Review

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How do RNA binding proteins trigger liquid-liquid phase separation in human health and diseases?

Ying Huai[1,2,3,4,§], Wenjing Mao[1,2,3,4,§], Xuehao Wang[1,2,3,4], Xiao Lin[1,2,3,4], Yu Li[1,2,3,4], Zhihao Chen[1,2,3,4,5,*], Airong Qian[1,2,3,4,*]

[1] Lab for Bone Metabolism, Xi'an Key Laboratory of Special Medicine and Health Engineering, School of Life Sciences, Northwestern Polytechnical University, Xi'an, Shaanxi, China;
[2] Key Lab for Space Biosciences and Biotechnology, School of Life Sciences, Northwestern Polytechnical University, Xi'an, Shaanxi, China;
[3] Research Center for Special Medicine and Health Systems Engineering, School of Life Sciences, Northwestern Polytechnical University, Xi'an, Shaanxi, China;
[4] NPU-UAB Joint Laboratory for Bone Metabolism, School of Life Sciences, Northwestern Polytechnical University, Xi'an, Shaanxi, China;
[5] Department of Obstetrics and Gynecology, Xijing Hospital, The Fourth Military Medical University, Xi'an, China.

SUMMARY RNA-binding proteins (RBPs) lie at the center of post-transcriptional regulation and protein synthesis, adding complexity to RNA life cycle. RBPs also participate in the formation of membrane-less organelles (MLOs) *via* undergoing liquid-liquid phase separation (LLPS), which underlies the formation of MLOs in eukaryotic cells. RBPs-triggered LLPS mainly relies on the interaction between their RNA recognition motifs (RRMs) and capped mRNA transcripts and the heterotypic multivalent interactions between their intrinsically disordered regions (IDRs) or prion-like domains (PLDs). In turn, the aggregations of RBPs are also dependent on the process of LLPS. RBPs-driven LLPS is involved in many intracellular processes (regulation of translation, mRNA storage and stabilization and cell signaling) and serves as the heart of cellular physiology and pathology. Thus, it is essential to comprehend the potential roles and investigate the internal mechanism of RPBs-triggered LLPS. In this review, we primarily expound on our current understanding of RBPs and they-triggered LLPS and summarize their physiological and pathological functions. Furthermore, we also summarize the potential roles of RBPs-triggered LLPS as novel therapeutic mechanism for human diseases. This review will help understand the mechanisms underlying LLPS and downstream regulation of RBPs and provide insights into the pathogenesis and therapy of complex diseases.

Keywords Biomacromolecule, phase transition, membrane-less organelles (MLOs), human diseases, therapeutic targets

1. Introduction

RNA binding proteins (RBPs) are a kind of protein family ubiquitously in eukaryotes and are considered key regulatory components and critical interaction partners for all cellular RNAs (*1*). In addition to the extensive physiological functions of RBPs, their defective expressions and intracellular mis-localization are contributed to a variety of human diseases, such as virus infection, cancer, aging-related diseases and neurodegenerative diseases (*2,3*). Generally, RBPs are characterized by the presence of RNA-binding domains (RBDs), through which they bind to target RNAs, thus regulating the fate or function of the bound RNAs. Moreover, some RBPs possess a high percentage of intrinsically disordered regions (IDRs) or prion-like domains (PLDs), which provokes great interest in deciphering the molecular mechanisms of RBPs in cellular compartmentalization and liquid-liquid phase separation (LLPS) (*4,5*). In other words, the unique structural features of RBPs allow them to assemble with RNAs/proteins to form dynamic liquid-like condensations *via* LLPS, thus controlling the RNA life cycle.

LLPS is a reversible and metastable de-mixing, where proteins and/or RNA spontaneously segregate to form two coexisting liquid phases (the condensed phase and the dilute phase) mediated by transient, multivalent interactions (*6*). LLPS is one of the important mechanisms by which organisms respond to external stimuli and protect themselves, regulating multiple physiological and pathological processes (*7*).

Moreover, LLPS acts as the foundation and driving force of the formation of membrane-less organelles (MLOs), which explains the self-assembly process of subcellular structures (*8*). MLOs, ubiquitous functional subunits of intracellular organization, are primarily responsible for localizing and regulating complex biochemical reactions intracellular, offering facile transport of substrates for cells. Additionally, numerous studies have shown that multiple aggregation-prone RBPs, such as Fused in sarcoma (FUS), Human antigen R (HUR), Ras-GAP SH3 domain binding protein 1 (G3BP1), TAR DNA-binding protein 43 (TDP-43) and T cell intracellular antigen-1 (TIA-1), spontaneously aggregate and develop MLOs by LLPS process (*9*). Notably, prominent MLOs condensed by RBPs, including stress granules (SGs), process bodies (P-bodies) and germ cells, are thought to orchestrate many important biological processes and in some cases drive diseases. However, there is still no systematic review of RBPs-triggered LLPS and their physiological and pathological functions. Therefore, in the present review, we will focus on the pioneering works of elucidating the molecular mechanism of RBPs-triggered LLPS and their physiological and pathological roles. This review will promote the current understanding of the molecular biology of RBPs-driven LLPS, provide new insights into the function of RBPs and offer future directions for RBPs and LLPS research.

2. Structural characteristics of RBPs involved in LLPS

RBPs are evolutionarily deeply conserved and generally ubiquitously expressed in eukaryotes, which just mirrors their central roles in the RNA life cycle. Structurally, RBPs are often characterized by the presence of RNA-binding domains (RBDs) and intrinsically disordered regions (IDRs) (*10*). Thereinto, RBDs are the functional units responsible for binding RNA in RBPs and primarily recognize their target sites through the sequences and shape of RNA. Moreover, RBDs of RBPs also serve important roles in the formation of membrane-less organelles (MLOs), thus participating in the compartmentalization, organization and stress response of the cells (*8*). On the other hand, IDRs in RBPs are repetitive and have a high content of glycine, arginine, lysine and tyrosine residues, which are commonly located in domains that interface with RNA. IDRs interaction is particularly important for assembly of RBPs and formation of multi-component MLOs (*11*). Interestingly, there is mounting evidence of RBPs containing RBDs and IDRs possess a particularly high LLPS propensity through complex interactions of multivalent protein-protein, protein-RNA and RNA-RNA (*5,12*). Moreover, RBPs also exert their regulatory roles through various post-translational modifications (PTMs), which are conducive to triggering LLPS to enable the cell to quickly and efficiently respond to stress stimuli (*3*). For example, multiple PTMs of FUS, such as serine and threonine phosphorylation as well as arginine methylation, can strongly influence the biophysical properties of FUS aggregation and LLPS (*13*). In a word, the unique structural composition and sequence characteristics of RBPs are conducive to their assembly and LLPS (Figure 1A).

3. LLPS underlies MLOs formation

As biological evolution, the emergence of diversification

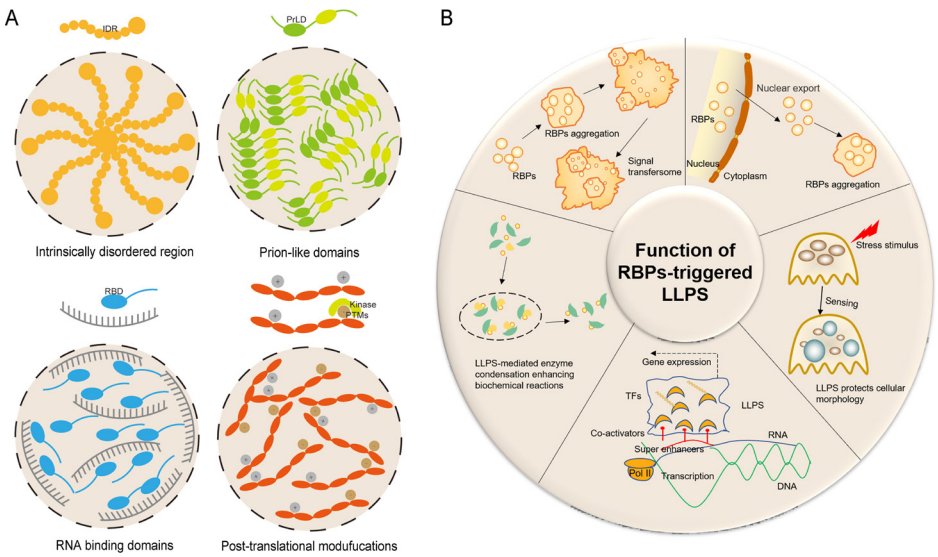

Figure 1. Structure characteristics of RBPs involved in LLPS and roles of RBPs-triggered LLPS in controlling RNA life cycle. A. The unique structures that drive the LLPS of RBPs. RBPs usually contain IDRs, PrLD, RBDs and PTMs which are vital for RBPs to undergo LLPS. **B**. The function of RBPs-triggered LLPS in RNA life cycle. RBPs and its LLPS are involved in regulating cellular mechanotransduction, intracellular biochemical reactions, cellular homeostasis, endocrine and gene transcription.

of organelles at the cellular level allows different biological reactions in specific organelles orderly. Similarly, MLOs are also involved in various cellular biological processes due to the concentrated nucleic acid and protein within them (*14*). LLPS is an important organizing principle and theoretical basis of MLOs, which explains the regulation mechanisms of MLOs in the assembly, composition and function.

Recently, numerous studies have indicated that these ubiquitously MLOs in eukaryotic cells modulate a diversity of physiological and pathological traits in multiple ways, which are closely related to the physical properties, types and intracellular localization of MLOs. Moreover, these MLOs formed by LLPS are distributed in the cytoplasm, nucleus, and on the membrane. Figure 2 displayed the various biomolecular condensates formed by LLPS and the assembly of stress granules (SGs). Cytoplasmic MLOs are dynamically assembled by the LLPS driven by the temporarily untranslated RNAs and proteins which coalesce into a concentrated state (condensed phase) in the cytoplasm. Prominent examples of cytoplasmic MLOs mainly include stress granules (SGs), processing bodies (P-bodies), RNA transport granules and germ granules. SGs are one of the predominant types of cytoplasmic MLOs formed by the crowded protein and RNA. Under stress, SGs immediately start to accumulate and regulate the mRNA utilization in eukaryotic cells, which is essential for maintaining cell integrity and intracellular homeostasis. Moreover, P-bodies are highly conserved cytoplasmic foci with properties of liquid droplets and have been observed in somatic cells originating from vertebrates and invertebrates, plants and yeast. P-bodies are formed by LLPS and are primarily composed of translation-arrested RNAs and RBPs related to mRNA decay, suggesting roles in post-transcriptional control. In addition, LLPS also appears to be important for driving the assembly of various nuclear-localized MLOs such as nucleoli, Cajal bodies and nuclear speckles, and underlie their biogenesis. Nucleolus, as the most prototypical and prominent MLO in nuclear, forms around regions of chromosomes containing stretches of tandem ribosomal DNA (rDNA) gene repeats, known as nucleolar organizer regions (NORs) (*15*). Nuclear speckles are another well-studied MLOs formed by LLPS in nuclear, exhibiting dynamic and irregular shapes. Nuclear speckles are subnuclear structures enriched in RBPs, particularly those involved in splicing, located in the interchromatin regions of the nucleoplasm of mammalian cells (*16*).

4. Well-studied RBPs involved in LLPS

Numerous RBPs, especially RBDs/IDRs-harboring RBPs, readily undergo concentration-dependent LLPS and mediate protein/RNA interactions to form MLOs (*17*). In eukaryotic cells, diverse stresses trigger coalescence and condensation of RBPs, which is an essential prerequisite for LLPS. Under stress stimuli, RBPs recruit the translation-stalled mRNA to form MLOs with liquid-like properties, which precisely support the fact that numerous dynamic MLOs (such as SGs) are rich in RBPs. The assemblies of condensates formed by phase transitions of RBPs are implicated in both health and disease, triggering interests in deciphering the molecular mechanisms of compartmentalization orchestrated by RBPs in both physiology and pathology. Until now, the LLPS of multiple RBPs, including TIA-1, FUS, G3BP1, HUR, poly (A)-binding protein (PABP),

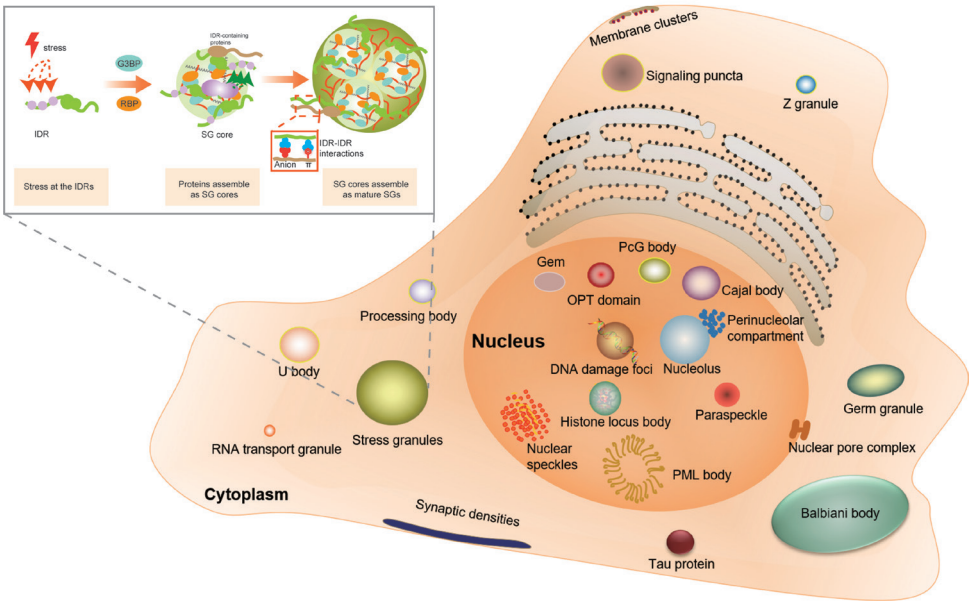

Figure 2. Types of membrane-less organelles (MLOs) in cells driven by LLPS. All these MLOs distribute in plasma membrane, cytoplasm and nucleus to perform their own duties. SGs are one of the most common MLOs in cytoplasm, whose formation process is driven through LLPS of RBPs.

Table 1. Overview of RBPs involved in the process LLPS.

RBPs	MLOs Association	Biological Function	Number of IDRs[a]	Overall percent disordered (%)[b]	Mechanisms of LLPS Occurrence
HUR	SGs (*34*)	RNA stability	8	19.33	The HNS domain makes it from the nucleus to the cytoplasm, accumulates aggregates, and colocalizes with G3BP1
TIA-1	SGs (*36*)	mRNA silencing	8	25.45	Self-association of the intrinsically disordered prion-like domain that facilitates LLPS
TDP-43	Nuclear gems (*90*); nuclear stress bodies (*91*); SGs (*24*); paraspeckles (*92*)	transcriptional and posttranscriptional regulation	8	35.78	The IDR, the unique structure of the C-terminal domain, and multiple RNA-binding sites for TDP-43 make it trigger LLPS
hnRNPA1	SGs (*93*); paraspeckles (*92*)	pre-mRNA splicing, mRNA stability	5	29.06	The 'prion-like' LC domain mediates hnRNPA1 self-association to trigger LLPS
FUS	Paraspeckles (*94*); Nuclear gems (*94*); SGs (20)	mRNA transport and translation, gene splicing	8	72.58	LC domain, C-terminal domain, and RRM, these self-assembling regions drive LLPS behavior, which is influenced by the various PTMs of FUS
G3BP1	SGs (*25*)	Ras signaling/marker of SGs	4	53.22	Three IDRs in G3BP1 interact according to the saturating concentration of RNA to change conformation and turn on LLPS
NCL	Nucleolus (*95*)	rDNA transcription, rRNA maturation, ribosome assembly	13	55.49	NLS enables it to enter the nucleus, where RBDs and RGG domains bind to other nucleolar components such as rRNA
PABP-1	SGs (*96*)	Translation stability	11	33.57	Unclear
EWSR1	DNA damage response foci (*97*); SGs; paraspeckles (*92*)	fuse with various partner genes	7	79.85	Interaction of the LC domain with the RGG domain contributes to self-association
TAF15	DNA damage response foci (*97*); SGs (*98*); paraspeckles (*92*)	Regulation of gene transcription	7	51.1	LC-RGG, LC-LC, and RGG-RGG interactions contribute to self-association
hnRNPA3	SGs (*99*)	pre-mRNA splicing, mRNA stability	7	38.76	LC region mainly drives self-association and RRM domain may function in the presence of RNA

[a], The number of IDRs within the proteins' sequences; [b], The proportion of disordered regions in the total protein sequence.

TDP-43, heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and nucleolin (NCL), have been well-studied. The detailed information of RBPs involved in LLPS was listed in Table 1.

4.1. Fused in sarcoma (FUS) drives LLPS

Fused in sarcoma (FUS) is a ubiquitously expressed and predominantly nuclear-localized RBP. Multiple well-established functions – mRNA transport and translation, gene splicing and gene expression – have been ascribed to FUS. Emerging evidence has shown that FUS undergoes a reversible dynamic phase transition between a dispersed state, liquid droplets, and hydrogels (*5,18*). Structurally, FUS maintains some self-assembly regions including prion-like low-complexity (LC) domains, C-terminal domains and RNA recognition motif (RRM), which are vital for driving LLPS behavior. Moreover, the various PTMs (*e.g.*, phosphorylation) of FUS also alter its localization, concentration and aggregation thus influencing the self-assembly or LLPS driven by FUS (Figure 3A). Monahan *et al*., showed that both phosphorylation and phosphomimetic variants reduce FUS propensity to aggregate and disrupt the LLPS and toxicity of FUS in the presence of RNA or salt (*19*). Collectively, the importance of FUS in the cellular life cycle, together with its facile self-assembled structures, raises the possibility that natural selection and evolution have preserved the LLPS propensity of FUS. The response of FUS to cellular stress also involves the formation of MLOs which are transient regulatory structures. For instance, under stress conditions like oxidative stress and hyperosmolar stress, FUS rapidly shuttles from the nucleus to the cytoplasm and assemble into SGs or P-bodies *via* LLPS (*20*). Upon removal of these cellular stress, liquid-like droplets of FUS disassemble in seconds. Noteworthy, excessive recruitment of FUS into phase-separated liquid-like droplets followed by aggregation has been proposed to drive disease. Taken together, proper assembly and aggregation of FUS through LLPS play essential roles in maintaining normal cellular physiological function. Inversely, aberrant LLPS driven by FUS mutants has been proposed as a cause of cellular dysfunction and

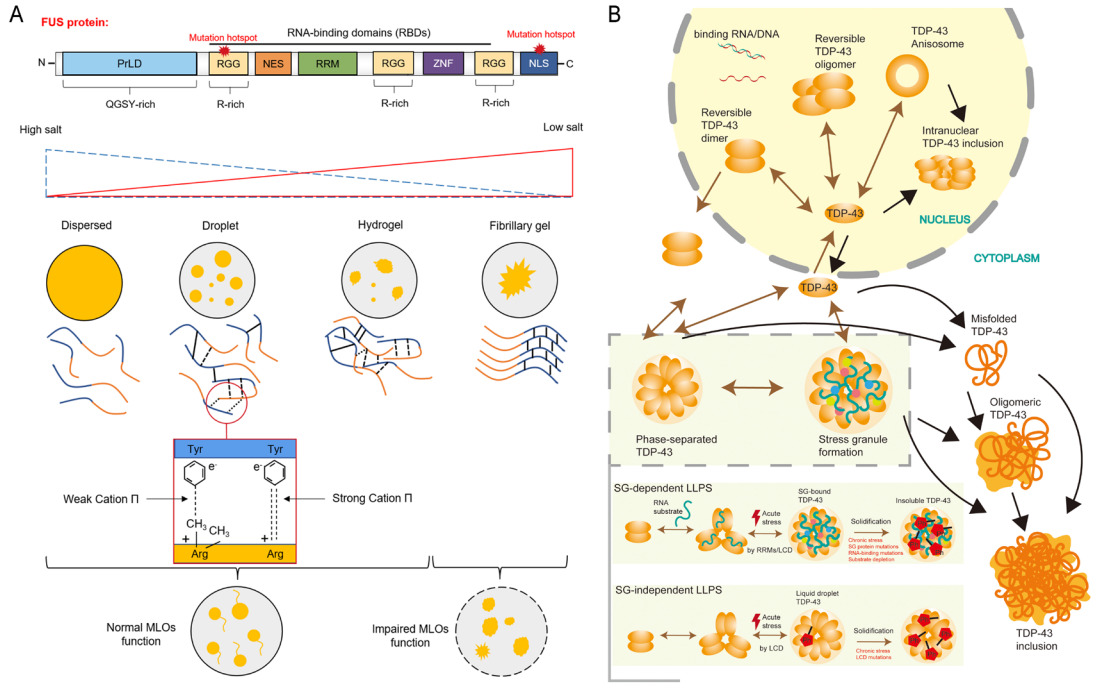

Figure 3. The molecular mechanisms of FUS- and TDP43-driven LLPS. A. FUS maintains self-assembly regions including prion-like low-complexity (LC) domains, C-terminal domains and RNA recognition motif (RRM), which are vital for driving LLPS behavior. **B.** TDP-43 can translocate from nucleus to cytoplasm under various stress conditions and form multiple MLOs through phase transition.

multiple severe diseases (be discussed in detail in the subsequent sections).

4.2. TAR DNA-binding protein 43 (TDP-43) drives LLPS

TAR DNA-binding protein 43 (TDP-43) is a highly conserved, ubiquitously expressed multi-domain RBP, belonging to the heterogeneous nuclear ribonucleoprotein (hnRNP) family (Figure 3B). TDP-43 is mainly implicated in the transcriptional and posttranscriptional regulation of mRNA transcripts that it binds (*21*). Under physiological conditions, TDP-43 is normally located in the nucleus of most cells. However, as a nucleocytoplasmic shuttling protein, TDP-43 also can translocate from nucleus to cytoplasm under various stress conditions, where it processes distinct cytoplasmic functions, including mRNA stabilization. Recently, several studies revealed that PrLD/IDR-containing TDP-43 undergoes LLPS and spontaneously develops membrane-less organelles. However, distinct from other RBPs, TDP-43 has no dominant LLPS motif in its intrinsically disordered C-terminal domain, how then can TDP-43 trigger LLPS? Structurally, there is a unique IDR with prion-like glutamine/asparagine (QN)-rich regions and central α-helical element at the C-terminus of TDP-43, which is critical for undergoing LLPS to form condensates. Specifically, the QN-rich domains in the IDR are predominately responsible for the aggregation of proteins. The α-helical element spans roughly 20 residues in the center of the domain and is involved in intermolecular interactions, that is just the reason why LLPS is controlled by fewer motifs in the C-terminal domain of TDP-43 (*22*). Moreover, this process of LLPS is primarily driven by three tryptophans in the α-helix, among which Trp-334 is the most important one and followed by Trp-385 and Trp-412. Notably, Trp-334 enables the α-helical element with a high intrinsic propensity for self-assembly, which enhances the intermolecular interaction and thus facilitates LLPS. On the other hand, several pieces of research showed that RNA binding also increases the liquid-like properties of TDP-43 condensates (*23*). The physiological interaction containing multiple binding sites of TDP-43 with RNAs significantly strongly nucleates TDP-43-driven multivalent LLPS and maintain its liquid properties. Collectively, the unique structures of IDR and C-terminal domain, as well as the multiple RNA binding sites of TDP-43, have prompted it more amenable to form various membrane-less organelles through LLPS.

Reportedly, TDP-43 forms different membrane-less organelles in various cell types, including Cajal bodies and paraspeckles in the nucleus as well as SGs in the cytoplasm (*24*). Given that TDP-43 is a nucleocytoplasmic shuttling protein, SGs are the most common type of membrane-less organelles formed by the accumulation of TDP-43 in the cytoplasm in response to stress. A variety of cellular stresses, such as heat shock, oxidative stress and osmotic, normally trigger TDP-43 to transiently localize to the cytoplasm and assemble into SGs. Moreover, localization of TDP-43 to SGs is generally mediated by both its RRM1 domain as well

as its C-terminal prion-like QN-rich domain. Notably, normal aggregation and assembly of TDP-43 into SGs are essential complexes that modulate RNA translation during stress. Nevertheless, sustained stress and ensuing TDP-43 misfolding or mislocalization are directly toxic and create aberrant SGs and pathogenic TDP-43 aggregates, which is a hallmark of a spectrum of neurodegenerative disorders (be discussed in next section).

4.3. G3BP1 drives LLPS

Ras-GAP SH3 domain binding protein 1 (G3BP1), one of the members of phosphorylation-dependent endoribonuclease that interacts with RasGAP, is a highly conserved multi-domain RNA binding protein involved in a variety of biological processes and diseases. Recently, G3BP1, acting as a molecular switch for the process of LLPS during the formation of MLOs (especially SGs), has drawn increasing attention among researchers (*25*). Several studies have revealed that G3BP1 is often involved in the initiation of SGs formation and is recruited to SGs in response to environmental stress (*26,27*). Therefore, G3BP1 is deemed as an essential determinant of the fate of mRNAs during cellular stress and a critical effector of SGs assembly. For example, Tourrière *et al*., showed that G3BP1 is rapidly recruited to SGs in cells exposed to sodium arsenite (SA), a well-recognized chemical stressor for inducing SGs (*28*). Moreover, Sun and colleagues observed that, upon newcastle disease virus (NDV) infections, endogenous G3BP1 was induced to present as punctate fluorescence and form stable SGs (*29*). Importantly, G3BP1 is often directly bonded by many viruses to specifically inhibit SGs formation thus evading the host's innate immune response (*27*). Another research also revealed that cells lacking both G3BP1 and G3BP2 cannot form SGs in response to p-eIF2α or eIF4A inhibition (*30*), indicating that G3BP is essential for the assembly of SGs initiated by p-eIF2α or eIF4A inhibition. Additionally, some other research holds the point that G3BP1 serves as the central node of the protein-RNA interaction network that triggers LLPS and subsequently assists SGs to assemble (*31*).

Generally, G3BP1 initiates and maintains the process of LLPS and SGs assembly *via* several well-recognized mechanisms. Specifically, one of the well-recognized mechanisms holds that the interplay between three IDRs in G3BP1 regulates its intrinsic propensity for LLPS and thus contributes to the SGs assembly (*25*). Interestingly, unlike the conventional IDRs, IDRs in G3BP1 have evolved to fine-tune the saturation concentration of RNA for LLPS. When RNA concentrations are low, the acidic IDR1 and the basic IDR3 favorably interact with each other to create a compact "closed" conformation. Above a threshold RNA concentration, RNA displaces IDR1 to bind IDR3, which permits the G3BP1 homodimer to adopt an expanded, "open" conformation, initiating LLPS. On the other hand, G3BP1 also facilitates and nucleates SG assembly by binding its RGG motif with 40S ribosomal subunits, which is also essential for G3BP1-mediated SGs formation. Moreover, the domain architecture of G3BP1 dimerization is another intrinsic property that influences the speed of LLPS and SGs assembly *in vitro*. Altogether, G3BP1 is a SGs-resident protein and acts as a tunable switch that triggers LLPS to nucleate SGs assembly through multiple interactions or peculiar structures.

4.4. Human antigen R (HuR) drives LLPS

Human antigen R (HuR), also known as HuA and ELAVL1 (embryonic lethal abnormal vision-like 1), belongs to the fourth member of the ELAVL family. As a well-established mRNA stabilizing RBP, HuR principally regulates the stability and translation of its target mRNAs to involve in multiple pathological processes. Although HuR appears predominantly localized in the nucleus, after exposure to specific stresses, it shuttles to the cytoplasm where HuR exerts its function in the stabilization of ARE-mRNA, which requires the HuR nucleocytoplasmic shuttling (HNS) domain (*32*). Recently, HuR has been repeatedly reported to assume essential roles in cellular stress responses, especially in the assembly of SGs. HuR aggregates and forms SGs in the cytoplasm under stresses, such as heat shock, oxidative stress and ultraviolet radiation (UV) (*33*). For example, Yoon *et al*., revealed that in human cervical carcinoma cells, sodium arsenite exposure enhances the accumulation of HuR in SGs and is accompanied by increased HuR binding to target transcripts SIRT1 and VHL mRNAs and by stabilization of these mRNAs (*34*). Moreover, heat shock treatment also promotes HuR to translocate from the nucleus into the cytoplasm, forming SGs, colocalizing with the SGs marker — G3BP1 protein, and regulating the translation of its binding mRNA (*35*). These studies indicated that HuR is an essential component of SGs and highlight that HuR presence in SGs associated with the fate of target mRNAs.

4.5. T cell intracellular antigen-1 (TIA-1) drives LLPS

T cell intracellular antigen-1 (TIA-1) is a prototypical prion-related RBP that consists of three N-terminal RRMs and a C-terminal intrinsically disordered low complexity domain (LCD), which play a central role in facilitating LLPS. TIA-1 is considered as one of the canonical scaffold proteins involved in nucleating LLPS, which regulates target mRNA translation in the cytosol under stresses *via* inducing a conformational change that favors LLPS (*36*). TIA-1 is a cellular stress response protein that shuttles into the cytoplasm promptly and facilitates the assembly of SGs upon stress. TIA-1 is a key component of SGs and makes cells

more sensitive to stress thus affecting SGs formation. As an RBP, TIA-1 often sequesters target RNAs into SGs that allow these RNAs to escape the unfavorable cellular stresses, such as heat shock, oxidative stress and hypertonic stress. Moreover, some evidence shows that TIA-1 overexpression is also sufficient to drive a spontaneous formation of SGs even in the absence of stress, presenting the importance of TIA-1 for SGs (*37*). Another study demonstrated that TIA-1 mutation slows the disassembly of SGs following heat shock in HeLa cells (*38*). How then does TIA-1 be involved in SGs assembly and disassembly? TIA-1 condenses and forms SGs *via* the self-association of its intrinsically disordered prion-like domain that facilitates LLPS. Besides, multiple RNA binding sites of TIA-1 are thought to induce aggregation of TIA-1 through LLPS, thus promoting the assembly and formation of SGs. The RRMs of TIA-1 are also essential for SGs formation (*37*). Specifically, interactions between folded RNA recognition motifs in granule proteins and RNA stimulate the formation of SGs.

5. RBPs-driven LLPS regulates physiological function

LLPS, a ubiquitous biophysical phenomenon of the cellular interior, governs multiple biochemical reactions therein. Notably, as key players in RNA metabolism and function, RBPs tend to aggregate and undergo LLPS to regulate various cell physiological activities and protect against extracellular stress perturbations. In this section, we will focus on the cellular physiologic function of RBPs-driven LLPS (Figure 1B).

5.1. Regulation of cellular mechanotransduction

Mechanical stimuli are essential for maintaining normal cell growth and development (*39*). Emerging evidence is revealing that LLPS plays essential roles in mechanosensing for regulating the mechanobiological signal coupling and mechanosensitivity of cells. For example, mechanical stretch regulates the SGs formation in smooth muscle cells thus affecting the protein translation and cell physiology (*40*). Another research revealed that biomacromolecule LLPS facilitates the assembly and organization of matured FAs, thus allowing for efficient mechano-transduction and cell migration. Moreover, the disturbance of LLPS can change the dynamics, mechanical sensitivity and durotaxis of cells (*41*). A new finding indicated that the mechanosensitive lncRNA Neat1 could undergo LLPS and form paraspeckle (an important nuclear MLO) under various mechanical stimuli, and subsequently enhances bone strength and promotes osteoblast function (*42*). These findings prompted that LLPS can respond to mechanical stimuli and thus regulate the mechanical sensitivity of cells, in turn, LLPS also requires mechano-transduction.

5.2. Regulation of intracellular biochemical reactions

LLPS is known to facilitate the subcellular compartmentalization (MLOs formation) and enrich proteins/RNAs locally, which allow the cells to carry out biochemical reactions smoothly and orderly. On the one hand, LLPS is in charge of concentrating biomacromolecules as MLOs thus promoting the biochemical reactions rate. On the other hand, LLPS can also inhibit some reactions by restricting molecular motion and sequestering the substrates from proteins (*43*). MLOs formed through LLPS, including SGs, nucleoli and P granules, are responsible for compartmentalizing the biochemical reactions in cells. Interestingly, RBPs concentration by LLPS also provide a highly cooperative mechanism to locally concentrate RNAs and promote cellular reactions (*44*). In addition, LLPS also regulate the cellular biochemical reactions through modulating the enzyme activities, which are important omnipresent catalysts of biochemical reactions. LLPS can selectively enrich or repel molecules, increase the molecular interactions, even modify the molecule conformations, and subsequently impart specificity to biochemical processes. For example, condensates formed by LLPS could condense an enzyme and its possible substrates to a specific subset, conferring specificity to the potentially promiscuous biochemical reaction (*8*). In summary, LLPS and the triggered RBPs condensation are crucial for cellular biochemical reactions.

5.3. Regulation of cellular homeostasis

Cells are continuously exposed to external stress such as salt concentration, pH, temperature and oxidative stress and are regulated by these external stressors. Accumulating evidence indicates that LLPS possesses both the ability to sense the external stress and the flexibility to respond to the changes, playing versatile roles in the maintenance of cellular homeostasis (*8*). Especially, RBPs condensation formed by LLPS can communicate with each other freely within the condensates and their surrounding solution, which enables cells to rapidly sense and respond to external stress signals. For instance, Riback *et al*., reported that the poly(A) binding protein Pab1, as an important RBP, synthesizes multiple thermal and pH signals into a unified quinary response through phase separation, thus enhancing cells to maintain cellular homeostasis and its adaptability to the environment (*45*). Moreover, LLPS of RBPs mediates multiple biological events related to redox maintenance by modifying the phase behavior of macromolecules, providing a membrane-less compartment that a cell can tap in response to oxidative stress. For example, Kato *et al*., reported that Pbp1 directly responds to redox imbalance, and the self-association of methionine-rich LC domain of Pab1 is readily oxidized upon oxidative stress induced by

dysfunctional mitochondria or H_2O_2 (*46*). Interestingly, the formation of LLPS is in turn modulated by these different external stress conditions. As the temperature is crucial for the cellular homeostasis and easily controlled *in vitro*, thermo-responsive biomolecular LLPS has been studied extensively. For example, both full-length FUS and its LC domain undergo LLPS in a temperature-dependent manner (*47*). LLPS of proteins such as folded egg-white lysozyme and the N-terminal intrinsically disordered regions (IDRs) of DEAD-Box helicase Ddx4 occurs only at temperatures below a critical temperature *Tc*, whereas LLPS of proteins like Pab1 and IDP α-elastin occurs at temperatures higher than *Tc* (*48*). Taken together, there is a reciprocal regulatory relationship existing between LLPS processes and the external stress the condensate environment in cells. LLPS is responsible for protecting against external stress perturbations in cells and maintaining cellular homeostasis during cellular stress.

5.4. Regulation of gene transcription

Transcription, as a vital contributor to functional cell states and intracellular gene expression, is strictly regulated by multiple factors including transcription factors (TFs), coactivators, enhancers as well as RBPs. RBPs-driven LLPS and the formed MLOs have emerged as a novel mechanism by which these factors regulate transcription (*49*). Transcription is driven by a type of protein termed RNA polymerases (Pol I, Pol II and Pol III), of which Pol II is responsible for producing messenger RNAs and non-coding RNAs and is considered the most important polymerase. Multiple RBPs prone to undergo LLPS are involved in the transcription regulation upon both normal and stress conditions. For example, representative RBP like FUS has been reported to be able to form phase-separated condensates thus recruiting the intrinsically disordered carboxy-terminal domain (ID-CTD) of Pol II to trigger the target gene transcription (*50*). Importantly, LLPS not only regulates transcription initiation but also modules the elongation phase of transcription. For instance, LLPS of the negative elongation factor (NELF) and positive transcription elongation factor b (P-TEFb) were found to modulate the transition from promoter-proximal pausing to transcription elongation (*51*). Specifically, P-TEFb, as the promotor of transcription elongation, translates from the paused Pol II into a condensate through phase separation to facilitate the phosphorylation of NELF and the CTD of Pol II, therefore forming elongation condensates and promoting transcription. In addition to RNA transcription regulation, LLPS also participates in DNA transcription regulation, particularly in the functions of CTD of Pol II and associated proteins, the disordered activation domains of transcription factors, and heterochromatin proteins (*52, 53*). The evidence reviewed here suggests that LLPS plays prominent roles in various stages of transcription regulation.

6. RBPs triggered-LLPS results in human diseases

In recent years, researchers have realized that the RBP-induced liquid-liquid separation process is the basis for the formation of MLOs, which is necessary to maintain normal cell functions. Nevertheless, there are emerging studies showing that abnormal assembly and LLPS of RBPs are also closely related to the pathogenesis of various human diseases, like neurodegenerative diseases, cancer and aging diseases. The following section introduces the roles of RBPs-triggered LLPS in human diseases (virus infection, cancer, neurodegenerative diseases and aging-related diseases) (Table 2 and Figure 4A).

6.1. Corona Virus Disease 2019 (COVID-19)

Recently, COVID-19 pandemic is becoming one of the largest global public health crises in modern history. As the etiologic agent of COVID-2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has also received widespread in society. Notably, SARS-CoV-2 nucleocapsid protein is an abundant RBP and is characterized by self-assembly, which is critical for viral genome packaging and transcription as well as viral replication (*54*). Lately, the mounting high-level studies have shown that SARS-CoV-2 nucleocapsid protein can undergo LLPS through its domain of dimerization *via* different ways and thus manage to outsmart host antiviral defense mechanisms. Factually, LLPS has been reported to serve as a scaffold for virus replication and accelerates viral assembly as well as virus production through proximity-dependent interactions (*55*). Here, we review and summarize the cooperative LLPS of the SARS-CoV-2 nucleocapsid protein and their roles during SARS-CoV-2 infection (Figure 4B).

For example, Chen *et al.*, have revealed that the interaction between SARS-CoV-2 nucleocapsid and single-stranded RNA (ssRNA) enables them to undergo LLPS in a Zn^{2+}-dependent manner, which further facilitated the viral assembly and transmission (*56*). Another study found that LLPS of SARS-CoV-2 nucleocapsid protein was mediated by the specific viral genomic RNA sequences and structures, which may be important for SARS-CoV-2 processes such as viral genome packaging and virus production (*57*). SARS-CoV-2 can assemble its nucleocapsid protein and genomic RNA through robust LLPS and then enters droplets formed by RBPs (FUS, TDP-43, hnRNPA2) associated with SGs formation, suggesting the essential roles of MLOs formed *via* LLPS in virus infection. Likewise, Wang and co-workers also found that the N-terminal IDRs are able to trigger the LLPS of SARS-CoV-2 nucleocapsid protein and enable them

Table 2. Pathological roles of RBPs Triggered-LLPS in human diseases

Type of disease/Specific type	Connection with LLPS	Substances involved	*Ref.*
Infectious diseases COVID-2019	LLPS manages to outsmart host antiviral defense mechanisms	SARS-Cov-2 nucleocapsid protein	(*56*)
Cancer leukemia	LLPS is essential for the development of leukemia with NUP98 fusion oncoprotein	NUP98 fusion oncoproteins (FOs)	(*60*)
Liver cancer	YAP and TAZ act as Hippo pathway effectors to inhibit liver cancer in mice through a competitive mechanism	YAP, TAZ	(*100*)
Neurodegenerative diseases Amyotrophic Lateral Sclerosis (ALS)	TDP-43 gene mutation induces ALS, Deposition of toxic protein aggregates containing RBPs (TDP-43, EWS, TAF15) can characterize related diseases	TDP-43, EWS, TAF15	(*101*)
Alzheimer disease (AD)	Abnormal deposition of Tau protein in the brain triggered by LLPS	Tau, amyloid-β	(*102*)
Parkinson	RBP induces LLPS to form pathological protein aggregates	FUS, α-synuclein	(*18*)
Aging-related diseases			
Diabetes	Islet amyloid polypeptide is gelled and aggregated by LLPS	Islet amyloid polypeptide	(*76*)

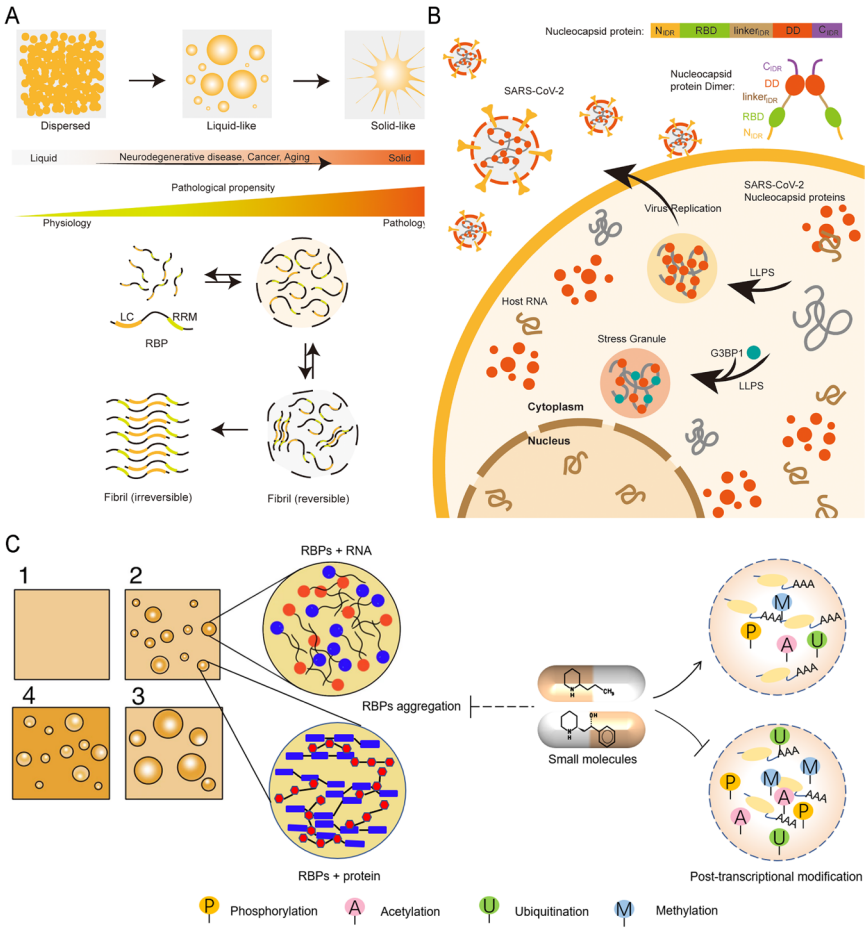

Figure 4. Pathological roles of RBPs-triggered LLPS in cells and human diseases. A. Abnormal assembly and LLPS of RBPs are also closely related to the pathogenesis of various human diseases, like neurodegenerative diseases, cancer and aging diseases. **B**. Roles of SARS-CoV-2-driven LLPS and SGs formation in virus infection of COVID-2019.

to assemble with G3BP1 into SGs to inhibit host cell innate immunity (*58*). However, differences of opinion lay behind the roles of SGs during virus infection. Some other studies showed that SARS-CoV-2 nucleocapsid protein enables to disrupt SGs assembly through interacting with G3BP1 and blocking the interaction between G3BP1 and other SG-related proteins to facilitate viral production. That is, well-assembled SGs are part of the antiviral responses during viral infection through sequestering host and viral mRNAs and proteins

(*59*).In general, LLPS of SARS-CoV-2 nucleocapsid protein may not only affect viral replication, genomic RNA as well as viral production, but also regulate host cell function to expedite viral transmission. More importantly, the LLPS activity of SARS-CoV-2 nucleocapsid protein may be a promising therapeutic target for pandemic COVID-2019 and new possibilities for the development of new antiviral drugs.

6.2. Cancer

Cancer is a pathological condition where cells uncontrollably divide and escape the regulatory bounds of normal homeostatic balance, which is maintained through precise spatiotemporal regulation. In fact, dysregulated LLPS and toxic aggregates of RBPs have been shown to regulate cancer cell pathology and are deemed as a hidden driver of oncogenic activity. In this part, we elaborate on how LLPS shapes the biochemical landscape of cancer cells.

Abnormal aggregation and assembly of RBPs lead to abnormal LLPS and drive carcinogenesis. For instance, IDRs-containing NUP98 (an important RBP) fusion oncoproteins (FOs) can undergo LLPS and thus result in aberrant transcriptional activity and transformation of hematopoietic stem and progenitor cells in pediatric leukemia (*60*). Similarly, NUP98-HOXA9, as the classical chimeric canceration protein, undergoes LLPS and forms droplet aggregates to further drives aberrant chromatin looping and cancer development (*61*). In addition, the virus-like domain of oncogenic EWS-FLI1 fusion protein enables it to undergo LLPS and assemble as condensates in Ewing sarcoma, which recruits unusual chromatin remodeling complex and promotes tumor gene expression (*62*). Besides, it has been verified that the intranuclear protein AKAP95 can undergo LLPS and regulate the splicing of cancer genes and tumor generation within the range of appropriate physical attributes. This discovery drives an unconventional idea for cancer treatment to inhibit cancer cells by controlling or interfering with biomolecule aggregates formed by LLPS.

In addition, LLPS triggered by RBPs have the function to regulate carcinogenic signals. A metabolic disorder is a typical feature of cancer cells, which is manifested in that cancer cells change the normal biosynthetic pathway to adapt to uncontrolled abnormal proliferation. LLPS serves as the main organizer of signal intervals to control carcinogenic signals. For example, PKA regulatory subunits undergo LLPS and form biomolecule aggregates rich in cAMP and PKA activity in liver cancer. Moreover, PKA fusion protein can block LLPS and induce abnormal cAMP signaling, which leads to abnormal cell activity (*63*). Besides, pathogenic SH2 mutant can lead to the alteration of the conformation of LLPS to trigger oncogene signal transduction and MAPK hyperactivation (*64*).

All in all, the close association between LLPS and cancer means that we are entering a new or exciting phase in cancer research. RBP-mediated LLPS are the hidden drivers of carcinogenesis, so it is of great research significance to regulate LLPS to directly control multiple processes in cancer. However, how the specific biochemical reactions of the aggregates formed by LLPS occur, and the exact functional relationship between the aggregates and cancer cell pathology need to be further expanded.

6.3. Neurodegenerative diseases

Neurodegenerative diseases are characterized by irreversibility and cause serious threats to human life. The toxic aggregate of RBPs and abnormal LLPS have been deemed as one of the pathological incentives and hallmarks for various neurodegenerative diseases (*65*). Therefore, abnormal RBPs and LLPS drive the exploration of new downstream mechanisms of neurodegenerative diseases.

Currently, multiple typical RBPs, such as FUS, TDP-43, TAU and they-driven LLPS are the key participants in neurodegenerative diseases (*66-68*). Specifically, genetic mutations in FUS and TDP-43 often lead to aberrant assembly condensates formation by LLPS (*68*). The prone-like LCDs of FUS enable its fibrillar amyloid assembly and thus result in amyotrophic lateral sclerosis (ALS) (*69*). The interaction of FUS with G4-RNA promotes its liquid-solid phase transition in ALS pathogenesis, which provides clues for the relationship between abnormal RBPs aggregation and ALS mechanism (*70*). Another study has revealed that ALS-FUS leads to decreased nucleocytoplasmic transport (NCT) and nucleoporin (Nup) density in the nuclear membrane of human neurons, which subsequently alters the phase separation characteristics and nucleocytoplasmic transport path in diseases (*71*). In addition, TDP-43 also participates in the pathogenic process of ALS. In 2006, mislocalization of TDP-43 was observed in the brains and spinal cord regions of ALS patients (*72*), which has been regarded as a symptom of most ALS. It was found that the excessive condensation of TDP-43 could affect its RNA networks in the context of disease (*73*). Notably, the pathological process of abnormal TDP-43 aggregation is associated with the formation of SGs. The accumulation of misfolded TDP-43 in the endoplasmic reticulum can activate PERK and phosphorylate elF2α to promote SGs formation (*74*). It has been speculated that a variety of neurodegenerative disease-related proteins are recruited to SGs when stress is applied, and are involved in the conformation of SGs. Besides, abnormal assembly of TAU and its solid phase accumulation are also involved in the development of neurodegenerative diseases (*67*). Phosphorylated free tau is mislocated and accumulates in dendrites and

somatic cells, which ultimately leads to the pathological features of neurodegenerative diseases (*75*). It has been demonstrated that aggregated TAU inhibits anterograde fast axonal transport, which supports the hypothesis that tau oligomers are toxic in neurodegenerative diseases and facilitates elucidation of the exact mechanism of tau-mediated neurotoxicity.

In summary, it is well established that aberrant RBP-mediated LLPS is one of the major causes of neurodegenerative diseases. The aberrant cellular functions are linked to the pathological liquid to solid phase transition of RBPs, which may pave the way for the potential treatment strategies of neurodegenerative diseases in the future.

6.4. Aging

Aging, an irreversible biological process, often manifests as a progressive loss of homeostasis in cells, which is characterized as RBPs aggregates. As the underlying mechanism of RBPs aggregates, LLPS is therefore believed to get involved in the pathological transitions during aging and drive the progression of age associated diseases. That is the changes of intracellular bioprocesses during aging can lead to the aberrant RBPs assembly and LLPS, in turn, these aberrant LLPS might promote aging and aging-associated diseases.

Generally, RBPs-driven LLPS can trigger the formation of mRNPs such as P-bodies and SGs to regulate the cellular functions in normal cells. As cells get aging, these mRNPs constantly aggregate and transformed into pathological accumulation that are harmful to cells. Therefore, some speculate that the abnormal phase transition of RBPs may be a vital pathogenesis for aging-related diseases. It is reported that islet amyloid polypeptide can undergo LLPS and induce hydrogelation and aggregation in amyloidogenic type II diabetes. During aging, the accumulation of misfolded polypeptides can lead to amyloidosis and affect LLPS-driven aggregation (*76*). RBP FUS converts into a gel-like state at a higher concentration level in cells and further transitions into solid-like fibrillar aggregates over time, which acts as the hallmarks of aging and related diseases (*77*). Moreover, the persistent SGs formed by RBPs irreversible aggregations and abnormal LLPS may also result in the pathogenesis of aging-related diseases. For example, PAB-1 and TIAR-2 excessively accumulate and form irreversible SGs and subsequently lead to the shorter lifespan of aged C. elegans (*78*). In addition, the irreversible amyloid-β oligomeric aggregates formed by TDP-43 disturb SG dynamics and thus cannot exchange materials with their surroundings and accelerate aging diseases (*79*). Another research showed that cellular senescence can lead to eIF2α hyperphosphorylation and disturb the formation of SGs in the stress response, indicating the interplay between aging and LLPS (*80*). Besides, SGs can recruit and assemble the pro-aging protein, plasminogen activator inhibitor-1 (PAI-1) thus performing their anti-aging effect (*81*). Therefore, irreversible RBPs aggregation by LLPS are believed to interfere with normal cellular functions during aging, which is worth investigating in the future.

Although the phase behavior existing in various diseases is not yet completely understood, it is still necessary to study phase separation in the future treatment of diseases. Some disease-related LLPS processes can be inhibited or promoted at the stage of separation, thereby changing the biological properties of related proteins. For example, treatment drugs taken for cancer affect the activity of medicine due to the formation of condensate (*82*). This situation can be improved by changing the mechanism of drug condensate formation to inhibit the formation of agglomerates. Therefore, this provides us with a new idea: controlling and regulating the LLPS procedure to change the drug activity for different diseases, this may be beneficial for disease treatment.

7. RBPs-driven LLPS as novel therapeutic mechanism

Nowadays, the broad physiological and pathological characteristics of RBPs-driven LLPS have raised interest of whether it can be a promising therapeutic mechanism and strategy for diseases. In this section, we draw attention to RBPs and they-induced LLPS as a new therapeutic area as well as introduce the therapeutic mechanisms of small molecules (including natural products) targeting RBPs (Figure 5).

At present, numerous small molecules have shown great potential to affect the process of RBPs aggregation and function to modulate LLPS-induced human diseases with different mechanisms. Especially, RBPs as the major components of SGs, small molecules targeting them can affect SG dynamics, including assembly, disassembly, maintenance and clearance. For example, a recent finding showed that targeting the liquid-like droplets formed by LLPS of SARS-CoV-2 nucleocapsid protein can restrain the virus replication and promote innate antiviral immunity (*83*). Troxerutin (also known as vitamin P4) has been shown to promote SG formation in a TIA-1-dependent manner (*84*). Another small molecule boric acid, promotes TIA-1 translocation from the nucleus to cytoplasmic SGs, thus exhibiting anticarcinogenic and bone-strengthening effects (*85*). As for mitoxantrone, it reduces the formation of TDP-43^{+} SGs and prevents the accumulation of mutant TDP-43 in SGs (*86*). Mitoxantrone also significantly suppresses the recruitment of FUS to SGs, and reduces the number and size of liquid FUS droplets formed *in vitro*, thus treating the neurodegenerative disease. Besides, compounds including lipoamide and lipoic acid also inhibit FUS accumulation and disrupt FUS-induced LLPS as well as

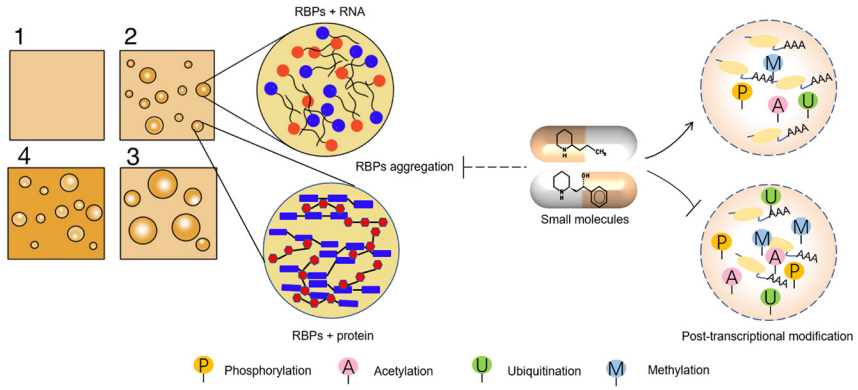

Figure 5. Small molecules targeting RBPs-triggered LLPS are the novel therapeutic strategy for human diseases.

SGs formation (*87*). It has been reported that the PARP inhibitor Olaparib delays the recruitment of TDP-43 and hnRNPA1 to SGs and delays SG assembly, which is beneficial in ALS and frontotemporal dementia (FTD) (*88*). Targeting the dysregulated PTMs which affect the function of RBPs is another therapeutic option for multiple diseases induced by abnormal LLPS. Some compounds target the dysregulated PTMs of RBPs to regulate the process of LLPS, thus alleviating diseases. For instance, multiple small compounds, such as silmitasertib, tetra bromo cinnamic acid and okadaic acid, could target G3BP1, the core component of SGs, to affect its dysregulated phosphorylation (*89*).

Altogether, targeting RBPs to inhibit their abnormal aggregations and restore the normal function of LLPS may be a novel therapeutic strategy in multiple diseases. RBPs-driven LLPS also open up a whole new field for the development of small molecule drugs. The huge drug discovery opportunities contained in the area LLPS have increasingly been recognized by researchers regardless of the exploration direction.

8. Concluding remarks and future perspectives

Ever since LLPS was first described, numerous studies have been dedicated to investigating its visible phase transitions, physicochemical properties, cellular functions and its possible involvement in human diseases. RNA binding proteins (RBPs) that contain IDRs, RBDs and LCDs are prone to aggregate, interact with other proteins/RNAs and undergo LLPS. In this review, we have tried to describe the whole development picture of LLPS and summarize the basic biological function of RBPs-driven LLPS and MLOs formation. As described in this review, aberrant RBPs aggregations and LLPS have become novel promising therapeutic targets for human diseases and opened up a whole new field for the development of small molecule drugs.

Despite such breakthroughs in the field of LLPS, our understanding of the LLPS is still in its infancy and a growing number of questions have also emerged. For example, what is the underlying mechanism in the regulation of RBPs condensates by their material properties during LLPS? How do disease-associated mutations of RBPs regulate the physical properties of their condensates? Whether RBPs which undergo LLPS can be used as therapeutic targets for diseases, and how to regulate the LLPS of RBPs to achieve the desired therapeutic effect still need to be further explored. Importantly, more quantitative tools or approaches need to be developed and applied to LLPS research.

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[§]These authors contributed equally to this work.
**Address correspondence to:*
Airong Qian, Lab for Bone Metabolism, Xi'an Key Laboratory of Special Medicine and Health Engineering, School of Life Sciences, Northwestern Polytechnical University, Xi'an 710072, Shaanxi, China.
E-mail: qianair@nwpu.edu.cn

Zhihao Chen, Lab for Bone Metabolism, Xi'an Key Laboratory of Special Medicine and Health Engineering, School of Life Sciences, Northwestern Polytechnical University, Xi'an 710072, Shaanxi, China.
E-mail: chenzhihao@nwpu.edu.cn

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