brangwynne, 2017).

In vitro studies show that the full-length Tau protein predominantly engages in homotypic LLPS (Figure 4A), a process largely influenced by the electrostatic forces between its negatively charged N-terminal region and the positively charged middle and C-terminal domains (Boyko et al., 2019; Kanaan et al., 2020). The ionic strength of the buffer significantly impacts this interaction. Moreover, Tau condensation exhibits minimal sensitivity to 1,6-hexanediol, which is also consistent with the prominent role of electrostatic interactions in this process (Boyko et al., 2019).

The six Tau isoforms in humans, resulting from alternative splicing (Wang and Mandelkow, 2016), also play a role in modifying Tau LLPS characteristics. For instance, the 2N3R isoform, which condenses relatively slowly, can effectively reduce the condensation of the 2N4R isoform, and consequently slow down the fibrillation process of the 2N4R isoform (Boyko et al., 2020). This finding indicates a complex interplay among different Tau isoforms, and sheds light on the mechanistic complexity of Tau LLPS and fibrillation.

The phase separation behavior of Tau is further complicated by its interaction with RNA, polyanions, metal ions, and various protein binding partners (Boyko et al., 2020; Lin et al., 2019; Najafi et al., 2021; Singh et al., 2020; Zhang et al., 2017) (Figure 4A). Proteins like TIA1 and α-Synuclein (α-Syn) have been identified as participants in the heterotypic LLPS of Tau, which exacerbates the pathological impacts of Tau (Ash et al., 2021; Siegert et al., 2021). Under normal physiological conditions, Tau engages in LLPS with its natural binding partner—tubulin (Hernández-Vega et al., 2017). The interaction with tubulin α/β dimers, which can segregate into Tau condensates and initiate polymerization, leads to the formation of stable microtubule bundles (Figure 4B). Intriguingly, Tau maintains these microtubules in a quasi-liquid state. This interaction may trigger Tau LLPS at concentrations significantly lower than those required in the absence of tubulin (Tan et al., 2019). Importantly, disease-related phosphorylation of Tau can influence the assembly of microtubule bundles within these condensates (Savastano et al., 2021), implying the potential association between dysregulation of Tau LLPS-mediated microtubule assembly and the pathogenesis of AD.

The aging and maturation of Tau dynamic condensates are characterized by a crucial transition from a liquid to a solid phase. Recent studies indicate that phosphorylated Tau within condensates quickly loses its dynamic properties after prolonged incubation and forms aggregates, as evidenced by binding to Thioflavin S (ThS), a dye specific to amyloid aggregates (Wegmann et al., 2018). This transition signifies a dramatic shift of Tau biophysical characteristics, correlating with AD

Table 1. Summary of PTMs and their impact on the condensation/aggregation of ALS/FTD-associated RBPs

Key RBP	Impact on protein condensation and aggregation	References
	Phosphorylation	
TDP-43	Phosphorylation at S403/S404 increases TDP-43 insolubility	Hasegawa et al., 2008; Nonaka et al., 2016
	Phosphorylation at S409/S410 promotes fibril formation of TDP-43	Hasegawa et al., 2008
	Phosphomimetic S409/S410D reduces aggregation of C25 and C15 fragments	Brady et al., 2011
	Phosphorylation at S393/S395 promotes TDP-43 aggregation	Nonaka et al., 2016
	Phosphomimetic S48D disrupts TDP-43 LLPS	Wang et al., 2018a
	Phosphorylation of the C-terminus of TDP-43 reduces LLPS and aggregation	Gruijs da Silva et al., 2022
FUS	Phosphorylation of the LCD reduces FUS LLPS and aggregation	Han et al., 2012; Murray et al., 2017;Monahan et al., 2017
hnRNP A1	Phosphorylation of the C-terminus induces cytoplasmic accumulation of hnRNP A1	Allemand et al., 2005
	Phosphorylation promotes the RNA binding and SG association of hnRNP A1	Guil et al., 2006
	Phosphorylation of the F-peptide region induces hnRNP A1 accumulation	Bhattarai et al., 2022
hnRNP A2	Phosphorylation of the LCD reduces hnRNP A2 LLPS and aggregation	Ryan et al., 2021
G3BP1	Phosphorylation at S149 diminishes G3BP1 aggregation	Sahoo et al., 2018; 2020
	Phosphorylation at S149/S232 reduces G3BP1 LLPS	Guillén-Boixet et al., 2020; Yang et al., 2020
TIAR	Phosphorylation of the LCD promotes TIAR-2 granule formation	Andrusiak et al., 2019
	Ubiquitination and SUMOylation	
TDP-43	Ubiquitination promotes TDP-43 aggregation	Hebron et al., 2013
	Ubiquitination at K263 enhances TDP-43 insolubility	Hans et al., 2014
	Ubiquitination decreases TDP-43-Q331K aggregation	van Well et al., 2019
	Ubiquitination of the RRM reduces TDP-43 condensation	Keiten-Schmitz et al., 2020
FUS	SUMOylation of the RRM inhibits FUS accumulation in SGs	Keiten-Schmitz et al., 2020
G3BP1	Ubiquitination of the N-terminus diminishes G3BP1 condensation	Gwon et al., 2021
	Ubiquitination inhibits G3BP1 LLPS *in vitro*	Yang et al., 2023
	Arginine methylation	
FUS	Arginine methylation modulates nuclear import and SG recruitment of FUS	Dormann et al., 2012; Tradewell et al., 2012
	Arginine methylation reduces SG formation and insoluble aggregation of FUS	Yamaguchi et al., 2012
	Inhibition of arginine methylation reduces cytoplasmic inclusions of FUS	Tradewell et al., 2012; Scaramuzzino et al., 2013
	Excessive inhibition of arginine methylation induces nuclear aggregation of FUS	Fujii et al., 2016
	Arginine methylation of the RGG motif reduces FUS LLPS	Hofweber et al., 2018; Qamar et al., 2018; Wang et al., 2022b
hnRNP A1	Arginine methylation of the RGG motif reduces hnRNP A1 LLPS	Wang et al., 2022b
hnRNP A2	Arginine methylation of the LCD reduces hnRNP A2 LLPS	Ryan et al., 2018
G3BP1	Arginine methylation of the N-terminus suppresses G3BP1 condensation	Tsai et al., 2016
	Arginine methylation at R435/R447/R460 suppresses G3BP1 condensation	Tsai et al., 2017
	Acetylation	
TDP-43	Acetylation at K145/K192 promotes TDP-43 LLPS and aggregation	Cohen et al., 2015; Wang et al., 2017; Wang et al., 2020; Yu et al., 2021a; Keating et al., 2023
	Acetylation of the C-terminal fragment reduces TDP-43 aggregation *in vitro*	Prasad et al., 2018
	Acetylation at K136 promotes TDP-43 LLPS and aggregation	Garcia Morato et al., 2022
FUS	Acetylation at K510 induces SG-like inclusions of FUS	Arenas et al., 2020
	Acetylation at K315/K316 reduces protein inclusions of FUS	Arenas et al., 2020
	Acetylation of the N-terminus facilitates LLPS and reduces aggregation of FUS	Bock et al., 2021
G3BP1	Acetylation at K376 reduces G3BP1 condensation	Gal et al., 2019
	PARylation and PAR binding	
TDP-43	PAR binding promotes TDP-43 LLPS and cytoplasmic accumulation	McGurk et al., 2018a; 2018b
	TDP-43 can be PARylated *in vitro* but its PARylation is not detected in cells	Duan et al., 2019
FUS	PAR binding promotes FUS LLPS	Altmeyer et al., 2015; Patel et al., 2015; Rhine et al., 2022
	PAR binding mediates FUS condensation at DNA damage sites	Rulten et al., 2014; Altmeyer et al., 2015; Singatulina et al., 2019
hnRNP A1	PARylation regulates nuclear export of hnRNP A1	Duan et al., 2019
	PAR binding promotes hnRNP A1 LLPS and co-LLPS with TDP-43	Duan et al., 2019
G3BP1/2	PARylation of the RGGs promotes G3BP1 condensation	Leung et al., 2011
	Reduction of G3BP1/2 PARylation suppresses SG formation	Jayabalan et al., 2021
	PAR binding of G3BP1/2 promotes SG assembly	Isabelle et al., 2012; Jayabalan et al., 2021
	Glutathionylation	
FUS	Glutathionylation at C477 promotes FUS LLPS and aggregation	Cha et al., 2022

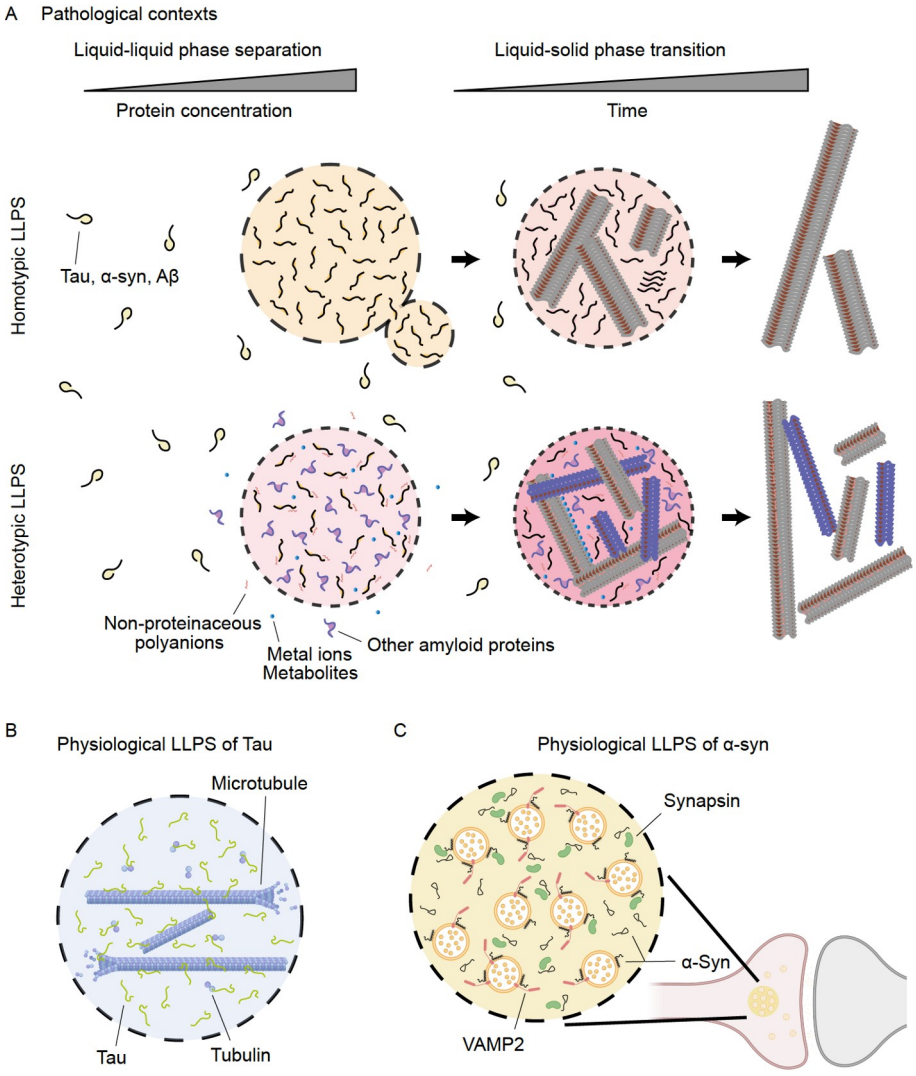


Figure 4. LLPS and liquid-solid phase transition of amyloid proteins under pathological and physiological conditions. A, Pathological phase transition of amyloid proteins. LLPS of amyloid proteins results in a dual-phase system with highly concentrated protein in the droplets. Amyloid proteins, such as Tau, Aβ, and α-Syn, are shown undergoing both homotypic (top) LLPS via self-association or heterotypic (bottom) co-LLPS in conjunction with other partners, accompanied by conformational changes. Over time, these dynamic droplets can undergo a transition from liquid to solid phase, a process referred to as droplet aging/maturation. These droplets may facilitate or nucleate the formation of amyloid fibrils. B, Tau undergoes LLPS via interaction with tubulin under physiological conditions, contributing to the promotion of microtubule polymerization. C, At presynaptic terminals, α-Syn forms liquid condensates with Synapsin and VAMP2. The co-LLPS results in the clustering of SVs into a concentrated liquid phase, thus playing a physiological role in synaptic function.

progression. Insight into Tau pathology is further gained by examining mutations associated with familial AD (Ingram and Spillantini, 2002). Recent studies on pathogenic Tau variants (G272V, ΔK280, and P301L) show that these mutations do not significantly change the inherent tendency of Tau to phase-separate compared to wild-type Tau (Boyko et al., 2020). However, they significantly accelerate fibrillation within the Tau condensates (Wegmann et al., 2018), which underscores the impact of genetic variations on Tau phase transition behavior and AD pathogenesis.

Tau LLPS is actively regulated by PTMs (Wang and Mandelkow, 2016). Phosphorylation, which increases the negative charge distribution of Tau, is particularly noteworthy. In AD, Tau exhibits extensive hyperphosphorylation at over 45 distinct sites, which profoundly influences the biophysical properties and activities of Tau (Arakhamia et al., 2020; Wesseling et al., 2020). The complex phosphorylation patterns of Tau notably increase the propensity of Tau to undergo LLPS (Ambadipudi et al., 2017; Kanaan et al., 2020; Savastano et al., 2021; Wegmann et al., 2018). Additionally, phosphorylation regulates the assembly of microtubules within Tau condensates, affecting the interactions of Tau with its physiological partners (Savastano et al., 2021). Another significant PTM of Tau is acetylation of lysine (Lys) residues, which neutralizes the positive charge. This diminishes both the homotypic and heterotypic LLPS of Tau, and specifically weakens its interaction with RNA (Ferreon et al., 2018; Ukmar-Godec et al., 2019).

Translating the *in vitro* findings on Tau LLPS to cellular and *in vivo* conditions, particularly in understanding their role and regulation in the human brain, remains a significant challenge. Progress in investigating Tau dynamic condensation and aggregation has been made using cell models, such as the introduction of GFP-tagged Tau in mouse primary cortical neurons and hippocampal neuronal lines (Wegmann et al.,

2018; Wu et al., 2021a). These models have facilitated detection of Tau-enriched cytoplasmic condensates, distinguished by their dynamic and fluid-like nature. A further study has utilized an optogenetic strategy, in which Tau was fused with light-activated self-associating proteins. This method triggered the formation of light-responsive cytoplasmic condensates in cultured neurons (Zhang et al., 2020b). Although these condensates were initially reversible, they hardened during prolonged light exposure, forming aggregates. Additionally, hyperphosphorylated Tau has been observed to gather in neuronal inclusions alongside various RNA-binding proteins, including TIA1 (indicative of stress granules) and HNRNPA2B1 (linked with N6-methyladenosine [m^6A] RNA) (Apicco et al., 2018; Jiang et al., 2021; Vanderweyde et al., 2016). The interaction between TIA1 and Tau in these condensates is thought to enhance Tau misfolding and its pathological effects. Despite the advancements in cellular studies, a significant hurdle remains in obtaining direct evidence of Tau LLPS in the tissues of either AD patients or healthy individuals, which is vital for understanding the phase transition of Tau from LLPS to solid aggregates in the related neurodegenerative conditions.

In contrast to the extensive studies on Tau LLPS, the LLPS tendency of Aβ, another crucial player in AD, has been less explored. Recent studies have shown that Aβ peptides can undergo LLPS, forming dense liquid droplets that may act as precursors to Aβ fibril formation. A noteworthy discovery reveals that under acidic conditions, polyphosphate (polyP) accelerates the amyloid fibrilization process of Aβ through heterotypic LLPS, leading to droplets filled with mature fibrils (Sudhakar et al., 2023). Furthermore, it has been observed that soluble Aβ oligomers can form liquid-like droplets *in vitro*. Hydrophobic interactions play a key role in the Aβ oligomer phase separation process (Gui et al., 2023). These findings suggest that LLPS could be an important mechanism in the transition of Aβ from the soluble to aggregated state.

Targeting the LLPS process and the maturation of Tau and Aβ condensates offers a potential new avenue for AD therapy. By influencing the phase separation behavior of Tau and Aβ, it may be possible to reduce the development of plaques and neurofibrillary tangles. Future research is required to identify the key molecular species (e.g., certain conformations of Tau and Aβ in condensates) within the LLPS pathway, and to develop drugs to effectively modify them. The LLPS of Tau and Aβ is known to be influenced by small molecules *in vitro*. Compounds like bis-ANS, myricetin, C1, and epigallocatechin gallate (EGCG) have the potential to alter these LLPS processes (Babinchak et al., 2020; Dai et al., 2021; Gui et al., 2023). Further exploration of these molecules may lead to new treatment strategies to counteract amyloid aggregation, opening new pathways for managing and potentially treating AD.

Protein condensation associated with Parkinson's disease

PD is a progressive neurodegenerative condition primarily known for its motor symptoms (Poewe et al., 2017). A crucial pathological aspect of PD is the buildup of Lewy bodies, mainly composed of α-Syn aggregates (Goedert et al., 2013; Spillantini et al., 1998; Spillantini et al., 1997). α-Syn is a small IDP found predominantly in the presynaptic terminals of neurons (Lashuel et al., 2013). Its precise physiological role is unclear, but it is thought to be involved in regulating synaptic vesicle trafficking. It interacts with the SNARE-protein Synaptobrevin-2/VAMP2 and exhibits non-classical chaperone activity, aiding in SNARE complex formation (Abeliovich et al., 2000; Burré et al., 2014; Burré et al., 2010). In PD, pathological misfolding and aggregation of α-Syn occur, leading to formation of fibrils, a key component of Lewy bodies (Goedert et al., 2013). This aberrant aggregation is a critical factor in PD progression, disrupting normal neuronal functions and leading to the disease's characteristic pathology.

Recent *in vitro* study demonstrates that α-Syn can undergo homotypic LLPS under molecular crowding conditions, forming dynamic condensates (Figure 4A) (Ray et al., 2020). These condensates exhibit a tendency to coalesce and show rapid fluorescence recovery after photobleaching, highlighting their dynamic nature. Over time, these α-Syn liquid-like condensates irreversibly transition into solid-phase β-sheet-rich amyloid aggregates, as indicated by increased fluorescence of Thioflavin T (ThT). This observation suggests that these liquid-like condensates could be precursors to PD-related fibrillar aggregates (Ray et al., 2020). The LLPS behavior of α-Syn is influenced by various factors, including pH, ionic strength, temperature, and PTMs. For instance, salts significantly impact the LLPS of α-Syn by neutralizing charges at its N- and C-terminal regions, and meanwhile enhancing hydrophobic interactions (Sawner et al., 2021). Metal ions including Ca^{2+}, Cu^{2+}, and Fe^{2+} have also been found to promote α-Syn condensation at low concentrations, even in the absence of molecular crowding (Huang et al., 2022b; Ray et al., 2020). Additionally, low pH conditions can also neutralize the C-terminal region, facilitating the involvement of both the N-terminal and central NAC regions in LLPS interactions. These findings highlight the important role of the C-terminal region in modulating α-Syn phase separation (Huang et al., 2022a; Ray et al., 2020).

Notably, hereditary PD mutations in α-Syn, such as E46K and A53T, play a significant role in modifying its LLPS properties and fibrillation (Ray et al., 2020; Xu et al., 2023). Recent studies showed that the LLPS of α-Syn is highly dependent on its sequence complexity (Mahapatra and Newberry, 2023). Even minor changes in the α-Syn primary sequence can markedly affect its phase separation behavior, thereby potentially influencing the pathogenesis of PD. In addition, the impact of PTMs on α-Syn phase separation behavior is noteworthy. For instance, N-terminal acetylation significantly delays the LLPS of α-Syn (Sawner et al., 2021).

α-Syn has also been shown to engage in heterotypic LLPS with a variety of binding partners. At presynaptic terminals, α-Syn LLPS is essential for the higher-order mesoscale assembly of synaptic vesicles (SVs) in a concentration-dependent manner (Sansevrino et al., 2023). Notably, the negatively charged C-terminal region of α-Syn accumulates in Synapsin condensates, which are key components of the matrix that cluster SVs into a liquid-like phase (Figure 4C) (Hoffmann et al., 2021). A recent study indicates that the molar ratio of Synapsin1 to α-Syn affects the phase separation of SVs, with higher α-Syn concentrations attenuating this process. Moreover, α-Syn LLPS is significantly enhanced through its interaction with the SNARE protein VAMP2 (Figure 4C) (Agarwal et al., 2023; Diao et al., 2013; Sun et al., 2019). Additionally, α-Syn engages in co-LLPS with various amyloid proteins, such as Tau, TDP-43, and prion proteins, resulting in the co-aggregation in disease contexts (Agarwal et al., 2022; Dhakal et al., 2023; Dhakal et al., 2021;

Siegert et al., 2021). Interestingly, α-Syn can also undergo co-LLPS with non-proteinaceous cofactors, including RNA and ATP. The resulting condensates exhibit unique liquid-to-solid phase transition behaviors (Lipiński et al., 2022). These findings collectively suggest that the heterotypic LLPS of α-Syn with other proteins and molecules plays a significant role in regulating its physiological functions and pathological activities in both normal and disease states.

Advanced techniques including NMR spectroscopy, cryo-electron microscopy (cryo-EM), and MS are pivotal in unraveling the phase behavior of α-Syn and its mutants (Iadanza et al., 2018; Scheres et al., 2023). While previous studies mainly focused on the α-Syn fibrillar state, recent studies have broadened the scope to examine its behavior during LLPS. A significant study combined cross-linking with MS to analyze the conformational dynamics of α-Syn during LLPS (Ubbiali et al., 2022). This study revealed a transition in α-Syn conformation from a "hairpin-like" structure to a more "elongated" shape, shedding light on how LLPS may facilitate the amyloid aggregation of α-Syn. Furthermore, the temporal evolution of α-Syn liquid droplets has been characterized using various high-resolution microscopic and spectroscopic techniques, enabling the examination of single droplets (Ray et al., 2020). These approaches together provide valuable insights into the molecular changes occurring during α-Syn phase transitions.

Like Tau and Aβ, finding direct evidence of α-Syn LLPS in the brain tissues of healthy individuals and PD patients is challenging. Investigating α-Syn condensates within neurons is critical to understand their potential roles in neurodegeneration. However, inducing α-Syn LLPS in cells in a pathologically relevant manner is difficult. One study managed to induce LLPS of overexpressed α-Syn in living HeLa cells using Cu^{2+} exposure (Mahato et al., 2023). Another investigation used a *C. elegans* model expressing human α-Syn to study the aging process of α-Syn condensates (Hardenberg et al., 2021). The early-stage model worms displayed liquid-like α-Syn inclusions sensitive to 1,6-hexanediol. However, in older worms, these inclusions became resistant to 1,6-hexanediol, suggesting a transition from a liquid to a more solid-like state. Using cellular and animal models is vital for observing the phase changes and corresponding pathogenicity of α-Syn during aging and disease progression. It should be noted that there is still a lack of observation for LLPS and liquid-solid phase transition of α-syn in PD-related *in vivo* contexts. Future work needs to find *in vivo* evidence to elucidate the role of α-syn's phase transition in the pathogenesis of PD.

Despite the many challenges, investigation of α-Syn LLPS behavior is opening new frontiers for therapeutic interventions in PD. There is an increasing interest in identifying molecules that can modulate α-Syn phase separation behavior. Compounds such as myricetin and the antimicrobial peptide LL-III are being explored for their abilities to inhibit the phase transition of α-Syn, potentially preventing the formation of harmful aggregates (Oliva et al., 2021; Xu et al., 2022b). Future studies should aim to distinguish between physiological and pathological LLPS of α-Syn. The challenge lies in developing strategies that specifically target pathological forms of α-Syn, thereby preventing or slowing the progression of PD, without interfering with the normal functions of α-Syn in neuronal processes. Furthermore, it is vital to investigate the effects of genetic and environmental factors on α-Syn LLPS, particularly in the context of PD.

Overall, the study of protein LLPS in the context of AD and PD is a rapidly advancing field that is significantly transforming our comprehension of related neurodegenerative disorders. Continued exploration in this area promises to uncover new therapeutic targets and strategies.

Phase separation and hearing loss

Syndromic and non-syndromic hearing loss

Hearing loss is a highly prevalent chronic condition in older adults, making it the most common sensory disorder in humans. The majority of prelingual hearing loss, with non-syndromic and syndromic hearing loss being the two classifications, is attributed to hereditary factors (Michalski and Petit, 2019; Shearer et al., 1993). Currently, more than 120 genes have been linked to non-syndromic hearing loss (for more details on any hearing loss gene, please refer to http://hereditaryhearingloss.org). These genes are identified either by the specific gene involved (e.g., *OTOF*-related deafness) or by the genetic locus and mode of inheritance (*DFN* for DeaFNess; *DFNA* for autosomal dominant; *DFNB* for autosomal recessive; *DFNX* for X-linked). Syndromic forms account for about 20% of prelingual genetic hearing loss, with about 650 entries documented in Online Mendelian Inheritance in Man (OMIM).

Usher syndrome (USH) stands out as the most common hereditary form of both hearing loss and blindness. It displays autosomal recessive inheritance and is clinically characterized by a combination of sensorineural hearing loss (SNHL), rod-cone dystrophy or retinitis pigmentosa, and variable vestibular dysfunction (Delmaghani and El-Amraoui, 2022; El-Amraoui and Petit, 2005). Usher syndrome exhibits clinical and genetic heterogeneity. Based on clinical phenotypes—such as symptom severity, disease progression, and age of onset—it has been classified into three distinct subtypes: USH1, USH2, and USH3. In terms of genetic diversity, the three subtypes are caused by mutations in nine specific genes: *USH1B*, *USH1C*, *USH1*, *USH1F*, *USH1G*, *USH2A*, *USH2C*, *USH2D*, and *USH3A*. These genes encode various proteins that play crucial roles in the development and function of the auditory system, as well as the maintenance of photoreceptors in the retina (Figure 5).

The cochlea is responsible for acquiring and digitizing sound signals through specialized cells called hair cells (Barr-Gillespie and Bement, 2015; Fettiplace, 2017; Liu et al., 2021c; Schwander et al., 2010; Tilney et al., 1992). Each hair cell is topped with bundles of hair-like structures called stereocilia, arranged in a staircase pattern. These stereocilia are interconnected by extracellular links known as tip links, top connectors, shaft connectors, and ankle links. Acting as mechanical sensors, the stereocilia are deflected by sound vibrations. Deflection triggers the opening of MET channels located at the tip of the stereocilia, resulting in hair cell depolarization. Any dysfunction in the hair cells can lead to issues in the auditory system and hearing-related brain functions.

In cochlear hair cells, the formation of biomolecular condensates through LLPS is critically involved in the development and proper functioning of stereocilia. These condensates contribute to the formation of protein-rich subcellular compartments, including the tip-link density, ankle-link density, tip complex density, and synaptic ribbon in hair cells. Understanding how these densities are formed and how the linkages are anchored to these densities is of great importance. This section

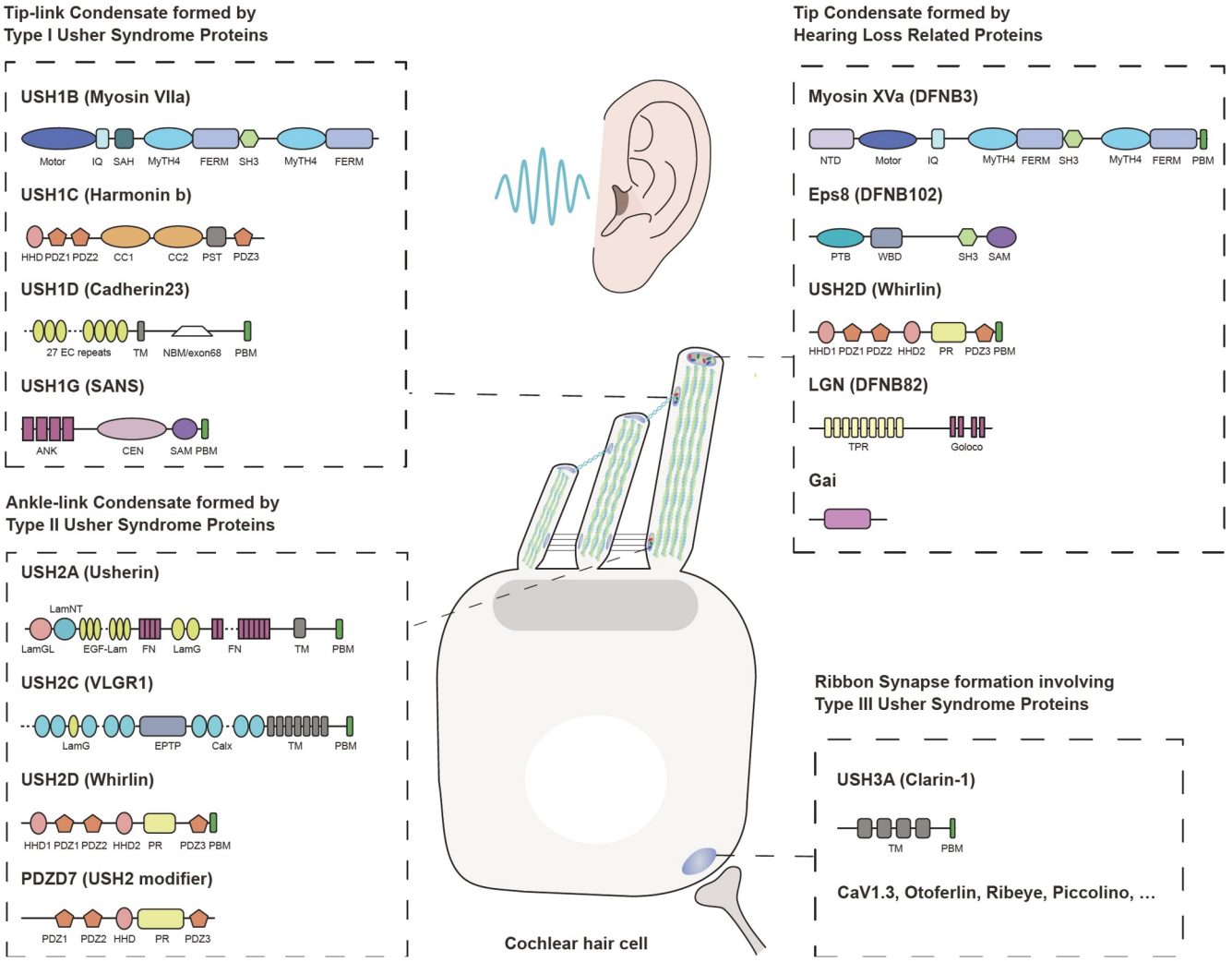


Figure 5. Schematic diagram showing a cochlear hair cell with stereocilia in a staircase pattern at the apical surface. The domain organizations of the core protein components forming the tip-link condensate, ankle link condensate, tip condensate, and ribbon synapse are illustrated.

focuses on the recent studies of LLPS-mediated condensates formed by proteins encoded by genes associated with both syndromic and non-syndromic hearing loss. Additionally, the co-assembling partners of these proteins are explored. The correlation between the clinical classification of hearing loss and the distinct condensations observed in cochlear hair cells will also be highlighted.

Formation of tip-link condensates by type I Usher syndrome proteins

Usher syndrome type 1 (USH1) is the most severe form of Usher syndrome, accounting for approximately 25-44% of all cases (El-Amraoui and Petit, 2005). It is characterized by severe-to-profound congenital sensorineural hearing loss, vestibular areflexia, and the onset of retinitis pigmentosa before puberty. USH1 is caused by specific genetic variations in five genes: *USH1B* (Myosin VIIa; USH1B), *USH1C* (Harmonin b; USH1C), *USH1D* (Cadherin 23; USH1D), *USH1F* (Protocadherin 15; USH1F), and *USH1G* (SANS; USH1G).

Four out of the five USH1 proteins—Myosin VIIa, Harmonin, SANS, and Cadherin 23—are crucial components of the upper tip-link density (UTLD) (Pan and Zhang, 2012). UTLD, observed as electron-dense plaques, anchors the tip link on the taller stereocilium with the mechanosensing channel at the other end. The protein encoded by *USH1B*, Myosin VIIa, serves as an actin-based motor protein. It has a motor head, five IQ motifs, a single α-helix, and a pair of Myosin Tail Homology 4 (MyTH4)-FERM domains separated by a SRC homology 3 (SH3) domain. Harmonin, encoded by *USH1C*, and SANS, encoded by *USH1G*, are scaffold proteins. Harmonin has an N-terminal Harmonin homology domain (HHD) and three PDZ domains, while SANS consists of Ankyrin repeats, a central region (CEN), and a SAM domain followed by a PDZ binding motif (PBM). Both Protocadherin 15, encoded by *USH1F*, and Cadherin 23, encoded by *USH1D*, are calcium-dependent adhesion proteins. They contain extracellular Cadherin repeats (ECs), a transmembrane helix, and an intracellular region with a PBM. Cadherin 23 and Protocadherin 15 interact with each other to form tip links between stereocilia, which are responsible for sound-induced force transmission to the mechanosensing channel.

UTLD formation is mediated by strong, multivalent interactions among USH1 proteins, driven by LLPS (He et al., 2019). Harmonin and SANS exhibit tight binding with a dissociation

constant (K_d) of a few nmol/L (Yan et al., 2010), mediated by binding of the HHD-PDZ1 domains of Harmonin with the SAM-PBM domains of SANS. SANS also interacts with Myosin VIIa with high affinity (K_d of approximately 50 nmol/L) (Wu et al., 2011), mediated by the CEN domain of SANS and the N-terminal MyTH4-FERM tandem domains. Further studies revealed that Harmonin PDZ3-PBM can bind with the C-terminal MyTH4-FERM domain with relatively low affinity (Li et al., 2017; Yu et al., 2017). The Harmonin HHD and PDZ2 domains respectively recognize an internal sequence and the PBM in the C-terminal region of Cadherin 23. These simultaneous interactions at multiple sites provide valency for LLPS and contribute to the assembly of upper tip-link densities. Overexpression of three USH1 proteins—Harmonin, Myosin VIIa, and SANS—was adequate to create condensed structures within cells (He et al., 2019). These condensates exhibit characteristic properties of LLPS, such as dynamic and liquid-like behavior. Furthermore, USH1-associated point mutations impact the LLPS behavior of USH1 proteins, diminishing their ability to form condensates in cells. Exploring the condensation process *in vivo* through animal model studies would be a powerful approach to uncovering further insights in this area.

Formation of ankle-link condensates by type II Usher syndrome proteins

Usher syndrome type 2 (USH2) is the most common subtype of Usher syndrome, accounting for 60% of all cases. It is characterized by moderate to severe congenital hearing loss, some balance problems, and the onset of retinitis pigmentosa during teenage years. USH2 is caused by three genes and a modifier: *USH2A* (Usherin; USH2A), *USH2C* (VLGR1; USH2C), *USH2D* (Whirlin; USH2D), and *PDZD7* (PDZD7; USH2 modifier) (Ebermann et al., 2010; Ebermann et al., 2007; Eudy et al., 1998; Weston et al., 2004). Whirlin and PDZD7 are scaffold proteins and both contain HHD and three PDZ domains that recognize specific PBMs to organize protein complexes. Usherin and VLGR1, on the other hand, are transmembrane adhesion proteins that form extracellular connections. All four USH2-associated proteins localize at the basal region of hair cell stereocilia and form the ankle-link complex, also known as the USH2 protein complex. Ankle links are a network of thin fibers that connect stereocilia at their basal regions, playing a crucial role in coordinating stereociliary development and facilitating hearing. In mice, these ankle links are only present from postnatal days 2 to 12. Additionally, a less prominent electron-dense structure can also be observed at the ankle-link region of stereocilia (Goodyear et al., 2005).

USH2 proteins undergo LLPS, resulting in the formation of condensates in ankle links (Wang et al., 2023b). The assembly of these protein condensates through LLPS is driven by multivalent interactions between Usherin, Whirlin, VLGR1, and PDZD7. At high concentrations, VLGR1 inhibits LLPS *in vitro*, suggesting a potential mechanism for the temporal disassembly of the ankle-link complex. The longer isoform of Whirlin is targeted to both the ankle-link and tip-link regions of stereocilia, and is responsible for the formation of ankle-link and tip condensates. The accurate targeting of Whirlin to the tip of stereocilia depends on its co-assembling partner (discussed in the following session). HHD1 of Whirlin plays a crucial role in targeting to the ankle-link condensate through interactions with Usherin and VLGR1. Disruption of the multivalent interactions essential for LLPS in USH2 proteins results in the loss of the typical distribution of the longer Whirlin isoform at the base of stereocilia in hair cells.

The biochemical characterization of LLPS offers alternative perspectives and methods for investigating the molecular mechanisms underlying hearing loss. Mutations associated with hearing loss can be categorized into two groups. The first group comprises mutations that significantly impact the protein's conformation or occur on the binding interface, disrupting interactions with target proteins and compromising the required multivalency for LLPS. For instance, the G103R mutation in PDZD7 disrupts its interaction with Usherin, leading to the disruption of condensation within the USH2 protein complex. The location of PDZD7 G103 in the binding groove implies its importance in target recognition. The effects of these mutants on phase separation align with the reductions in their binding affinities, and highlight the high sensitivity of LLPS capabilities in USH2 complexes to the binding affinities of the multivalent interaction network. The second group of mutations comprises changes that may only cause minor perturbations in protein structures. They are often overlooked or considered variants of unknown significance. The A64D and R223H mutations in Whirlin, even though they do not affect binding to Usherin or VLGR1, lead to deafness by impairing the phase separation of USH2 condensates. Therefore, phase separation emerges as a powerful mechanism for understanding variants found in hearing-related disorders.

Ribbon synapse formation involving type III Usher syndrome proteins

Usher syndrome type 3 (USH3) is the least common subtype of Usher syndrome, representing around 2-4% of all cases. Individuals with USH3 exhibit progressive post-lingual SNHL along with variable vestibular dysfunctions and retinitis pigmentosa. *USH3A* (Clarin 1; USH3A) is the only gene known to be associated with USH3. Clarin 1 is a protein with four transmembrane domains and has recently been identified as a crucial component of the hair bundle and ribbon synapse in hair cells (Ogun and Zallocchi, 2014). Clarin 1 was proposed to play roles in both pre- and postsynaptic functioning of the ribbon synapse (Dulon et al., 2018). In the presynaptic region, it is suggested that Clarin 1, together with Harmonin and Cav1.3 channels, forms a molecular platform associated with F-actin in the active zones of the hair cell ribbon synapse. The absence of Clarin1 also results in postsynaptic defects, including abnormal distribution of postsynaptic AMPA glutamate receptors and structural changes in afferent dendrites. Sequence alignments suggest that Clarin1 shares similarity with Stargazin, a calcium channel subunit that is involved in the clustering and mobilization of AMPA receptors at neuronal synapses. Clarin 1 may contribute to the assembly of the ribbon synapse, similar to how Stargazin is involved in condensation and AMPA receptor synaptic transmission via LLPS (Zeng et al., 2018). However, the exact molecular mechanisms involving USH3A protein and its role in USH3 pathology are yet to be fully understood.

In the mammalian cochlea, the ribbon synapses between sensory inner hair cells (IHCs) and postsynaptic spiral ganglion neurons play a crucial role in ensuring precise and reliable encoding of auditory signals (Chakrabarti and Wichmann, 2019; Voorn and Vogl, 2020). These synapses, known for their high

throughput, are characterized by an electron-dense projection called the synaptic ribbon. The synaptic ribbon acts as a structural scaffold and anchors a large pool of synaptic vesicles. Ribeye is identified as the core component protein of the synaptic ribbon and clusters voltage-dependent calcium channels at the base of the presynaptic plasma membrane. Additionally, Piccolino acts as a multi-protein interaction hub essential for ribbon morphology. It not only connects with Ribeye but also interacts with other synaptic target proteins, including Otoferlin, Cav1.3, and Clarin 1.

Many presynaptic condensates have been shown to interact with lipid membranes, forming various mesoscale structures through different modes of organization between membraneless condensates and membranous organelles (Cai et al., 2021; Wu et al., 2020; Wu et al., 2019; Wu et al., 2021c). These mesoscale interactions have shed light on the long-standing puzzle of mobilization, exocytosis, and retrieval of synaptic vesicles (Feng et al., 2021; Wu et al., 2020). However, for the ribbon synapse, with its unique characteristics, key questions remain unanswered, such as the mechanisms underlying the formation of the ribbon density, and how the condensates coordinate the assembly of protein components and vesicles.

Formation of tip condensates by non-syndromic hearing loss proteins

The tip complex is responsible for the electron-dense regions at the tips of stereocilia and plays essential roles in promoting actin polymerization and bundling (Schwander et al., 2010). The tip complex mainly consists of Myosin XVa, Whirlin, and EPS8 (Manor et al., 2011; Zampini et al., 2011). Myosin XVa is an actin-based motor, containing a motor head, two IQ motifs, and two MyTH4-FERM domains separated by an SH3 domain. Whirlin is the PDZ-containing scaffold protein, also involved in the formation of ankle-link densities as described above (5.3). EPS8, an actin-capping protein, is composed of a PTB domain, an SH3 domain, and a SAM domain. Recent studies have shown that two planar cell polarity factors, LGN and Gαi, may together play an important role in defining row 1 stereocilia (the tallest cilia in the "staircase") (Mauriac et al., 2017; Tadenev et al., 2019). The LGN-Gαi complex, enriched at the tips of row 1 cilia, colocalizes with Myosin XVa, Whirlin, and EPS8. LGN, also known as GPSM2 (G-protein-signaling modulator 2), comprises eight tetratricopeptide repeats (TPRs), an unstructured linker, and four GoLoco (GL) motifs which are well known for regulating spindle orientation during asymmetric cell division. The GL motifs of LGN can bind to the GDP-bound form of the α subunit of heterotrimeric G protein and act as a guanine nucleotide dissociation inhibitor (GDI) (Willard et al., 2004; Zhu et al., 2011).

Similar to the UTLD assemblies mentioned in 5.2, the tip complex is also formed through multivalent interactions and undergoes LLPS as reported (Lin et al., 2021; Shi et al., 2022). PDZ3 of Whirlin interacts with PBM of Myosin XVa. The Whirlin HHD region binds to EPS8 in the region between its PTB and SH3 domains, named as the Whirlin-binding domain (WBD). Via its PTB domain, EPS8 also interacts with the C-terminal MyTH4-FERM domain of Myosin XVa. In addition, the proline-rich (PR) region of Whirlin has the ability to bind to the TPR region of LGN. The GL motifs of LGN bind Gαi in a typical binding mode. The self-association properties of Whirlin, EPS8, and LGN contribute to the overall valency. All five components—Myosin XVa, Whirlin, EPS8, LGN, and Gαi—form the tip complex density. The SAM domain of EPS8, along with the other components under LLPS conditions, robustly promotes the bundling of F-actin. This mechanism potentially coordinates polarity cues and elongates the tallest stereocilia in the hair cell.

In contrast to condensates formed by subtype-specific proteins in distinct density regions of the hair cell, the components of the tip condensate show heterogeneity in terms of clinical phenotypes caused by variants in related genes. *MYO15A*, the third most significant gene associated with hereditary sensorineural hearing loss following *GJB2* and *SLC26A4*, has a mutation frequency of approximately 6% in patients with autosomal recessive non-syndromic hearing loss (Farjami et al., 2020). A total of 192 recessive *MYO15A* variants are associated with *DFNB3* (Rehman et al., 2016). Individuals with *MYO15A*-related hearing loss may experience varying types and severity of impairment, ranging from mild to profound hearing loss. A hearing loss mutation in *MYO15A* disrupts condensate formation, consequently impairing actin bundling (Lin et al., 2021). Pathogenic variants in the *EPS8* gene (*DFNB102*) lead to non-syndromic hearing loss (Abbasi et al., 2023). *WHIRLIN* is one of the USH2 genes. Variants of *LGN* are associated with *DFNB82* non-syndromic hearing loss (Walsh et al., 2010) and Chudley-McCullough syndrome (CMS). CMS is a rare autosomal recessive disease characterized by severe to profound sensorineural hearing loss and partial agenesis of the corpus callosum (Doherty et al., 2012). A truncated mutant form of LGN lacking both the linker and GL motifs, found in CMS, fails to form condensates (Shi et al., 2022). Further investigations will help us to understand the mechanisms underlying the diverse pathogenic processes of hearing loss.

Membraneless organelles in cancer

Cancer is a genetic disease coupled with immune and metabolic dysfunction. Increasing evidence implicates the importance of biomolecular phase separation in cancer initiation, progression, and drug resistance. The focus here is on the mechanism of formation and impact of phase-separated condensates on tumor signal transduction and drug resistance, and potential preventive strategies based on targeting these oncogenic condensates.

Initiation of tumorigenesis by condensation of oncoproteins generated by gene fusions

A distinctive hallmark of cancer is unrestricted growth driven by the constitutive activation of oncogenic signals. Gene fusion, via chromosomal rearrangement, is one mechanism for initiating cellular transformation and proliferation. The encoded fusion proteins form condensates, within which they act as scaffold proteins to recruit multiple cofactors (Table S1). A notable instance of an oncogenic fusion event involves the abnormal activation of the RTK/RAS/MAPK signaling cascade (Lin et al., 2023). The conventional platform for RTK/RAS/MAPK cascade activation is the plasma membrane, where RTK (receptor tyrosine kinase) signal transduction typically occurs. However, this pathway can be activated in the cytoplasm by an EML4-ALK oncoprotein, generated by fusion of the *EML4* and *ALK* genes. The EML4-ALK fusion protein forms membraneless biomolecular condensates within the cytoplasm (Qin et al., 2021; Tulpule et

https://doi.org/10.1007/s11427-024-2661-3

al., 2021), which have the ability to recruit RAS-associating proteins such as GRB2, GAB1, and SOS1 (Figure 6A). This recruitment results in the activation of cytosolic RAS, bypassing the regulatory capacity of native plasma membrane-bound RTKs. Subsequent signaling cascades are amplified, culminating in a robust transcriptional output, which dramatically promotes cell proliferation and facilitates tumorigenesis and malignancy. Remarkably, this mode of action has been extended to other oncogenic RTK-related fusions, including CCDC6-RET, EML4-RET, and CCDC6-ALK (Tulpule et al., 2021). In some cases, the products from gene fusion events do not form *de novo* oncogenic condensates but rather they interfere with normal condensation. This mechanism is exemplified by the DnaJB1-PKAcat fusion protein. Under physiological conditions, the type I regulatory subunit of cAMP-PKA (RIα) forms condensates and concentrates cAMP, whereas the DnaJB1-PKAcat fusion abolishes RIα condensation and leads to abnormal cAMP signaling, which supports tumorigenesis through strengthened cellular proliferation and transformation (Zhang et al., 2020a).

In addition to participating in oncogenic signal transduction in the cytoplasm, fusion proteins also mediate the formation of oncogenic transcriptional condensates in the nucleus. Recent studies have delved into the crucial role of fusion proteins in driving the formation of oncogenic condensates. One example involves the fusion proteins YAP-MAMLD1 and C11ORF95-YAP, which give rise to nuclear, punctum-like, membraneless condensates dependent on the IDR of YAP (Hu et al., 2023). Once these fusion oncoproteins partition into condensates, they coalesce with transcription factors and co-activators. This co-condensation initiates an oncogenic transcriptional program, thereby driving ependymoma tumorigenesis. Chromosomal translocations involving the Nucleoporin 98 (*NUP98*) gene and various partners result in diverse oncogenic fusion proteins frequently observed in hematological malignancies (Terlecki-Zaniewicz et al., 2021). The NUP98-HOXA9 fusion is a well-characterized example, where the N-terminus of NUP98 and the C-terminus of HOXA9 combine. The N-terminal IDR of NUP98 mediates LLPS, inducing CTCF-independent chromatin loops which are enriched at oncogenes (Chandra et al., 2022). This potentiates an oncogenic transcriptional output, leading to the subsequent transformation of hematopoietic cells. The EWS-FLI1 fusion oncoprotein, prevalent in Ewing's sarcoma, results from chromosomal translocations which juxtapose the N-terminus of EWSR1 and the C-terminus of FLI. The N-terminal prion-like domains of EWSR1 mediate condensation, recruiting the BAF complex to oncogenic enhancer sites for pro-carcinogenic transcription (Boulay et al., 2017; Zuo et al., 2021).

However, controversy exists regarding the impact of transcriptional condensates on transcription regulation. Chong et al. reported that the phase separation of EWS-FLI1 led to a decline in transcription (Chong et al., 2022). Their single-molecule imaging analysis of endogenous EWS-FLI1 transcription events revealed that the degree of LCD-LCD interactions determines whether the fusion protein activates or inhibits target gene expression. Moderate LCD-LCD interactions led to transcription hub formation and upregulation of transcriptional target genes. However, further triggering of phase separation inhibited transcription, as transcription factors were sequestered in the condensates. These results, along with other studies, suggest that LCD-LCD interactions are a key driving force for gene activation independent of phase separation (Chong et al., 2018; Trojanowski et al., 2022). Therefore, it is crucial to elucidate the functions of phase separation in transcription by visualizing and quantifying them *in vivo* under endogenous and multiple context-dependent conditions.

In summary, these discoveries indicate that the abnormal condensation of fusion oncoproteins fuels unchecked growth, culminating in the development of tumors. The fusion proteins

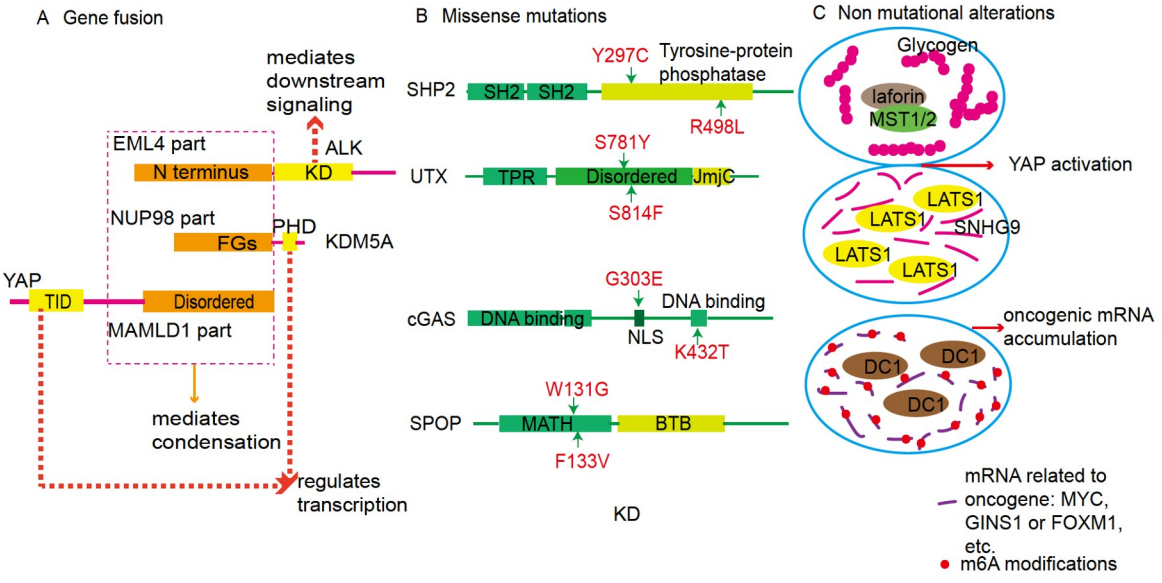


Figure 6. The formation of oncogenic condensates via gene fusion, mutations, and non-mutational alterations. (a) EML-ALK, NUP98-KDM5A, YAP-MAMLD1, and EML-ALK fusion proteins, generated by gene fusion events. Through their respective molecular functions, these fusion proteins mediate condensation and activate downstream signaling pathways or transcription. (b) Disease-associated mutations give rise to aberrant condensation. Mutations in SHP2 (Y297C and R498L) and in UTX (S781Y and S814F) lead to reinforced condensation and trapping of tumor suppressors, while mutations in cGAS (G303E and K432T) weaken normal condensation and dampen the anti-tumor response. These mutations finally contribute to tumorigenesis. (c) Non-mutational alterations influence condensation and exert pro-tumor effects. Pro-oncogenic YAP signaling is induced by accumulation and condensation of glycogen, which traps MST1/2 (top), or by interaction of the lncRNA *SNHG9* with LATS1, which results in YAP-activating condensates. m^6A on oncogene mRNAs like *MYC*, *GINS1* or *FOXM1* promotes YTFDC1 phase separation, thus protecting these mRNAs from degradation and promoting their accumulation.

serve a dual role, contributing to both condensate formation and the activation of oncogenic signals across various cell types. Importantly, it is worth noting that some oncogenic properties associated with these oncofusion events may not be exclusively ascribed to the capacity of fusion proteins for condensate formation. Multiple sources of evidence support this nuanced perspective (Chong et al., 2022; McSwiggen et al., 2019a; McSwiggen et al., 2019b).

Promotion of tumor growth by mutations that affect condensate formation

Aside from chromosomal rearrangements, genetic mutations are another driver of oncogenic condensation. In some instances, a single residue replacement in a proto-oncoprotein is adequate to initiate tumorigenesis due to changes in the biophysical properties of condensates. Notably, in juvenile myelomonocytic leukemia patients, mutations in the non-receptor protein tyrosine phosphatase SHP2, including Y279C and R498L, endow the protein with a stronger propensity for condensation than wild-type SHP2 (Figure 6B). The mutant versions form phase-separated condensates that recruit wild-type SHP2, leading to pro-carcinogenic MAPK hyperactivation (Zhu et al., 2020a). These results indicate that localization of SHP2 protein within condensates can increase its activity. More work is needed to clarify the causal relationship between condensation and tumorigenesis in animal models and patient samples.

Another example involves the histone reader ENL. ENL drives phase-separated condensation of the super elongation complex (SEC) for swift transcriptional activation (Guo et al., 2020). Hotspot mutations in ENL lead to self-reinforced recruitment, resulting in increased chromatin occupancy and gene activation. These mutations underlie the oncogenic potential of ENL in acute myelocytic leukemia (AML) (Wan et al., 2020; Wan et al., 2017). Another study indicates that the outcomes differ depending on the expression level of mutant ENL. More specifically, at endogenous levels, mutant ENL forms condensates at chromatin sites for excessive transcriptional activation, whereas overexpression of mutant ENL gives rise to larger condensates away from chromatin which cannot activate transcription (Song et al., 2022). This may be a sign of the dose-dependent properties of condensates.

In addition to mutations in oncoproteins, mutations in tumor suppressor proteins can disrupt condensate function, thereby promoting tumorigenesis. UTX/KDM6A is a tumor suppressor protein with high mutation frequency in human cancers. It forms condensates and concentrates MLL3 for chromatin regulation. However, several cancer-associated mutations in the core IDR of UTX, including S781Y, S814F, and the 5M cluster (S674Y, S781Y, H808Y, S814F, and S818L), enhance its condensation. This leads to a reduction in the fluidity of biocondensates, ultimately abolishing the tumor-suppressing activity of UTX (Shi et al., 2021). SPOP, another tumor suppressor, harbors mutations associated with prostate, breast, and other cancers. SPOP mediates the degradation of oncogenic substrates like DDAX and AR via LLPS. Mutations such as W131G and F133V in SPOP weaken condensation and disrupt SPOP-substrate colocalization. This disruption results in the accumulation of oncogenic substrates (Bouchard et al., 2018). Additional research indicates that mutations in SPOP diminish its ability to post-translationally modify SQSTM1, enhancing SQSTM1 condensation and leading to KEAP1 inhibition and subsequent oncogenic NFE2L2 activation (Gao et al., 2022b).

Aberrant phase separation may disrupt the immune responses to tumors. Upon binding to DNA, cGAS (cyclic GMP-AMP synthase) forms liquid-like droplets, becomes activated, and initiates innate immune signaling (Du and Chen, 2018). Tumor-associated mutations in cGAS (G303E and K432T) impair its ability to form biocondensates, thus impairing the antitumor response (Xie et al., 2019). Another example involves a novel effect of the mutated tumor suppressor NF2 (Neurofibromin 2) with missense mutations in the FERM domain. When activated by upstream signals, IRF3 (interferon regulatory factor 3) binds and induces the mutant NF2 to form biocondensates containing the PP2A complex and TBK1 (TANK binding kinase 1). The PP2A complex deactivates TBK1, thereby attenuating the antitumor response (Meng et al., 2021).

In conclusion, multiple different mutations converge on a pro-carcinogenic trajectory by facilitating the formation of oncogenic condensates or disrupting the formation and function of tumor suppressive condensates. Unlike condensates in normal physiological conditions, these oncogenic condensates are irreversible and comprise multiple components, thereby driving continuous tumor growth.

Promotion of condensation and tumorigenesis by non-chromosomal alterations

The Hippo signaling pathway has been established as a tumor-suppressing machinery. Several components of the Hippo pathway have been found to form or be recruited into condensates (Figure 6C). Recent research discovered that accumulated glycogen forms phase-separated condensates wherein Laforin-MST1/2 form a complex which traps MST1/2, thus activating YAP indirectly for oncogenic transcription and tumorigenesis (Liu et al., 2021b). In another example, the lncRNA *SNHG9* imprisons LATS1 by driving its LLPS, thereby activating YAP for cellular proliferation (Li et al., 2021). m^{6}A modifications on mRNA promote phase separation of YTHDF-family m^{6}A-binding proteins to mediate multiple processes (Ries et al., 2019). Similarly, m^{6}A encourages nuclear YTHDC1-m^{6}A condensation, which reduces degradation of oncogene mRNAs like *MYC*, *GINS1*, and *FOXM1*. This machinery sustains AML cells in the undifferentiated state (Cheng et al., 2021).

Altogether, the evidence suggests that altered condensation guides cancer cells away from tumor-suppressing mechanisms and towards a higher grade of malignancy.

Condensates and resistance of tumors to drug therapies

In addition to influencing tumor initiation and progression, accumulating evidence supports the role of biomolecular condensates in mediating tumor drug resistance. One example is the promotion of condensation of androgen receptor (AR) mutants, arising from mutations or splice variants, in a ligand-independent manner in castration-resistant prostate cancer (CRPC) patients (Xie et al., 2022). These condensates of AR elicit antiandrogen therapy resistance and can be reversed by the compound ET516, which specifically disrupts the formation of AR condensates. In colorectal adenocarcinoma patients, elevated SENP1 expression augments RNF168 deSUMOylation, weakening RNF126 phase separation. This intensifies the DNA damage

repair response and confers chemoresistance on cancer cells (Wei et al., 2023).

Despite promising progress in immunotherapy, resistance is still a significant challenge in solid tumors (Sharma and Allison, 2015). IFN-γ (Interferon-gamma) plays a dual role in the immunotherapy of solid tumors: on one hand, it can activate T cells to eliminate cancer cells; on the other hand, prolonged exposure to IFN-γ can lead to immunotherapy tolerance. The underlying mechanisms are not fully understood (Benci et al., 2016). Two recent reports indicate that transcriptional condensates mediate the pro-tumor effects of IFN-γ. Firstly, IFN-γ can activate the oncoprotein YAP to form nuclear condensates, initiating the transcription of a series of genes, including the immune checkpoint gene *CD155*, which inhibits T cell attacks (Yu et al., 2021b). Secondly, IFN-γ can induce the formation of transcriptional condensates of KRT8 and IRF1, leading to high-level expression of PD-L1 and ultimately resulting in resistance to treatment (Wu et al., 2023).

Overall, the evidence suggests that aberrant condensation may provide cancer cells with a defense against therapies, making targeting of phase separation an appealing strategy. Some studies have demonstrated promising efficacy in preclinical models by targeting oncogenic transcriptional condensates. For instance, a small peptide (2142–R8) was developed to suppress KAT8–IRF1 condensation and PD-L1 expression, thereby improving anti-tumor immunity in a preclinical model (Wu et al., 2023). GSK-J4, an H3K27 demethylase inhibitor targeting HOXB8 (a participant in the core regulatory circuitry of super enhancers), disrupts condensates, thus reducing metastasis and chemoresistance in osteosarcoma (Lu et al., 2021). Future directions include further development of more efficient phase separation inhibitors and subsequent clinical testing.

Phase separation in immunity

Immunity is an essential function in all living organisms. The activities of many sensors, receptors, and adaptor proteins, which first evolved in prokaryotes, are coordinated to create multiple signaling pathways that control immune responses to pathogen infection, cellular stress, and cancer in animals (Slavik and Kranzusch, 2023). Importantly, activation of the two branches of the animal immune response—innate and adaptive—requires the formation of higher-order signaling complexes, which are termed "immune signalosomes", "supramolecular organizing centers" (SMOCs), or "immune synapses" (Kagan et al., 2014; Shi et al., 2020; Wu and Fuxreiter, 2016; Xia et al., 2021; Xiao et al., 2022). In many classical forms of immune signalosomes, the orderly self-assembly of specific domains results in the formation of stable and solid-like supramolecular complexes; for example, necrosomes, inflammasomes, myddosomes, and T-cell receptor nanoclusters (Wu and Fuxreiter, 2016; Xia et al., 2021). The formation of these solid-like assemblies was traditionally thought to be the most common mechanism involved in immune signaling. However, recent studies have revealed the assembly of immune complexes to be more complicated, with some signalosomes being defined as more "liquid" than "solid" in form. Liquid-like condensates exist in both the innate and adaptive immune signaling pathways (Du and Chen, 2018; Su et al., 2016). Building on these findings, recent evidence further demonstrates that much of the core machinery of immune signaling is organized as a liquid condensate rather than in the solid form, suggesting that LLPS may be a common form of regulation in the immune system (Mehta and Zhang, 2022; Xiao et al., 2022) (Figure 7).

Innate immune responses play a central role in the defense against invading pathogens. Normally, the innate immune response relies on a signaling cascade and follows this multistep paradigm: (1) sensor proteins directly recognize molecular signatures of infection; (2) the sensors transmit signals to downstream adaptor proteins; and (3) this ultimately induces the expression of an immune gene program (Harapas et al., 2022). Growing evidence suggests that LLPS is a widespread cellular mechanism in all three of these steps. Hallmark examples of LLPS in innate immunity include the mammalian DNA sensor cGAS (Du and Chen, 2018; Sun et al., 2013). Upon direct binding to DNA, cGAS undergoes a conformational change and becomes enzymatically active, allowing it to synthesize the nucleotide second messenger 2′3′-cGAMP (cG[2′–5′]pA[3′–5′]P) (Gao et al., 2013; Zhou et al., 2018). 2′3′-cGAMP can then bind to and activate the downstream adaptor protein STING (stimulator of interferon genes). Upon activation, STING translocates from the ER to the Golgi, where it recruits the kinase TBK1, the transcription factor IRF3 and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells). These signal transduction events result in the expression of type I IFN and interferon-stimulated genes (ISGs) that orchestrate broad antiviral effects (Ablasser and Chen, 2019). Key molecules in the cGAS-STING signaling pathway—including cGAS, STING, TREX1 (three prime repair exonuclease 1), IRF3, TRIM5 (tripartite motif protein 5), and MxA (myxovirus resistance protein A)—were recently reported to undergo LLPS (Li and Gao, 2023) (Figure 7A). In the case of DNA sensing, cGAS and DNA molecules readily concentrate into liquid-like droplets and allow the efficient activation of downstream signaling (Du and Chen, 2018). cGAS-DNA LLPS is driven by multivalent interactions between cGAS and DNA (Xie et al., 2019; Zhou et al., 2021). Surprisingly, recent evidence has demonstrated that cGAS-DNA phase separation is regulated independently of cGAS enzymatic activity and is not directly required to control 2′3′-cGAMP production (Zhou et al., 2021). Instead, cGAS-DNA phase separation functions as a selective filter in cells. Specifically, cGAS, its substrates (e.g., DNA, ATP, GTP, Mg^{2+}), and positive regulators (e.g., G3BP1, USP15, Ku80/Ku70, ZCCHC3, ZYG11B) are permitted to rapidly enter and diffuse throughout the liquid environment, whereas negative regulators (e.g., TREX1, BAF) are restricted to the outer shell of cGAS-DNA droplets (Li and Gao, 2023; Zhou et al., 2021). However, in the case of STING, phase separation is used to downregulate STING signaling. A high concentration of 2′3′-cGAMP is capable of driving the formation of the STING-cGAMP condensate, also known as the STING phase-separator (Yu et al., 2021c). It is proposed that the STING-cGAMP condensate behaves as a specific environment that traps and restrains the activity of TBK1 and, as a result, prevents immune activation (Yu et al., 2021c). In addition to the regulation of DNA sensing in the cytosol, LLPS can also control the immune response when the transcription factor IRF3 arrives in the nucleus. It was recently reported that upon nuclear translocation by cGAS activation, nuclear IRF3 undergoes LLPS with the promoter of the *IFNB1* gene, stimulating the expression of type I IFN (Qin et al., 2022). As a result, type I IFNs activates an antiviral signaling program by controlling the transcription of numerous ISGs. In addition, recent studies suggest that the

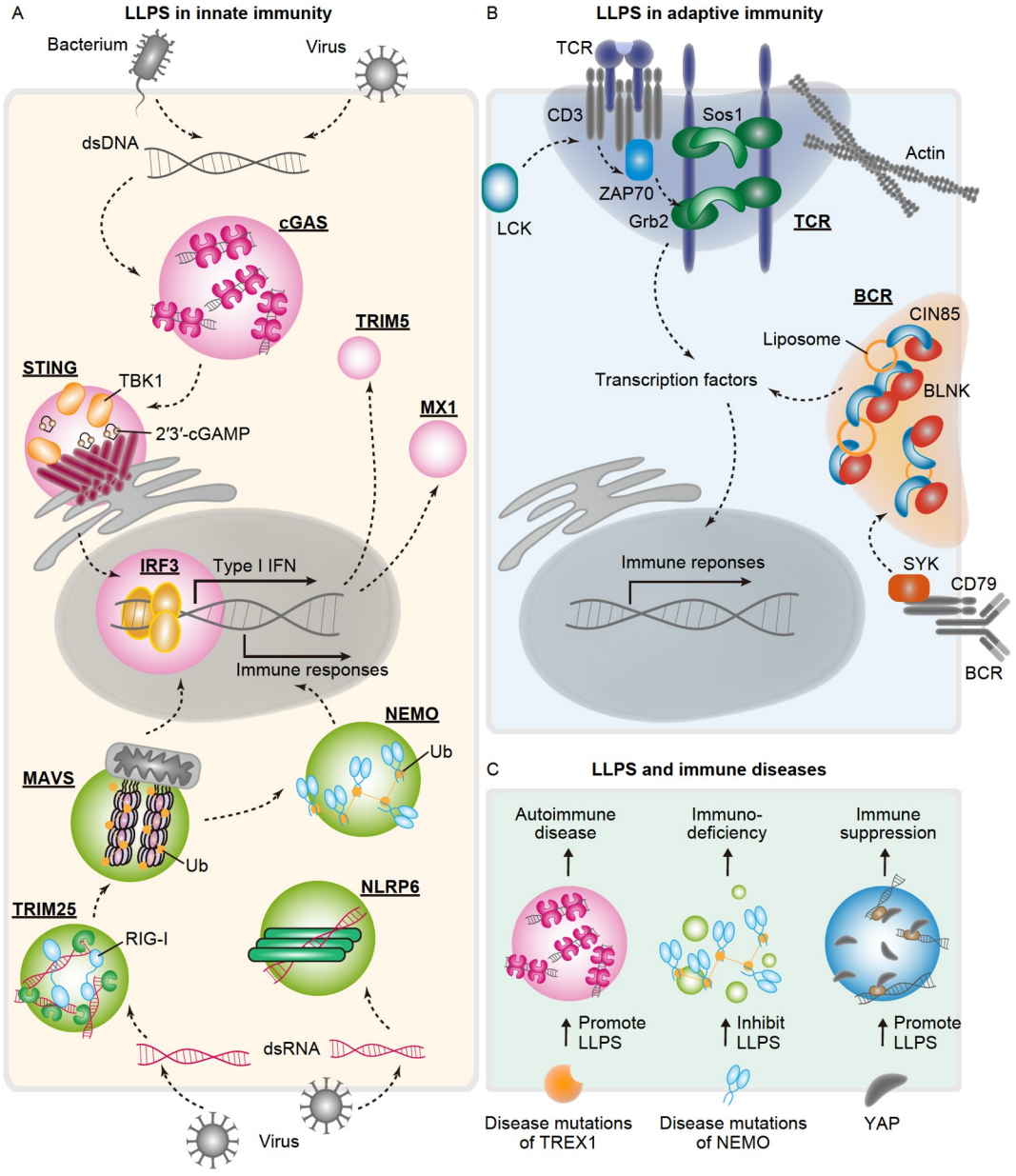


Figure 7. Phase separation in immune signaling and disease. (a) Phase separation in innate immunity. Recent evidence has demonstrated that much of the core machinery of innate immunity behaves as liquid condensates. The key immune response proteins that drive LLPS formation—cGAS, STING, IRF3, TRIM5, MX1, TRIM25, MAVS, NEMO, and NLRP6—are underlined. Abbreviations: dsDNA, double-stranded DNA; cGAS, cyclic GMP-AMP synthase; STING, stimulator of interferon genes; TBK1, TANK binding kinase 1; 2′3′-cGAMP, cG[2′–5′]pA[3′–5′]P; IRF3, interferon regulatory factor 3; IFN, interferon; TRIM5, tripartite motif protein 5; MX1 (also known as MxA), myxovirus resistance protein A; dsRNA, double-stranded RNA; RIG-I, retinoic acid-inducible gene I; TRIM25, tripartite motif protein 25; MAVS, mitochondrial antiviral-signaling protein; Ub, ubiquitin; NEMO, NF-κB essential modulator; NLRP6, NOD-like receptor family pyrin domain containing 6. (b) Phase separation in adaptive immunity. Both the TCR and BCR signaling pathways depend on the formation of liquid-like condensates, which function as signal hubs that recruit signaling proteins and activate downstream immune responses. Abbreviations: TCR, T-cell receptor; CD3, cluster of differentiation 3; LCK, lymphocyte-specific protein tyrosine kinase; ZAP70, zeta-chain associated protein kinase 70 kD; GRB2, growth factor receptor-bound protein-2; SOS1, son of sevenless 1; BCR, B-cell receptor; CD79, cluster of differentiation 79; SYK, splenic tyrosine kinase; BLNK, B-cell link protein, also known as SLP65; CIN85, Cbl-interacting protein of 85 kD. (c) A schematic of the potential relationship between the dysregulation of LLPS and immune-related diseases. Human TREX1 disease-causing mutations alter interactions with cGAS-DNA droplets, which is a new mechanism to explain autoimmune disease. Similarly, NEMO mutations associated with human immunodeficiency impair phase separation and NF-κB signaling. In addition, IFN-γ induces the nuclear translocation of YAP, which causes the formation of YAP condensates. YAP liquid droplets are selective environments that promote the expression of many immunosuppressive genes. Abbreviation: YAP, yes-associated protein.

antiviral functions of ISGs can also be regulated by LLPS. For example, TRIM5 and MxA, two well-known viral restriction factors, were recently reported to undergo LLPS (Davis et al., 2019; Haubrich et al., 2020).

Like DNA, cytosolic double-stranded RNA (dsRNA) is a potent danger signal in animal cells and can trigger innate immune responses. Recent studies revealed that LLPS functions as a direct regulator of RNA-sensing pathways to play important roles in the response to a broad spectrum of RNA viruses. Indeed, a common role for LLPS is to control the ubiquitination of several key components in RNA immune signaling pathways, including the E3 ligase TRIM25 (tripartite motif protein 25),

adaptor protein MAVS (mitochondrial antiviral-signaling protein), and NEMO (NF-κB essential modulator) (Figure 7A). RIG-I (retinoic acid-inducible gene I) is a key sensor of dsRNA. Upon dsRNA recognition, K63-linked ubiquitination of RIG-I by TRIM25 effectively induces RIG-I oligomerization and signal transduction (Rehwinkel and Gack, 2020). A recent study showed that TRIM25 can form liquid droplets with RNA, which promotes the ubiquitination activity of TRIM25 (Haubrich et al., 2020). Similarly, K63-linked polyubiquitination and SUMOylation of MAVS promote liquid droplet formation and enhance antiviral responses (Dai et al., 2023; Wang et al., 2021b). Condensed MAVS further functions as a signal hub to activate downstream IRF3- or NF-κB-dependent immune programs. In the canonical NF-κB pathway, NEMO binding to polyubiquitin chains leads to the formation of liquid droplets that are required for the activation of NF-κB (Du et al., 2022). In addition to the RIG-I-MAVS pathway, new discoveries further expand the role of LLPS in the inflammasome. NLRP6 (NOD-like receptor family pyrin domain containing 6) is central to host defense, and viral RNA is able to induce LLPS of NLRP6 both *in vitro* and in cells. Phase separation of NLRP6 promotes inflammasome activation and innate immune responses to RNA viruses (Shen et al., 2021).

The adaptive immune system uses antigen-specific receptors on T and B cells to drive signalosome formation and to stimulate antibody- and cell-mediated immune responses (Xiao et al., 2022). Like in the innate signaling pathway, adaptive immune signaling is transduced via immune receptors, downstream adaptors and effector proteins. The binding of antigens to immune receptors, including TCRs (T-cell receptors) and BCRs (B-cell receptors), activates multiple signaling cascades and leads to multiple outcomes to defend against pathogens and cancer. Remarkably, emerging evidence has demonstrated that LLPS is a conserved mechanism in adaptive immune signaling that enables naïve T and B lymphocytes to rapidly sense diverse molecular cues related to pathogen infection and cellular stress (Figure 7B). Upon antigen engagement, TCRs quickly form microclusters with CD3 on the cell membrane, which recruit the kinase LCK (lymphocyte-specific protein tyrosine kinase). LCK then phosphorylates the ITAMs (immunoreceptor tyrosine-based activation motifs) of CD3 to license the recruitment of ZAP70 (zeta-chain associated protein kinase 70 kD), which in turn phosphorylates the key adaptor protein LAT (linker for activation of T cells). Phosphorylated LAT functions as a platform that further recruits downstream adaptor proteins (such as GRB2 and SOS1) through an LLPS mechanism. The liquid-like condensate of LAT allows the penetration of signal kinases while excluding phosphatases from the droplet exterior, leading to T-cell receptor signal transduction (Su et al., 2016). Similar droplet patterns were reported for BCR signaling. The scaffold protein BLNK (B-cell link protein), also known as SLP65, is a key component that mediates BCR signal transduction and has been shown to participate in activating B cells via LLPS (Wong et al., 2020a). *In vitro* and in live B cells, effective B-cell activation requires tripartite phase separation of SLP65, CIN85 and lipid vesicles. After antigen stimulation, BCRs cluster with CD79 and activate cytoplasmic SYK (splenic tyrosine kinase). SYK then phosphorylates the scaffold protein BLNK, which in turn drives the formation of liquid-like condensates and promotes signal activation (Wong et al., 2020a).

Recent rapid growth in super-resolution imaging, structural biology and functional studies has illuminated the foundation of immune signalosomes and their important roles in immune responses. Formation of ordered, solid-like, large supramolecular complexes—once thought of as solely relevant to high-order assemblies—is now known to be one of the assembly mechanisms of immune signalosomes. Meanwhile, LLPS—once thought to be dispensable for immune signalosomes— has now been observed for many immune signaling proteins with important roles in immune activation. Taken together, data in the field show that phase separation functions as a new form of regulation in immunity. Strikingly, emerging evidence suggests that aberrant phase separation is linked to immune-related disorders. For example, genetic mutations in *TREX1* and *NEMO* impair phase separation, which provides a new mechanism to explain autoimmune disease and immunodeficiency; and YAP forms a liquid-like condensate in the nucleus to enhance the expression of key immunosuppressive target genes and cause immune resistance (Du et al., 2022; Yu et al., 2021b; Zhou et al., 2021; Zhou et al., 2022) (Figure 7C). Understanding how phase separation controls physiology and pathology remains one of the most important open questions in immunology.

Chemical modulation of phase separation and potential therapeutic mechanisms

We have discussed in length about how phase separation, as a physiochemical phenomenon, take part in inducing various pathological conditions, leading to diseases. The formation of intracellular condensates, which involves dynamic and multivalent interactions between biomacromolecules, will be affected by the surrounding environment (Banani et al., 2017; Maruri-Lopez and Chodasiewicz, 2023). However, small molecules like metabolites or drugs are governed by the same principles, and some of them have the ability to change the surrounding subcellular conditions, or to interact directly with phase-separating proteins. Thus, small molecules can contribute to the organization or sequestration of vital cellular components through regulation of condensation (Kilgore and Young, 2022). These properties allow us to design novel drugs to potentially treat phase separation-related diseases by various means. Drugs modulating the physical properties, macromolecular network, composition, dynamics, and functions of biomolecular condensates to prevent diseases are termed condensate-modifying therapeutics (Mitrea et al., 2022). In this section, we provide a brief summary of drug interactions with phase-separated biomolecular condensates and how they might be beneficial in developing potential therapies.

Chemical regulation of the concentrations of phase-separating proteins

Phase-separating proteins concentrate at certain subcellular regions to create a higher local concentration and form droplets, which serves as an important physiochemical mechanism of membraneless condensate formation. The most direct way small molecules can have an impact on biomolecular condensates is to target and promote the degradation of the proteins that act as scaffolds within the condensates. This is where proteolytic targeting chimera (PROTAC) technology shines. By linking proteins to E3 ligases, PROTACs lower the concentration of crucial proteins forming condensates, thus alleviating the

manifestations of related diseases (Zou et al., 2019). An example of this is ARV-825, a heterobifunctional PROTAC. ARV-825 can recruit BRD4, one of the scaffolds of transcriptional complexes, to E3 ubiquitin ligase, resulting in reduced expression of a wide range of cancer genes (Lu et al., 2015). Wheeler et al. also screened small molecules and revealed that lipoamide can selectively dissolve stress granules, which points to a possible therapeutic method for cancers and other diseases with excessive phase separation of pathological condensates (Wheeler et al., 2019). An opposing strategy is to promote the formation of normal condensates. For instance, the plant-derived compound sulforaphane can inhibit the formation of KEAP1-NRF2 complexes, hence promoting the nuclear accumulation of NRF2, which slows the progression of autosomal dominant polycystic kidney disease (Lu et al., 2020). Sulforaphane has also been tested in clinical studies for a range of chronic diseases and cancer (Houghton, 2019). Together, these studies provide mechanistic insights into the effects of many small molecules on phase-separated condensates and their components. By regulating the concentration of scaffold proteins, small-molecule therapeutics might prevent pathological outcomes.

Partitioning of compounds into condensates

Rather than disrupting the condensates, some small molecules are found to selectively concentrate in condensates to exert pharmacological effects (Klein et al., 2020). This partitioning requires specific chemical patterns to cope with the environment within the condensates (Kilgore and Young, 2022). Kilgore et al. developed and trained a model to predict partitioning of small molecules *in vivo*. Using immunofluorescence to confirm the results, they successfully predicted the partitioning of mitoxantrone, a potent chemotherapeutic, into nucleoli, and of tryptanthrin, an alkaloid, into chromocenters (Kilgore et al., 2024). This marks a breakthrough in integrating machine learning into investigating the "chemical grammar" which underlies the partitioning of small molecules into condensates with vastly different chemical environments than the surrounding cytoplasm. Such mechanisms can also be utilized by cancer cells to induce drug resistance. An example of this is the tamoxifen resistance of the breast cancer cell line TAMR7. TAMR7 cells overexpress MED1—one of the scaffolding proteins of transcriptional complexes—which expands the volume of the condensates. When tamoxifen was delivered to TAMR7 cells, it was sequestered within the MED1 condensates, and therefore its effective concentration and anti-cancer activity were reduced in TAMR7 cells compared to other breast cancer cell lines (Klein et al., 2020). In summary, small molecules require specific structures to partition into condensates to perform their effects, and the same mechanism can lead to beneficial pharmacological outcomes on one hand or to drug resistance on the other hand. Researchers should pay attention to these partitioning effects when designing drugs targeting biomolecular condensate components.

Direct targeting of biomolecular phase separation by small molecules

There are also small molecules which can target the phase-separating proteins directly. For instance, treatment with SHP099, an allosteric inhibitor of the ubiquitous non-receptor phosphatase SHP2, causes the shrinkage of SHP2 puncta. A more potent allosteric inhibitor, ET070, exhibits even stronger inhibition of SHP2 LLPS, which suggests a possible pharmacological mechanism to tackle SHP2-related diseases like Noonan syndrome (Zhu et al., 2020a). IDRs are seldom considered as possible targets due to their lack of structural features, yet the small molecule EPI-001 is able to target the IDR of the androgen receptor, whose condensation is closely related to castration-resistant prostate cancer. Research has shown that EPI-001 can attenuate the progression of the disease (De Mol et al., 2016). PTMs might also be potential targets requiring more attention from researchers. Previous reports on FRMP (fragile X mental retardation protein), whose C-terminal LCD can be phosphorylated and methylated, indicated opposing outcomes of these two modifications: phosphorylation promotes LLPS while methylation inhibits LLPS (Tsang et al., 2019). The formation of phase-separated FRMP bodies has an impact on the inhibition of translation (Blackwell et al., 2010). These reports suggest novel pharmacological mechanisms for therapy of phase separation-related diseases.

The studies mentioned above demonstrate how individual small molecules can interfere with phase separation to potentially treat diseases. Nevertheless, researchers are usually faced with a large number of possible compounds that need screening and filtering before narrowing down to a small number of potential candidates. A recent study introduces a novel high-throughput method called DropScan to screen condensate-regulating small molecules. With high-content image screening, researchers were able to screen 1,777 anticancer drugs using high-content image screening to monitor condensate counts at multiple checkpoints in a time-dependent manner (Wang et al., 2023d). High-throughput methods will be useful for screening more phase separation-related small molecules.

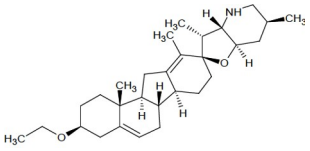
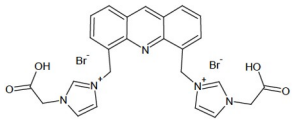
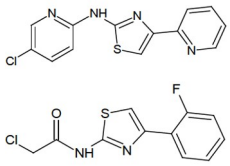
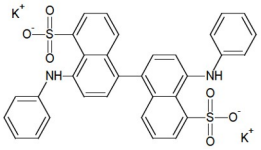
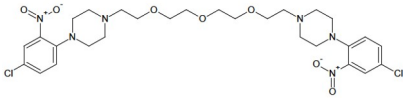
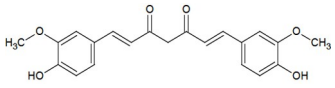
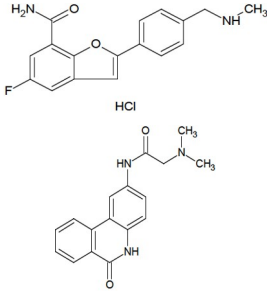
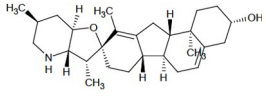
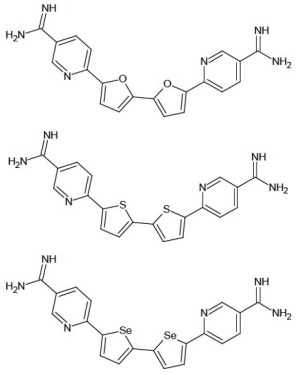
Although there are still many questions regarding the causality of aberrant condensates and disease mechanisms, this conceptual framework, based on intervention by small molecules, provides a new paradigm for identifying innovative therapeutic targets. Condensate formation is driven by multivalent interactions among IDRs or IDPs, multiple copies of interaction domains, and RNAs, which are not conventional drug targets (Hofmann et al., 2021; Mathieu et al., 2020; Tsang et al., 2020). In Table 2, we summarize the emerging proof-of-concept examples of small molecules that directly target phase separation.

Chemical modulators of LLPS through targeting of IDRs

Up to 25% of disease-associated missense mutations locate to IDRs; however, these mutations have long been disregarded or annotated as variants of unknown biological significance (Vacic and Iakoucheva, 2012). Following the discovery that phase separation homeostasis is disrupted by IDR mutations, researchers have been inspired to uncover small-molecule modulators of IDRs.

Although 1,6-hexanediol can disrupt intracellular or *in vitro* condensates within minutes, there are concerns regarding its selectivity and toxicity when used at high concentrations (over 3.5%) (Kroschwald et al., 2017). Therefore, it is necessary to identify compounds with enhanced potency and selectivity for potential application in the treatment of neurodegenerative

Table 2. Emerging small-molecule modulators of biomolecular phase separation

Compound ID	Structure	Target	Activity	References
A3E		RSV–M2-1 in inclusion bodies	Antiviral IC_{50}: 1.0 ± 0.3 μmol/L	Risso-Ballester et al., 2021
AIM4		IDR of TDP-43 in SGs	K_d > 150 μmol/L	Girdhar et al., 2020; Prasad et al., 2016
BAY 249716; BAY 1892005		Full-length p53 in p53 condensates	Antiproliferation IC_{50} 2–10 μmol/L; BAY 1892005: covalent binder	Lemos et al., 2020
bis-ANS		LCD of TDP-43 in SGs	Biphasic	Babinchak et al., 2020
BiTud		Tudor domain of TDRD3 in SGs	K_d with Tandem Tudor chimeras: 6 μmol/L; Inhibitory concentration of SG growth: 5–10 μmol/L	Fan et al., 2024
curcumin		α-Synuclein in Lewy bodies	IC_{50}: ~50 μmol/L	Xu et al., 2022a
CVL218, PJ34		LLPS formed by full-length N protein of SARS-CoV-2	K_d: 4.7 μmol/L (CVL218), 696 μmol/L (PJ34); Concentration in LLPS assay, 20 μmol/L	Zhao et al., 2021a
Cyclopamine (CPM)		RSV–M2-1 in inclusion bodies	Antiviral IC_{50}: 36 nmol/L	Bailly et al., 2016
DB1246; DB1247; DB1273		G-quadruplexes in RNA foci	K_d: 200–400 nmol/L	Simone et al., 2017

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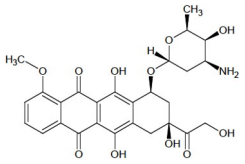
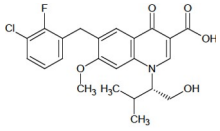
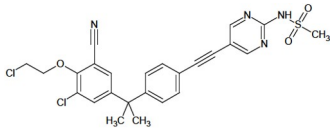
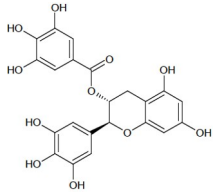
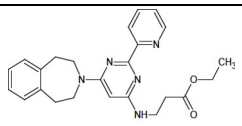
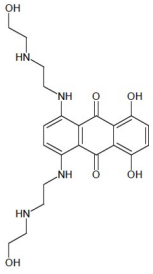
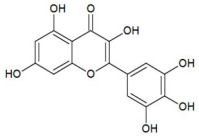
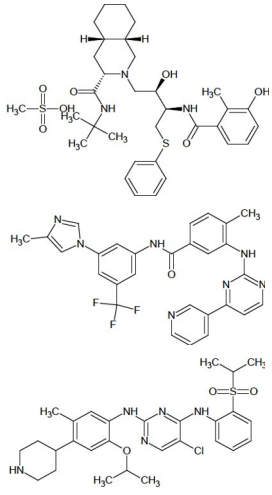
Compound ID	Structure	Target	Activity	References
Doxorubicin		47×CAG nuclear foci	Concentration to disrupt RNA foci in cells: 2.5 μmol/L LLPS concentration: 1 mmol/L	Jain and Vale, 2017
Elvitegravir (EVG)		IDR of SRC-1 in transcriptional condensates	IC_{50}: 8–86 μmol/L	Zhu et al., 2021
ET516		IDR of AR in DHT-stimulated condensates	Inhibits ARE reporter activity, K_d: 25 μmol/L IC_{50}: 0.7 μmol/L (transcription), 0.2 μmol/L (condensation)	Xie et al., 2022
GCG		SARS-CoV-2 N protein	Antiviral IC_{50}: 44.4 μmol/L	Zhao et al., 2021b
GSK-J4		IDR of HOXB8 in CRC condensates	K_d: 420 μmol/L	Lu et al., 2021
Mitoxantrone		TDP-43 in SGs	IC_{50} (SG condensation): 5–10 μmol/L	Fang et al., 2019
Myricetin		Tau in SGs / α-Synuclein in Lewy bodies	Single dose, 10 μmol/L (Tau toxicity), 100 μmol/L (α-Syn amyloid aggregates)	Dai et al., 2021; Xu et al., 2022b
nelfinavir mesylate; nilotinib; LDK378		SARS-CoV-2 N protein in cytoplasm puncta	IC_{50}: 21 μmol/L (nelfinavir mesylate)	Jack et al., 2021

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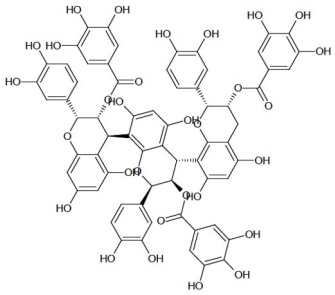
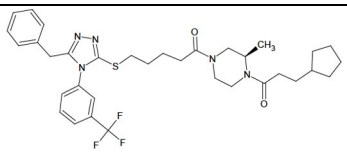
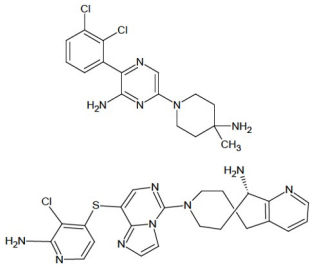
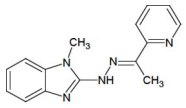
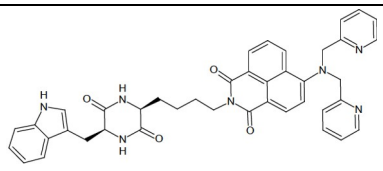
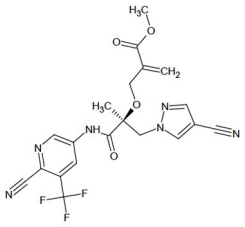
Compound ID	Structure	Target	Activity	References
PCG		IDR of BRD4 in BRD4 nuclear condensates	Single dose: 50 μmol/L (hardens BRD4 condensates)	Wang et al., 2022a
R-huezole		Tubulin in microtubules	K_d: 3.5μmol/L; IC_{50} (anti-proliferation): 4.4 μmol/L	Ado et al., 2022
SHP099; ET070		Folded domain of SHP2 mutants in cytoplasm puncta	Single dose: 20 μmol/L (LLPS), 10 μmol/L (in-cell condensation)	Zhu et al., 2020a
SI-2		IDR of SRC-3 in SRC-3 foci	Improves the sensitivity to BTZ treatment; reduces SRC-3 aggregation; suppresses or even halts tumor growth in most cases at 25 nmol/L	Liu et al., 2021a
SMM1c		Tau (IDP) in Zn-induced tau LLPS	K_d: 3.95 μmol/L	Ramesh et al., 2022
UT-143		AR-V7 (IDP) in condensates (predominantly nuclear, some cytoplasm)	IC_{50}: 150 nmol/L	Thiyagarajan et al., 2023

diseases and cancers. For instance, AIM4 binds to the prion-like domain of TDP-43 at an affinity of over 150 μmol/L, and inhibits the aggregation and phase separation of TDP-43 (Girdhar et al., 2020; Prasad et al., 2016). Furthermore, a series of small molecules with planar structures (e.g., mitoxantrone) were identified through high-content screening. These compounds modulate the dynamics of stress granules, and prevent accumulation of cytoplasmic TDP-43 with a single-digit micromolar IC50 (Fang et al., 2019). Curcumin reduces the aggregation of wild-type and disease-related α-Syn with an IC50 of about 50 μmol/L (Xu et al., 2022a). A natural antioxidant flavonoid, myricetin, inhibits LLPS of full-length Tau *in vitro* to delay the liquid-to-solid phase transition towards amyloid aggregation, at a potency of approximately 10 μmol/L (Dai et al., 2021). A rationally designed small molecule, based on a cyclic dipeptide, has single-digit micromolar binding affinity with Tau, and significantly inhibits zinc-mediated Tau phase separation at 10 μmol/L (Ramesh et al., 2022). A biphasic modulator, bis-ANS, enhances LLPS of the TDP-43 LCD at a low concentration of 5 μmol/L and inhibits LLPS at high concentrations (Babinchak et al., 2020).

Besides these chemical modulators targeting neurodegeneration-linked proteins, compounds have been identified with potential anti-cancer effects. GSK-J4, an H3K27 demethylase inhibitor, attenuates core regulatory circuitry condensates by binding to the IDR of the super enhancer HOXB8 at a K_d value of 420 μmol/L. Although GSK-J4 demonstrates inhibitory efficacy

in osteosarcoma cell proliferation, metastasis and re-sensitivity to chemotherapy drugs in a patient-derived xenograft model, it remains uncertain whether this efficacy should be ascribed to inhibition of HOXB8 condensation or H3K27 demethylase activity (Lu et al., 2021). A natural product, PCG, directly binds to the IDR of BRD4. This binding turns dynamic BRD4 condensates into static aggregates, albeit at a relatively high PCG concentration of 50 μmol/L (Wang et al., 2022a). In another study, a phase-separation-based phenotypic screen identified ET516, which binds to the disordered N-terminal domain of AR with a K_d of 25 μmol/L. ET516 dose-dependently inhibits AR transcriptional activity and formation of condensates by the AR F877L/T878A mutant with an IC50 of 0.7 and 0.2 μmol/L, respectively. ET516 reduces the proliferation of AR-positive prostate cancer cells and growth of LNCaP-AR(F877L/T878A) xenograft tumors (Xie et al., 2022). To enhance the potency of IDR inhibitors, another feasible strategy is to discover compounds which undergo irreversible covalent linkage with the target protein. For instance, UT-143 covalently binds to the AR AF-1 region at residues C406 and C327, which are critical for chromatin condensation and the AR-V7 interactome. UT-143 inhibited AR transactivation with an IC50 of 150 nmol/L, and eventually suppressed prostate cancer cell proliferation and tumor growth (Thiyagarajan et al., 2023).

Modulation of LLPS by inhibitors targeting structured domains

Despite the encouraging examples presented above, it remains challenging to develop potent and selective IDR inhibitors to mediate LLPS. An alternative and fruitful approach is to target the structured protein domains that are key to LLPS. For example, the aminothiazole BAY 1892005 covalently binds to the R175H and Y220C mutants of p53, and induces dissolution of p53 mutant condensates (Lemos et al., 2020). A self-assembling compound, huezole, forms liquid droplets to sequester tubulin (K_d=3.5 μmol/L) *in vitro* and in cells, thus preventing cell mitosis by forming tubulin-concentrated condensates. This indicates that cellular processes can be exogenously tuned by inducing condensates enriched in specific components (Ado et al., 2022). In another study, the SHP allosteric inhibitors SHP099 and ET070 stabilize SHP2 in its auto-inhibited conformation. Treatment with these two inhibitors attenuates the formation of SHP2 puncta (Zhu et al., 2020a).

The assembly of biomolecular condensates is considered to be driven by multivalent weak non-covalent interactions among biomolecules which induce the formation of liquid droplets. The potency of a small molecule that usually targets a monovalent interaction is therefore limited by the nature of these multivalent weak interactions within MLOs. A new strategy has been developed in which two monovalent domain inhibitors are chemically linked together. The bivalent inhibitor BiTud (K_d of 6 μmol/L) exhibits over 10-fold affinity enhancement relative to the monovalent one. BiTud directly disrupts the multivalent interaction between the SG hub protein G3BP1 and TDRD3, a reader of asymmetrically dimethylated arginine. BiTud exhibits inhibitory efficacy on SG growth (Fan et al., 2024).

Several LLPS inhibitors have been discovered to target a full-length protein with unknown epitopes. For instance, the steroidal alkaloid A3E suppresses human respiratory syncytial virus (RSV) replication, with an IC50 of 1.0 μmol/L, by hardening virus-induced condensates called inclusion bodies (Risso-Ballester et al., 2021). A cellular study demonstrated that compound SI-2 eliminates the LLPS of SRC-3, probably by disrupting the binding of NSD2 to the SRC-3 IDR. SI-2 at 25 nmol/L sensitized multiple myeloma to bortezomib treatment (Liu et al., 2021a). The anti-HIV drug Elvitegravir, which was identified from a cell-based YAP reporter screen, binds to SRC-1 and inhibits SRC-1 phase separation and proliferation of a variety of lung cancer cells (Zhu et al., 2021). The PARP (poly ADP-ribose polymerase) inhibitors CVL218 and PJ34 bind to the N protein of SARS-CoV-2 with K_d values of 4.7 and 696 μmol/L, respectively. Treatment with 20 μmol/L CVL218 or PJ34 increases the condensate size and diffusion rate of the N protein (Zhao et al., 2021a). GCG, a polyphenol from green tea, disrupts RNA-triggered LLPS of SARS-CoV-2 N protein and inhibits virus replication (Zhao et al., 2021b). Similarly, nelfinavir mesylate, nilotinib, and LDK378 modulate the number, size, and shape of the N protein condensates (Jack et al., 2021). Nevertheless, the exact binding sites of these inhibitors remain to be mapped by further biophysical or biochemical assays.

Modulation of LLPS by RNA binders

Mediation of LLPS by RNA binders represents a novel approach which takes into account the essential roles of RNAs in ribonucleoprotein condensates. For example, repeat-containing *C9orf72* RNA forms G-quadruplexes, which are bound by compounds DB1246, DB1247, and DB127 with K_d values of 200–400 nmol/L (Simone et al., 2017). Treatment with these compounds reduces RNA foci in the neurons of patients. A DNA intercalator, doxorubicin, blocks the formation of CAG RNA gels and dissolves the 47×CAG nuclear foci in cells. However, the doxorubicin concentration required to disrupt RNA foci in cells is 2.5 μmol/L, much lower than 1 mmol/L used *in vitro* (Jain and Vale, 2017).

In a nutshell, phase separation is not only a common mechanism for physiological and pathological processes, but it can also be utilized in drug design and screening since small molecules also behave according to the physiochemical mechanisms of biomolecular condensate formation. Notably, phase separation can be a possible mechanism for drug resistance. Researchers are encouraged to investigate the details of these mechanisms so as to design drugs with better precision in targeting phase separation. With the help of machine learning and phase separation-related databases (Hou et al., 2023; Li et al., 2020; Wang et al., 2023d), we expect to see more drugs dealing with phase separation-related diseases in the future.

CONCLUSIONS AND PERSPECTIVES

As discussed above, mounting evidence depicts a scenario in which intracellular spaces are packed with numerous different types of MLOs, fulfilling distinct physiological processes. Recent studies also suggest close relationships between aberrant LLPS and distinct pathologies. Understanding the role and regulatory mechanisms of LLPS of biomolecules under both physiological and pathological conditions has great significance for uncovering the pathogenic processes of these diseases and providing clues for therapeutic strategies. For example, approaches aimed at preventing and/or disaggregating the pathological MLOs can be beneficial. These approaches may involve developing small molecules, peptides, or antibodies that can modulate LLPS of biomolecules, prevent pathological condensation, or promote the clearance of toxic aggregates. Therapeutic strategies targeting

these mechanisms are still in the early stages of development. Further research and clinical studies are needed to identify the most effective approaches to translate these findings into effective treatments for the devastating diseases described above. Techniques for detecting and probing LLPS, combined with bioinformatic methods for predicting LLPS, offer powerful tools for the discovery of unknown MLOs and the exploration of their biological roles. Therefore, there is an urgent need to develop and improve these techniques in the future.

Compared to the biophysical properties underlying LLPS, which have been extensively analyzed and discussed, our understanding of MLOs themselves remains limited. There is scant knowledge about the mechanisms by which MLOs participate in various biological processes, as explored by existing studies. Recent research has revealed that the distinct material properties of different MLOs are closely linked to their physiological and pathological functions, thus playing a crucial role in achieving the spatial and temporal precision of diverse biological processes (Wang et al., 2022c). A multidisciplinary approach—integrating theories and techniques from biology, physics, mechanics, chemistry, and other fields—is urgently needed and could prove effective in advancing this research direction. For instance, one study revealed that cellular proteins can function as surfactants, modulating the interfacial tension of transcriptional condensates and thereby regulating their DNA affinity and transcriptional activity (Wang et al., 2023e). Applying surfactant adsorption theory from interfacial chemistry to MLO research is a key aspect of this work, along with the use of a micropipette-based interfacial tension measurement technique derived from soft matter physics. Investigating the relationship between the material properties of MLOs and their biological functions is crucial for uncovering the mechanisms underlying the physiological and pathological roles of MLOs involved in diverse life processes, and this area merits increased attention in future research. Such studies may also uncover potential strategies and targets for treating diseases associated with MLOs. For example, Wang et al. found that the material properties of biomolecular condensates can be targeted and modulated by small-molecule compounds, which alter the activity of the condensates and the consequent cellular pathway (Wang et al., 2022d).

Another issue of concern is the subdivision of MLO architecture. All biomolecular condensates can be divided into internal and interfacial structures. A vast number of studies have revealed the importance of the internal structure of MLOs. In contrast, the importance and significance of the interfacial structure of MLOs have not been fully explored or received enough attention. In fact, as the unique feature that distinguishes MLOs from non-phase-separated biomolecular solutions, the interfacial structure deserves to be regarded as one of the essential characteristics of MLOs. Several studies have preliminarily revealed the irreplaceable roles of the interface and its material properties in determining the architecture of multiple MLOs (e.g., the nucleolus), packaging of autophagosomes, modulating the activity of transcriptional condensates, and so forth (Feric et al., 2016; Agudo-Canalejo et al., 2021; Wang et al., 2023e; Milovanovic et al., 2018; Ma and Mayr, 2018; Wan et al., 2018; Shin et al., 2018; Wu et al., 2021b; Wu et al., 2021c). Computational research has revealed that the interfacial biomolecules at the surface of phase-separated MLOs adopt a distinct molecular conformation compared to internal biomolecules. Thus, the interface possesses unique material properties that are different from the internal part (Farag et al., 2022). The unique molecular conformation and material properties may have significant implications for the special biological function of the MLO interface. This area deserves deeper and more systematic analyses in the future. Moreover, MLO interfaces are also involved in disease pathogenesis. Some recent studies found that formation of amyloid fibrils by hnRNPA1 and FUS in ALS/FTD occurs at the interface instead of the interior of these protein condensates (Linsenmeier et al., 2023; Shen et al., 2023). Drugs such as small-molecule compounds targeting the interfaces may be a promising strategy for preventing amyloid fibril formation.

In conclusion, research into LLPS and MLOs may be experiencing its golden age. Various physiological and pathological processes have been found to be intimately associated with LLPS and MLOs, and studies on LLPS and MLOs, in turn, are pushing these biological and medical fields to a whole new level. In addition to continuous and deeper exploration of the correlation between LLPS and biological processes, research into the functions of MLOs also deserves more attention. Techniques for the qualitative or quantitative study of LLPS and MLOs, especially within *in vivo* environments, are also urgently needed, which will help to expand the blueprint for the field of LLPS. With these efforts, we believe that LLPS and MLOs, once again, will bring a revolutionary advance in the disciplines of biology and medicine.

Compliance and ethics

The authors declare that they have no conflict of interest.

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