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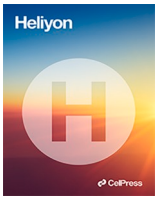

Review article

Phase separation is regulated by post-translational modifications and participates in the developments of human diseases

Weibo Zhang *, Zhengfeng Li, Xianju Wang, Ting Sun

Faculty of Health Sciences Building University of Macau E12 Avenida da Universidade, Taipa, Macau, China

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ABSTRACT

Liquid-liquid phase separation (LLPS) of intracellular proteins has emerged as a hot research topic in recent years. Membrane-less and liquid-like condensates provide dense spaces that ensure cells to high efficiently regulate genes transcription and rapidly respond to burst changes from the environment. The fomation and activity of LLPS are not only modulated by the cytosol conditions including but not limited to salt concentration and temperture. Interestingly, recent studies have shown that phase separation is also regulated by various post-translational modifications (PTMs) through modulating proteins multivalency, such as solubility and charge interactions. The regulation mechanism is crucial for normal functioning of cells, as aberrant protein aggregates are often closely related with the occurrence and development of human diseases including cancer and nurodegenerative diseases. Therefore, studying phase separation in the perspective of protein PTMs has long-term significance for human health. In this review, we summarized the properties and cellular physiological functions of LLPS, particularly its relationships with PTMs in human diseases according to recent researches.

1. Introduction

The conception of phase separation of intracellular biomacromolecules was first proposed by Brangwynne et al. in the article "Germline P granules are liquid droplets that localize by controlled dissolution/condensation" in 2009 [1]. The P granules displayed liquid-like properties in *Caenorhabditis elegans* embryonic cells, occasionally fusing with one another and flowing and dripping like water droplets when induced by shear stresses. Furthermore, P granules could rapidly dissolve and condense, meaning components of the P granules could communicate with constituents of the cytoplasm [1]. Since this first report, additional research on phase separation has gradually been conducted. In 2012, Pilong Li et al. reported that phase separation in vivo was reproducible in a cell-free system provided the biochemical conditions were appropriate [2,3], which was a notable breakthrough in phase separation research. In recent years, continuous technological advancements have led to the generation of increasing evidences confirming the existence of biological phase transition.

In this review, we discuss the composition and biochemical properties of phase separation, which is strictly monitored by cells through a series of physiological regulations. Phase separation is related to numerous physiological and pathological activities of cells, such as RNA transcription, DNA damage repair, and so on. The role of aberrant phase separation in various diseases, such as neurodegenerative diseases, cancer, and so on is also discussed.

* Corresponding author.
E-mail addresses: yb67606@umac.mo (W. Zhang), lzfeng528@126.com (Z. Li), yb67631@umac.mo (X. Wang), yc17622@umac.mo (T. Sun).

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2. Biochemical properties and composition of protein liquid-liquid phase separation (LLPS)

Phase separation is a physicochemical process that leads to macromolecules, such as proteins and nucleic acids, compartmentalizing into condensed and dissolved phases [4](Fig. 1A). This process is an important mechanism for functional partitioning in cells through specific aggregation of biological macromolecules [5,6]. Phase separation creates the initial conditions required for the formation of membrane-less organelles with various biological functions [7,8]. In terms of protein LLPS, multiple protein molecules associated with the same function rapidly converge on each other to form highly concentrated complexes, like a drop of oil in water [7, 9–12]. To ensure a prompt response to complicated and volatile environments, phase separation is characterized by rapid aggregation and dissolution [13,14]. Simultaneously, proteins within the condensed phase can communicate with molecules dissolved in the cytosol [6,15](Fig. 1A). Meanwhile, the condensate also has a boundary that selectively permits certain macromolecules; thus, it can function as a compartment [16]. The ability of these protein-protein or protein-DNA/RNA molecules to interact and form dynamic condensates is based on a network of weak and transient interactions [17].

2.1. *Intrinsically disordered regions are crucial driving forces for protein LLPS*

The primary driving force for phase separation is the multivalency from intrinsically disordered regions (IDRs), including pi-pi conjugation, cation-pi, electrostatic, hydrophobic function, and hydrogen-bond interactions [8,18–22]. IDR composition exhibits a bias for some specific minority amino acids (AA) as IDRs usually contain polar and charged AA including arginine (R), lysine (K), proline (P), glutamine (Q), glycine (G), serine (S), and glutamic acid (E) [23–25]. Aromatic residues, such as phenylalanine (F) and tyrosine (Y), can also participate in LLPS regulation, and are related to the uniformity of distribution in IDRs [21]. IDRs are often composed of biased amino acids and a proportion of simple repetitive sequences, and specific subsets of these IDRs are also known as low complexity regions (LCRs), which are characterized by a lack of stable secondary and tertiary structures. The prion-like domains (PrLDs), often found in RNA binding proteins, have a much narrower definition than IDRs and LCRs. In addition to containing regions of low sequence complexity, PrLDs have a large proportion of glycine and uncharged polar amino acid residues such as glutamine, serine, tyrosine, and asparagine [26,27]. Ure2 and Sup35 from budding yeast are relatively earlier studied two prion proteins, which are found to form self-propagating aggregates. In human, more than 200 proteins were screened to contain PrLDs homologous sequences using the prion-like amino acid composition (PLAAC) algorithm, including FUS and TDP43 that have been confirmed to phase separate in cells [28–32]. Numerous studies have shown that IDRs/LCRs/PrDs are intrinsic elements of proteins to promote phase separation [33–36]. For instance, deletion of the N-terminal IDR of LAF-1, a DDX3 RNA helicase, abolished the occurrence of droplets in vitro and P granules disappeared in the early embryo [37]. Nevertheless, not all IDR-containing proteins undergo phase separation, even under appropriate physiological conditions, including suitable temperature, pH, protein concentration, and so on [18].

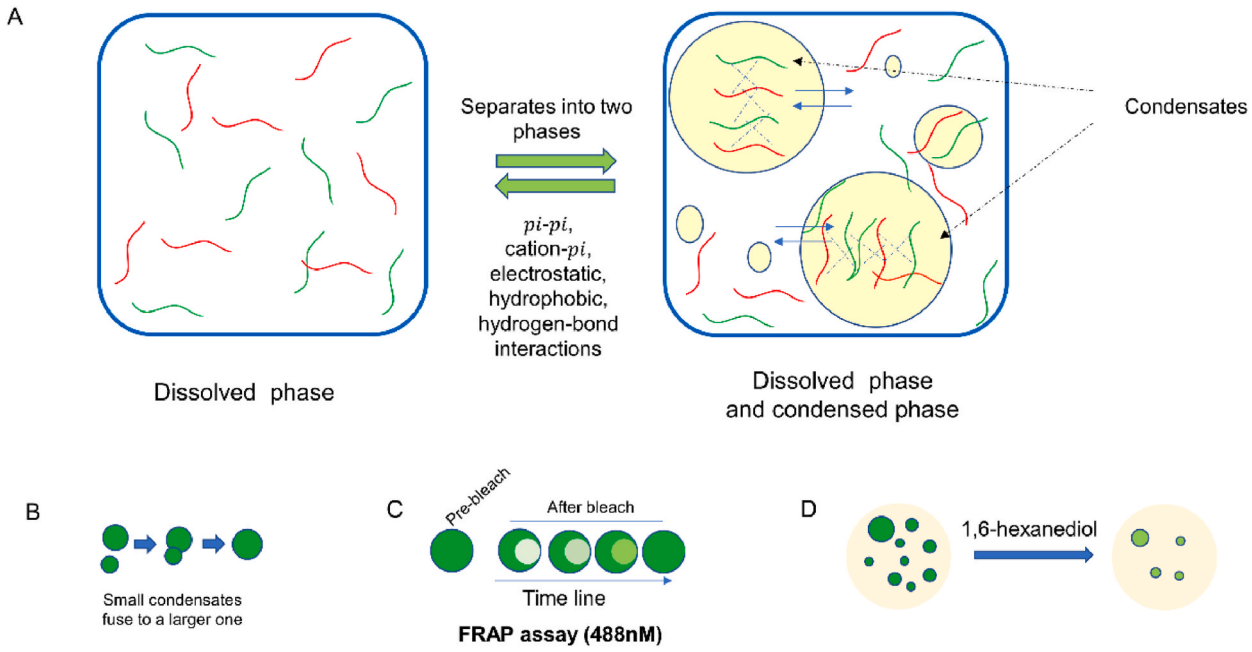

Fig. 1. The models showing the reversible process of liquid-liquid phase separation formation and the liquid behavious. (A) Diagram indicating the dynamic process of LLPS formation through weak multivalent interactions. (B) Diagram indicating two neighbouring small condensates could gradually fuse to a bigger one in minutes. (C) Diagram indicating the fluorescence recovery after photobleaching (FRAP): liquid-like condensate could recover after bleached by 488 nm laser. (D) Diagram indicating 1,6-hexanediol could disrupt the hydrophobic interactions and dissolve condensates.

Furthermore, some proteins lacking the long IDR can still experience LLPS through oligomerization. For example, GIT1 contains a coiled-coil domain mediating its dimerization, and its N-terminal can specifically bind its C-terminal, both of which are highly conserved. This unique three-dimensional conformation allows GIT1 to form protein polymers and undergo phase separation without IDR [38].

2.2. *Interactions with nucleic acids and proteins affect the formation of LLPS condensates*

Protein-nucleic acids interaction. LLPS condensates formed by electrostatic forces can be regulated by nucleic acids due to the negative charge arising from their phosphate group, and the amount of negative charge is directly proportional to the length of the molecule [39–41]. In general, redundant RNA suppresses LLPS. PUB1 is an RNA-binding protein that formed stress-related condensates in budding yeast when the pH was lowered. However, addition of RNA decreased the size and number of Pub1 condensates triggered by lowered pH or high temperature [42]. A study from the laboratory of Richard A. Young [43] demonstrated that LLPS of transcriptional complexes was regulated by a negative feedback loop. Briefly, low levels of small noncoding RNA enhance electrostatic interactions of transcriptional condensates in the early stage of transcription. Then, since the charge of RNA is proportional to its length [39], when enough mRNA is synthesized, the electrostatic interactions are destroyed, and in turn, transcriptional condensates are dissolved (Fig. 2).

DNA also influences LLPS morphology and behavior. NUP98-HOXA9 is a fusion protein comprising the IDR of NUP98 as the N-terminal and the DNA-binding domain of HOXA9 as the C-terminal. The Kriwacki laboratory reported that the puncta of NUP98-HOXA9 formed free of DNA were significantly smaller in size but larger in number compared with the puncta formed with the addition of DNA [44]. DNA also enhanced the mobility of the NUP98-HOXA9 condensates, as the fluorescence recovery after photobleaching (FRAP) assay (Fig. 1C) showed the mobile fraction of the condensates without DNA was reduced relative to that of the condensates with DNA.

Protein-protein interaction. In some conditions, the formation of LLPS condensates is chaperone dependent. For example, the heat-stress-induced solid-like aggregates of PUB1 require the assistance of Hsp104 for dissolution [42]. Dorothee Dormann's research group validated that methylation modification of the C-terminal RGG3-PY domain and RGG of FUS, an RNA-binding protein, was a suppression signal for phase separation. Nuclear import receptor Transportin (TNPO1) acted with methylated RGG3-PY domain as an essential chaperone for nomal cells, which suppressed phase separation and SG formation of FUS independent of its nuclear import activity. One mechanism confirmed by Dormann group was that TNPO1 competitively interacted with the RGG3-PY domain of FUS then prevented RNA binding and facilitation of phase separation. Another mechanism proposed by the same group was that the binding of TNPO1 to the RGG3-PY domain of FUS may interrupt the formation of electrostatic interactions from arginine-related charged residues, which are required for phase separation [45].

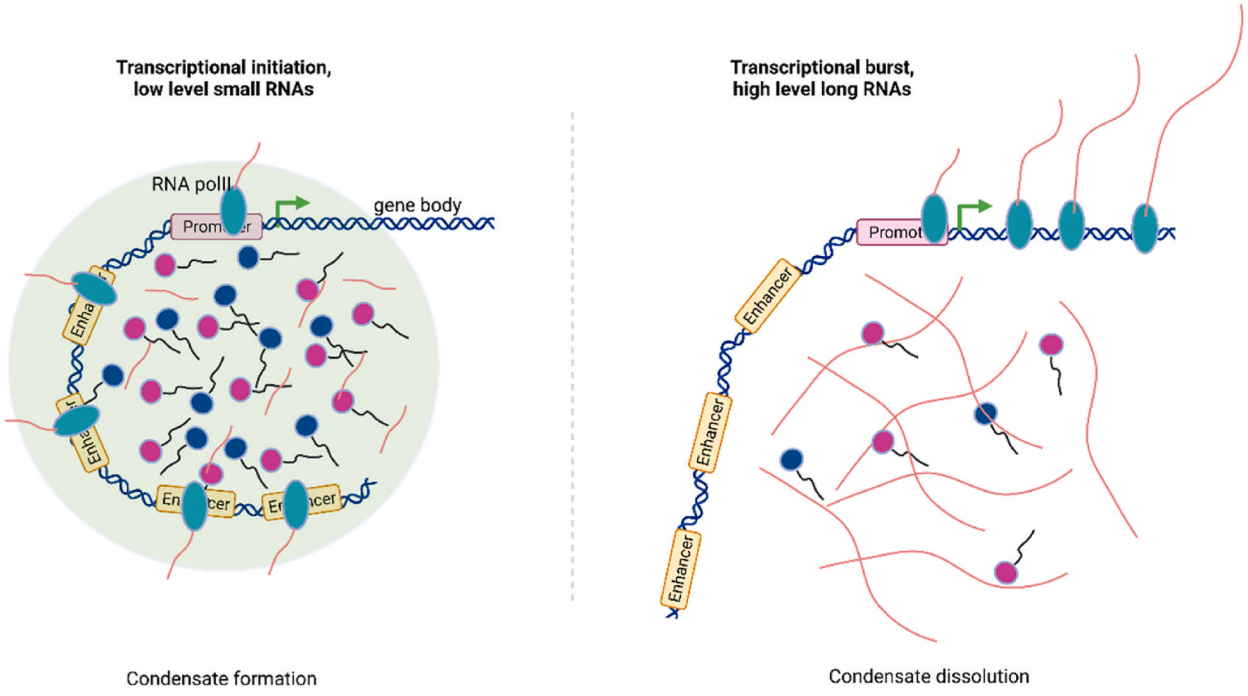

Fig. 2. A model for RNA-mediated feedback control of transcriptional condensates. The low levels of RNA promote condensate formation at the early stage of transcription; as more and more RNAs are produced, high levels of RNA in turn promote the dissolution of condensate.

2.3. Phase separation is dynamic

Liquid droplets are a critical physical state in which the components can rapidly and easily exchange with each other or with the surroundings [6]. Condensed phase droplets are generally spherical in cells and in vitro because of their molecular fluidity [46]. Confocal laser microscopy indicates that the diameter of LLPS complexes is generally on the nanometer scale in cells, and on the micrometer scale in cell-free systems [47]. These small droplets can further assimilate proteins from the cytosol (Fig. 1A) and integrate with each other to form larger complexes (Fig. 1B); the liquid properties of the droplets also allow them to incorporate other "tenant" proteins [1,48,49].

2.4. Phase condensates can experience aging

In some diseased cells, aberrant phase separations may occur that are not controlled by cells and can convert from liquid-like droplets to solid aggregates irrepressibly [2,50]. An important mechanism revealing this phenomenon is that the formation of normal LLPS complexes is dependent on multivalent, weak, and transient interactions, while the correlative proteins are mutated in abnormal cells and the interactions become much stronger and sustained. For example, with the protein FUS, the wild-type condensates are homogeneous amorphous, and lack ordered structures in normal cells. However, in the cells from patients with amyotrophic lateral sclerosis (ALS), G156E-mutated FUS formed solid-like fibrous aggregates that were characterized by heterogeneous, transversely arranged, fiber-like structures (Fig. 3 A and B). Furthermore, photobleaching experiments revealed that the condensates from both wild-type and G156E-mutated FUS recovered to almost the same degree after bleaching at 0 h. However, 8 h later, condensates of mutated FUS no longer recovered, while the wild-type FUS recovered to the same degree as at 0 h. Thus, the condensates of mutated FUS converted from a dynamic state to solid aggregates [46]. The same phenomenon occurred with the protein hnRNPA1, whereby the hnRNPA1-D262V mutant developed fibrosis within a few minutes, forming insoluble amyloid-like fibrils, but wild-type hnRNPA1 did not exhibit this behavior [15].

Redundant RNA could accelerate the aging of phase separation. For example, RNA can facilitate the phase formation of SOP-2, then promoted the muturation of droplets: A transition from liquid-like to hydrogel and solid [51].

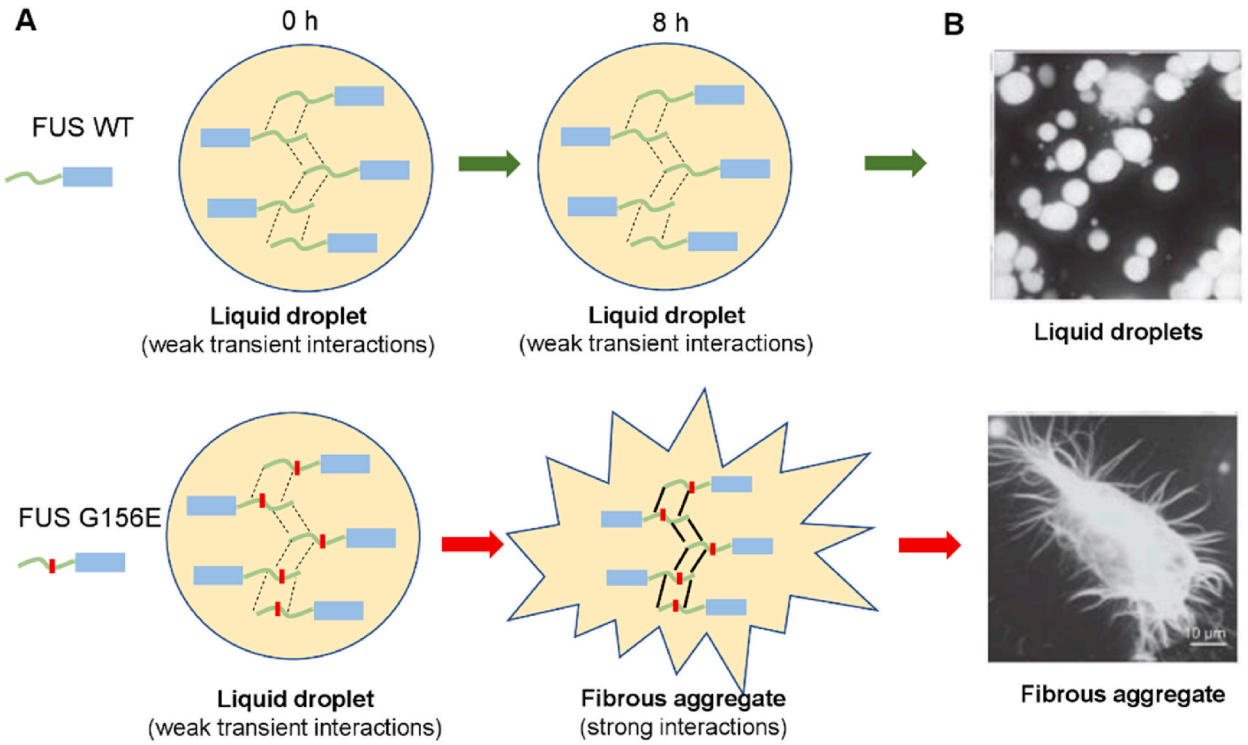

Fig. 3. Models and mechanisms showing the transition from liquid droplets to aggregated state. (A) Diagram indicating the liquid droplets formation of WT or G156E FUS and the transition into fibrous aggregates. Wild-type FUS protein forms reversible liquid-like condensates depending on transient weak multivalent interactions; while mutated FUS forms irreversible fibrous aggregates due to strong interactions. (B) Morphology images of WT and G156E FUS formed droplets in vitro through microscopy (From Simon Alberti [46]).

3. Physical factors influencing the formation, dynamics, and aging of LLPS condensates

The IDR (LCR or PrD) is necessary but insufficient for LLPS induction. The internal components of cells are crowded and complex, and whether phase separation occurs depends on the physiological and biochemical needs of the cells. The primary structures of proteins capable of phase separation are fixed, therefore cells determine whether phase separation occurs by regulating the internal environment, including protein concentration, pH, salt concentration, and so on [52]. Factors influencing LLPS are summarized in Table 1.

3.1. Protein concentration

Protein concentration is a key determinant of whether phase separation can occur. The concentration of most proteins in a cell is at a low level and the proteins dissolve evenly in the cytosol. When the protein concentration exceeds its saturation concentration (cS), these protein molecules will spontaneously aggregate to form small droplets under suitable conditions, such as an appropriate temperature and pH [50,75]. At this point, proteins in the cell exist in two phases—one phase is when the proteins are dissolved in the cell cytosol, the other is when the proteins are aggregated into small droplets by weak, multivalent interactions. Proteins in the two phases can communicate with each other [1,53].

3.2. Salt concentration

As reviewed above, electrostatic interactions are an important driving force for LLPS [6,76]. Therefore, factors affecting protein charge, such as salt concentration, may play a role in phase separation. High concentrations of salt ions can be involved in electrostatic shielding. Thus, for some condensates formed by hydrophobic and electrostatic interactions, conditions of low salt concentration may be more favorable for phase separation, such as with BRD4 and MED1 [4]. This is because the IDR of MED1 is enriched in serine, which can be phosphorylated by a variety of kinases and thus becomes charged. Phase separation of hnRNPA1 depends on electrostatic interactions and lowering the NaCl concentration can weaken the electrostatic shielding effect, leading to LLPS at a lower concentration of hnRNPA1 [15].

Some proteins that undergo phase separation independent of electrostatic interactions can still be affected by salt concentration, such as FUS, which phase separates by cation-pi interaction provided by RGG repeats of the IDR. In vitro, FUS droplets were not observed when the NaCl concentration was 150 mM, but as the salt concentration was reduced to 50 mm, FUS phase-separated into

Table 1
Factors influencing phase separation formation [58,62–64,66,68,69,72,73].

			Influence mechanisms	References for enhancing (+)/ inhibiting (-)
External influencing factors	protein concetration		multivalent interactions	lower (-); higher (+)[7, 9, 53, 54]
	salt concentration		electrostatic screening	lower (+)[4, 15]; higher (+) [55]
	pH value		electrostatic screening, conformation alteration	lower PH (+)[42, 56]; conformation change[56, 57]
	temperature		entropy	lower (+)[15, 58]; higher (+)[42, 55, 59]
	crowding agent		mimic intracellular environment	Ficoll (+)[15, 60], PEG8000 (+)[4, 61]
	inhibitors (1,6-hexanediol)		hydrophobicity interrupting	(-)[4, 62]
	RNA/DNA		electrostatic interaction	(-)[42, 43]; (+)[44]
	chaperone		competitive inhibition,	(-)[45]; (+)[42]
Internal influencing factors	IDR/LCR/ PrD	biased amino acid composition	multivalent interactions	[8, 18, 20-22]
	Ordered structure	α-helix	oligomerization and multivalent interactions	(+)[55, 63]
	PTMs	phosphorylation	biorecognition, conformal changes and electrostatic interactions	(-)[64, 65]; (+)[66]; transformation[67]
		ubiquitylation/ sumoylation	oligomerization and multivalency	(-)[55]; (+)[51, 68-70]
		acetylation	disruption of charges	hyperacetylation (-)[54, 71, 72]
		methylation	disruption of hydrophilicity and dimerization	(-)[45, 73, 74]

hundreds of micron-scale droplets [77]. The specific mechanism of this process requires further exploration. In contrast, high salt concentrations can lead to easier phase separation of some proteins such as Tau. The C-terminal fragment of Tau, namely Tau187, experiences LLPS only when the NaCl concentration is higher than 4 M. Furthermore, addition of 4 % 1,6-hexanediol to disrupt the weak hydrophobic interactions (Fig. 1D) completely dissolved the LLPS condensates of Tau187, suggesting electrostatic screening enhanced the hydrophobic interactions [59].

3.3. pH

Changing the pH of the cytosol or in vitro solution can also affect phase separation through altering the charge interactions within proteins [56]. Simon Alberti and colleagues [42] treated budding yeast with 2-deoxyglucose (2-DG, an inhibitor of glycolysis) and antimycin A (AntA, an inhibitor of mitochondrial respiration) to alter the pH of the cytosol from 7.5 to 5.7, and the lower pH significantly induced the reversible formation of SG. In addition, changes in pH affected the conformation of a protein leading to re-distribution of hydrophobic and hydrophilic surface residues, suggesting a role in LLPS through protein solubility [56,57].

3.4. Temperature

Mammalian cells usually exist in a narrow temperature range environment that appears to be unconnected with phase separation. However, temperature does affect the occurrence of phase separation and aging whether in cells or in vitro. For example, for the protein hnRNPA1, a relatively lower temperature is more conducive to phase separation in vitro [15]. Songi Han's research group recorded the phase transition process of Tau protein after repeated heating-cooling cycles at high concentrations of NaCl (3.5 M) [59]. The turbidity remained around zero when the temperature was below 35 °C but rose to 0.4 when the temperature increased to 40 °C, suggesting a higher temperature is more favorable for LLPS of Tau. However, the phase transition caused by high temperature is partially irreversible as some turbidity remained in the sample when the temperature was decreased.

Budding yeast alleviates the detrimental effects of a changeable living environment, such as glucose starvation treatment, by forming SGs [42]. Simon Alberti and colleagues [42] reported that both heat stress and lower pH could significantly drive the formation of SGs. Heat stress does not induce SG formation through lowering of the cytosolic pH because incubation of the yeast at 46 °C caused only a slight decrease in the pH of the cytosol and this was insufficient to induce SG formation. However, temperature-induced SGs were affected by salt concentration and pH [42].

3.5. Crowding agents

The cytoplasm is a crowded environment because the cell interior comprises not only the cytoskeleton and various organelles but is also filled with thousands of biological macromolecules, which account for approximately 40 % of the cell volume [78]. Under normal physiological conditions, the biological macromolecular density of the cytoplasm can reach 200 mg/mL [79]. Macromolecular crowding can affect phase behavior, so Ficoll-400 and polyethylene glycol 8000 (PEG8000) are often used to simulate the intracellular environment when studying phase separation in vitro [60,61,67]. Furthermore, although crowding reagents are not necessary for LLPS in some cases, they can enhance phase behavior [15,60]. The addition of crowding reagents can significantly reduce the protein concentration required for phase separation in vitro [15]. However, further work is necessary to elucidate how these crowding agents promote phase separation.

4. Protein LLPS is regulated by post-translational modifications

Although phase separation is affected by the above physical conditions, many of these influencing factors are relatively stable within living cells compared with in vitro, such as temperature, pH, and salt concentration. Therefore, these influencing factors are virtually invalidated in living cells, meaning cells must have evolved alternative ways of regulating LLPS. These alternative regulatory approaches include various post-translational modifications (PTMs). The IDRs responsible for LLPS activity do not have a complex high-level structure, thus are more vulnerable to post-transcriptional modifications.

4.1. Phosphorylation

Protein phosphorylation is one of the most ubiquitous intracellular post-translational modifications (PTMs) by which cells regulate protein activity, conformation, charge and stability, subcellular localization and so on [65,80–85]. At present, it has been revealed that protein phosphorylation can regulate phase separation by at least three ways, including biorecognition, conformal changes and electrostatic interactions [86–89].

The C-terminal domain (CTD) of RNA polymerase II (Pol II) was highly intrinsically disordered and could be phosphorylated by CDK7/9 during the transition from initiation to elongation [90,91]. CTD phosphorylation by either CDK7/9 caused a reduction in CTD incorporation into mediator or MED1 droplets. In another way, hyper-phosphorylated CTD by CDK7/9 was incorporated and condensed into RNA splicing factor SRSF1 or SRSF2 formed droplets. The results form Richard Young elucidated that the phosphorylation of IDR region affected Pol II transforming from transcription to elongation ralated phase separation [67]. The phase separation of heterochromatin protein HP1α highly depends on the inter-dimer contacts. Study show that phosphorylation drives the phase-separated droplets formation of HP1α, because the phosphorylation of serine residues riched N-terminal of HP1α promotes the

switch from a compact to extended state which is required for the higher-order oligomerization [92]. Phosphorylation also affects phase-separation propensity through exaggerating the negative charge densitiy. *In vitro* experiment showed that the CKII mediated LCR phosphorylation of Fragile X Mental Retardation Protein (FMRP), an abundant neuronal granule protein, made it phase-separate at a lower protein concentration than unphosphorylated FMRP. To gain insights into the mechanism, the authors analyzed the charge patterns of phospho- and unphospho- FMRP LCR, they found that serine phosphorylation increased the negative charge density, promoting the multivalent electrostatic interactions for LLPS [93].

4.2. Ubiquitylation and sumoylation

Protein ubiquitination and sumoylation could affect multivalency and thus participate in the regulation of phase separation, too. Recently, Yasushi Saeki lab revealed proteasome formed lysine 48 (K48)-linked ubiquitin chain dependent LLPS under acute hyperosmotic stress in colon cancer cells, which majorly degraded ribosomal proteins that failed to properly assemble [94]. The autophagy receptor protein p62 could combine with K63 polyubiquitin-tagged proteins and formed aggregates, then degraded by autophagosomes to flush mis-folded proteins [95]. Recombinant p62 protein could not undergo droplet formation in cell-free system, but when added K63 polyubiquitin chains p62 phase separation occurred [70]. Sumoylation could ehhance the liquid property of SOP-2 droplet in vitro, as the sumoylated SOP-2 formed droplets were larger in size and more abundant in amount. The FRAP assay showed that sumoylated SOP-2 has a slightely increased recover rate which reflected the internal fluidity [51].

Besides, protein ubiquitination also destroys LLPS. UBQLN2 (Ubiquilin2), as a proteasomal shuttle factor, formed LLPS depending on its LCR and C-terminal UBA, a helical structure that could oligomerize or be poly-ubiquitinated to K48-linked chain. Carlos A. Castañeda lab [55] found UBQLN2 was involved in the formation of human cell stress particles which was associated with ALS and other diseases [96–98]. They found that UBQLN2 forms LLPS through the multivalent interactions provided by IDR and UBA through NMR technology, and the oligomerization of UBA is an important prerequisite for phase separation. The condensates gradually dissolved when the UBA region was K48 poly-ubiquitinated; and significantly, both the mutated ubiquitin and ΔUBA abrogated the disruption from K48 poly-ubiquitination on multivalent interactions in UBA region [55].

4.3. Acetylation

Protein acetylation or deacetylation affects proteins solubility and valency, which regulates phase formation and assembly of stress granules in response to various stresses [71]. Stress granules (SGs) are typical non-membranous organelles, which are mainly composed of untranslating mRNAs and translation initiation related proteins, and the suppression of translation initiation promotes the formation of SGs [12]. RNA helicase DDX3X was revealled as an important constituent for SG formation [99]. Patrick Matthias lad found the acetylation of DDX3X disorded N-terminal (IDR1) impairs its LLPS formation and SG maturation. The acetylation of DDX3X-IDR1 region could be stimulated by various stress conditions including ER stress (thapsigargin), oxidative stress (sodium arsenite, H2O2 and diethyl maleate), heat shock and osmotic stress (sorbitol), energy depletion (CCCP and clotrimazole), translation inhibition (puromycin) and proteasome inhibition (MG132). In this process, histone acetyltransferases CBP plays an important role in acetylating DDX3X antagonizing the deacetylation function of HDAC6, another important SG component. Stresses induce the activation of CBP then DDX3X acetylation, then HDAC6 deacetylated DDX3X and promoted a robust LLPS and SG formation [71]. IDR hyper-acetylation dramatically dissolved the droples of Tau, a protein that underwent phase separation partially by electrostatic interaction. Hyper-acetylation neutralized the positive charges from lysines motifs and thereby interrupted the positive-negetive electrostatic interactions with microtubules [54].

4.4. Methylation

The arginine residues in RGG/RG structure of IDR sequence are protonated and positively charged in the physiological pH condition, which empowered them to interact with DNA/RNA. But some research showed the Arg-methylation mainly affected proteins hydrophilicity through increasing the hydrogen bonding capacity (strength of cation−π interactions) insdead of electrostatic interactions, for example FUS protein [45,][100,101]. To date, most studies on arginine methylation have shown its inhibiting effect on phase separation. Arg-methylation of RGG/RG motifs suppress LLPS and SG partitioning of FUS and ehanced internal mobility within droplet [45]. In addition, Nicolas L. Fawzi lab showed that arginine methylation of hnRNPA2 RGG motif reduded LLPS through disrupting preferential contacts of arginine with aromatic residues [102].

Arg-methylation also could affecte phase formation by preventing protein self-binding. Yeast protein Scd6 (Suppressor of Clathrin Deficiency 6) contains a C-terminal RGG-motif rich domain, and Purusharth I Rajyaguru lab found the involvement of Scd6 in phase separation formation is regulated by its dimerization which is affected by RGG methylation [74]. Hmt1 (hnRNP methyltransferase 1) is the predominant Arg-methyltransferase in yeast, which has been convinced to methylate Scd6 at its RGG-motif [103]. The knock-out of Hmt1 in yeast cells decreased the RGG methylation and increased the dimerization level of SCD6 sequentially, which resulted in the increased localization of Scd6 to granules [74]. Although arginine methylation can increase protein water solubility and inhibit phase separation [45], it is necessary for phase separation maintenance properly for some protein. Processing bodies (P-Bodies) are canonical cytoplasmic mRNP granules related to mRNA decay in eukaryotic cells, and experiments show that P-bodies are LLPS droplets [104–106]. RNA-associated protein 55 (RAP55A) has been validated participating in the formation of P-bodies and SGs in cultured human Hep-2 cells, and knock-down of RAP55A will result in loss of P-bodies [106]. Ken Matsumoto lab found that arginine methyltransferases PRMT1 asymmetrically dimethylated the second RGG motif of RAP55A in HeLa cells, and knock-down of PRMT1 will

result in a translocation of RAP55A from P-bodies [107].

5. The physiological functions of LLPS

Under normal physiological conditions, LLPS is flimsy and reversible, and highly sensitive to all kinds of environmental factors [7,46,50,108,109]. LLPS has been elucidated as an important mechanism for cells responding to complicated and changeable environment pressures then rapidly and dynamically adjast intracellular structure in recent years [12,50,110].

5.1. Transcription activation

LLPS mediates super-enhancer formation. The conception of super-enhancer (SE) was firstly proposed by Richard A. Young in the year 2013 [111]. SEs are different from the traditional-sense enhancers in DNA length, transcription factors (TFs) and apparatus density, epigenetic modifications levels with transcription efficienty and sensitivity to perturbation [111,112]. As we know, enhancers are DNA *cis*-acting regulatory elements, which could recruit TFs, coactivators and RNA Polymerase II (Pol II) to regulate genes transcription [113]. Usually, a SE consists of clusters of enhancers that are high densely occupied by the transcription apparatus and coactivators compartmentalized and concentrated by LLPS of coactivators [4,111]. LLPS let the enhancer elements and promoters with long linear distance much spatially closer in the densed phase, and these elements have high interaction frequencies with each other (Fig. 4) [114–116]. Richard A. Young believes that SEs usually occur for the robust expression of some important genes with prominent roles in cell identity, such as key mESC-identity gene klf4 [4,111,112,117,118].

Phase separation is involved in the regulation of various physiological functions of cells, one of the most valuable and common functions of LLPS is to robustly enhance genes transcription on the physiological need. In mouse ESC, the expression of stemness related gene klf4 is regulated by coactivator mediated LLPS. In this client-scaffold model, Mediator condensates concetrated hundreds of molecues of transcription apparatus and RNA Pol II (RNA polymerase II) at SEs. The subunit of Mediator, MED1, act as acaffold. BRD4 is necessary for the release of RNA Pol II from the transcription initiation site. And the TF is responsible for precise positioning at *cis*-elements through its structured DNA-binding domain [4,67].

There are hundreds of TFs containing ADs in a mammalian cell, but it's a considerably small number of coactivators interacting with these TFs [119]. Although these ADs share little homologous sequence, the possession of IDR is their common character. For exmple, Med1 has been verified to incorporate MYC, P53, NANOG, SOX2, RARa, GATA and ER and other TFs in vivo and in vito [61]. MED1 acts as the scaffold of phase, TFs are responsible for the targetting to SEs. When deregulate OCT4 protein level in cell, MED1 no longer forms condensates at SEs. Most TFs could form droplets alone in droplet formation buffer, such as MYC and NONOG, in spite of some TFs could not form alone, such as OCT4 and P53. But both kinds of TFs will be concentrated into MED1 droplets when added with MED1-IDR [4,61].

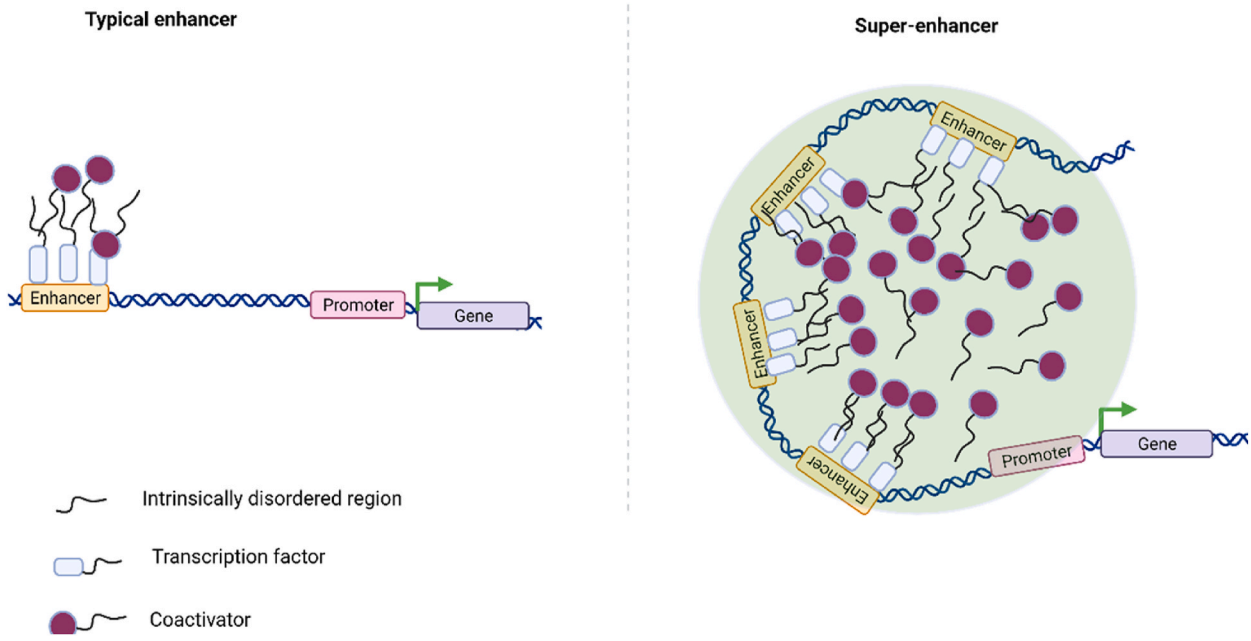

Fig. 4. The diagram of typical enhancer and super enhancer by LLPS. Typical enhancers are DNA *cis*-elements recruiting transcription factors, coactivators and the transcription apparatus to enhance gene transcription. Super-enhancers are clusters of enhancers achieved by the phase separation, recruiting high concentration of coactivators, transcription factors and transcription apparatus, which is mediated by IDRs depending on the multivalent interactions [4].

LLPS may also enhance cancer associated genes expression. In a proportion of T-cell acute lymphoblastic leukemia (T-ALL) cases, for example Jurkat, the mRNA level of oncogene TAL1 is markedly higher than some other T-ALL cell lines, such as DND-41. A. Thomas Look [117] found the reason is DNA heterozygous insertion mutation at the noncoding ~8 kb upstream site of TAL1 oncogene introduded an SE that mediated a robust transcription of TAL1. Besides, LLPS of NUP98 fusion oncoproteins (FOs) is a critical pusher of leukemogenesis. NUP98 FOs have been confirmed to be closely associated with about 5 % pediatric acute myeloid leukemia (AML) and chemotherapy-resistance [120–124]. NUP98 FOs usually are composed of an IDR containing N-terminal portion of the NUP98 protein and a DNA-binding domain containing C-terminal region from other proteins [125], which is conincide with the character of most LLPS proteins. Recently, Richard W. Kriwacki and his colleagues revealled the recombinant NUP98-HOXA9 induced aberrant HOX and other genes expression through LLPS [44].

5.2. Transcription inactivation

Not all phase separations enhance expression, and sometimes LLPS are formed to inactivate transcription. The activationg-puncta were composed of Pol II and TFs [4,43,126], but what if they are replaced with repressors? The downregulation of flowering repressor gene Flowering Locus C (FLC) is the important for vernalization of most flowering plants, such as Arabidopsis. Experiments showed that the stable gene suppression of FLC closely correlated with Vernalization 1 (VRN1) mediated dimethylation of H3K27 and H3K9 [127–129]. Recently, Luhua Lai team confirmed VRN1 protein underwent LLPS in nucleus and in vitro [130]. VRN1 protein consists of an IDR of 115 residues flanked by two B3 DNA-binding domains. VRN1 liquid-like puncta through multivalent protein–DNA interactions in Arabidopsis cell nuclei, suggesting it may suppress FLC transcription by LLPS. However, this speculation remains to be further researched.

Another study on Arabidopsis revealled that phase separation indirectly inhibits transcription through the epigenetic modification of intermediates. It has been demonstrated the alternative 3′-end polyadenylation processing of lncRNA COOLAIR (the antisense transcript of FLC) could suppress the transcriptional initiation and elongation of FLC [131]. And the RNA-binding protein FCA is nessary for the polyadenylation of COOLAIR [132,133]. Caroline Dean group [11] demonstrated FCA repressed FLC transcription through LLPS. First, they found the ectopic expression of FCA significantly repressed FLC expression in cells. FCA protein is composed of two RNA-binding domains at the C-terminal, and two PrD at the C-terminal [134], suggesting it has a potential to undergo LLPS. They found FCA protein formed dynamic liquid-like nuclear bodies in cells. Crosslinked nuclear immunoprecipitation showed that FCA bodies could incoporate nascent transcripts of COOLAIR and polyadenylation related factors, such as FY [135] and RRM-containing protein FPA [136]. Collectivelly, FCA condensates compartmentalize COOLAIR and polyadenylation factors, and inactive the expression of FLC by the robust polyadenylation of COOLAIR.

A confirmed mechanism of transcriptional inhibition is the heterochromatin silencing mediated by phase separation. LLPS mediated by heterochromatin protein 1 (HP1) is important for chromatin compaction. H3k9me3 recruited HP1, and HP1 condensates promoted the conformational change of histone octamer, to accomplish the compression of heterochromatin [137,138].

In addition to the above functions, modulating protein level [139], chromatin remodeling [140,141], DNA repair [142], protein degradation [94,143], nuclear pore transport [144–146], stress response [12,50] and so on, are closely related with LLPS.

6. Relationships between phase separation and diseases

LLPS provides an efficient regulatory mechanism inside the complicated and precise cytosol, which must be closely monitored by the cell at any time. As LLPS performs multiple functions in living cells, LLPS dysfunctions, such as overformation or occurrence at an inappropriate space-time, must inevitably lead to a disorder of cellular function and even the disease, such as cancer and neurodegenerative diseases [108].

6.1. Cancer

Phase separation (PS) is a promising target for cancer treatment as abberant aggeregates promote tumor occurrence and development through lose-function of suppressor proteins, enhancement of onco-proteins including gain-IDR mechanism and up-regulation of PS proteins [147]. For example, the tumor suppressor p53 protein experiences acetylation and ubiquitylation modifications mediated by PML (Promyelocytic leukemia) body in a "client-scaffold" model to induce cell cycle arrest and senescence [148,149]. However, p53 frequently occures missense R248Q mutation at DNA binding domain (DBD) in breast cancer, making it more prone to non-liquid enrichment, namely amyloid fibrils [150]. Mutated p53 loses the cancer-suppressor function replaced by gain-of-function (GoF) of irreversible and more rapid solid-like aggregations, like the R249S mutant in liver cancer and M237I mutant in glioma [151, 152]. Interestingly, Magzoub lab used ADH-6, a tripyridylamide, to dissolve p53 R248W mutant fibrils which recovered transcription activity with tumor inhibition effect of p53 mutant [153].

Unlike p53 inactivation mechanism, some suppressors have lost their IDRs in cancer cells instead. For example, histone demethylase UTX (also named KDM6A) supperesses AML cells proliferation through forming phase condensates. The wildtype UTX protein contains an IDR sequence from amino acid 594–848, unfortunately its aa555 often undergoes terminational mutations in cancer cells leading to the expression of a truncated UTX (1–554) which loses aggregation function thoroughly [140].

Cunning cancers also assemble oncoproteins by gainning IDR fragments. As mentioned above, NUP98-HOXA9 fusion protein simultaneously integrated IDR from NUP98 and transcriptional activation DBD from HOXA9 as a super-transcription factor (TF) to pediatric leukemias through puncta formation [154]. The N-terminal LCDs from FET (FUS/EWS/TAF15) family proteins can fuse with

DBD of transcription factors, such as Fli-1 (ETS transcription factor) in Ewing sarcoma, to form super-TF [155]. Super-TF EWS-FLI1 undergoes puncta at DNA "GGAA" repeats adjacent to transcription start sites, which recruits Pol II, MED1 and BRD4, to powerfully and globally drive oncogenes expression including ERG2 [156]. Similarly, the fusion proteins like BRD4-NUT, NONO-TFE3 and SS18-SSX are in the same mechanism [157–159].

The overexpression of onco-PS proteins is another cruicial point for cancer development. In neuroblastoma Kelly cells, N-myc protein undergoes LLPS in a high concentration dependent manner to promote oncogene transcription [160]. In breast cancer stem cells, the upregulated TAZ and NANOG enrich in liquid-like puncta at the promoter regions of Sox2 and Oct4 to regulate cancer stemness and chemoresistance [161].

6.2. Neurodegenerative disease

Abberrant phase separation of proteins controlling movement in nerve cells led to abnormal deposition of relevant proteins and gradually develop into a solid state, namely aggregates, which was one of the important pathogeneses of ALS [46]. Protein aggregates has been widely accepted and regarded as pathological hallmark of multiple neurodegenerative diseases by the medical community, such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), as ALS/FTD patients often contain abnormal redundant stress granules or ribonucleoprotein (RNP) granules. TDP43 and FUS are two main components of protein aggregates in ALS and FTD. In normal cells, TDP43 and FUS are widely expressed in the nucleus, but in ALS and FTD patients, TDP43 and FUS are rarely present in the nucleus, instead, they go to the cytoplasm and participate in the formation of cytoplasmic aggregates [110]. Post-mortem brains showed that up to 95 % of ALS and 50 % of FTD patients have a redistribution of TDP43, and about 5 % ALS and 10 % FTD ratio fo FUS protein [14].

Mutation and aberrant expression of prion-like protein FUS are directly involved in the occurrence and development of ALS, as variant FUS protein formed abnormal aggregations [46,162]. Simon Alberti [46] found that wild type FUS protein formed dynamic condensates possessing liquid properties in normal cell, but in ALS, FUS was mutated as G156E or R244C. Both two mutations accellerated the transition from LLPS to solid aggregates. Not like LLPS, the fibrous aggregates have little liquidity, which seriously hinders the normal physiological function of cells (Fig. 3 A and B). Diffusion in time of cell compartments is necessary for physiological order. Just imagine the compartments formed for active genes expression refuse to dissolve, cells will face a disastrous.

6.3. Drug discovery

Developing small molecule drugs targeting pathological condensates has a broad prospect as phase separation makes the proteins difficult to target more accessible. Some undruggable proteins, typically transcription factors and phosphatases, perform their functions through aberrant enrichment [163]. Theoretically, using LLPS platform to develop drugs has four advantages at least: 1, LLPS is more sensitive to protein concentration regulation; 2, LLPS may amplify the functions through enrichment effect of small molecules by multivalency; 3, The multivalent interactions supporting LLPS are more fragile; 4, Aberrant aggragates densed with proteins experienced mutations or PTMs made the targets more definite.

The drug strategies are deployed around modulating condensates including protein-protein interactions (PPI) and protein levels [164]. For example, AR and AR-V7 (lost C-terminal LBD) promote chemoresistance in CRPC through LLPS mediated by NTD. Jidong Zhu lab used phase-separation-based platform to screen a compound ET516 which specifically disrupted puncta of AR and AR-V7 by binding the NTD. Interestingly, ET516 not only dose dependently inhibited enzalutamide resistant CRPC proliferation but also retarded CRPC xenografts growth in mice [165]. Proteolysis-targeting Chimera (PROTAC) is a powerful tool to reduce LLPS components, as Yu Rao lab used BRD4 degrader ZXH-3-26 to delete BRD4-MED1-Pol II condensates at super-enhancer [166]. Encouragingly, the AR PROTAC degrader ARV-766 from Arvina showed a significant effect on CRPC inhibition in phase 1/2 clinical trials [167]. However, due to tumor suppressor p53 mutant forms loss-of-function fibril aggretates in cancer, PROTAC seems not applicable to rescue p53 activation. Not only ADH-6 is effective for rescuing mutant p53 activity as mentioned above, aminothiazole compounds BAY 249716 and BAY 1892005 covalently binds with mutant p53 (R175H, R282W and Y220C) then dissolve nuclear condensates markedly [168]. Since the enrichment effect, LLPS condensates affect drug concentration and activity, which provide a chance for new use of old drugs, such as cisplatin disrupts MED1 puncta at super-enhancers in colon caner HCT116 [169]. So, PS-targeting drugs will surely bloom a hundred flowers in the future.

7. Summary and perspective

In recent years, the research results and articals about LLPS spring up like the bamboo shoots, which provides new perspective for understanding human physiological processes and pathological mechanisms. We can preliminarily clarify the physiological and biochemical composition, occurrence and development mechanism of phase separation. The relationship between phase separation and major diseases is also in steady and orderly research. But these are far from in-depth, and many outstanding issues have not been resolved. For example, at present, we only know that phase separation depends on multivalent interactions between macromolecules and can verify them by means of salt dissolution and salting out. However, we can't figure out the specific multivalent diagram, nor can we calculate the multivalent accurate value. During the development of cancer, the incidence and prevalence of specific gene segregation have not been calculated systematically. For disease, is phase separation a cause or a result? And how to realize the treatment of diseases through interference phase separation? As mentioned above, LLPS related diseases are often caused by protein overexpression, mutation or abnormal modification. Can we design drugs for specific proteins in the future to achieve the purpose of

regulating phase separation and turn fatal diseases into chronic diseases?

In general, although phase separation is a new field, it develops rapidly and provides great significance for biological study. It is undeniable that with the deepening and improvement of LLPS research, it will conquer an important position for human diseases and have more clinical significance.

CRediT authorship contribution statement

Weibo Zhang: Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Zhengfeng Li:** Writing – review & editing. **Xianju Wang:** Writing – review & editing. **Ting Sun:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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