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REVIEW PAPER

14-3-3 Proteins are Potential Regulators of Liquid–Liquid Phase Separation

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Abstract

The 14-3-3 family proteins are vital scaffold proteins that ubiquitously expressed in various tissues. They interact with numerous protein targets and mediate many cellular signaling pathways. The 14-3-3 binding motifs are often embedded in intrinsically disordered regions which are closely associated with liquid–liquid phase separation (LLPS). In the past ten years, LLPS has been observed for a variety of proteins and biological processes, indicating that LLPS plays a fundamental role in the formation of membraneless organelles and cellular condensates. While extensive investigations have been performed on 14-3-3 proteins, its involvement in LLPS is overlooked. To date, 14-3-3 proteins have not been reported to undergo LLPS alone or regulate LLPS of their binding partners. To reveal the potential involvement of 14-3-3 proteins in LLPS, in this review, we summarized the LLPS propensity of 14-3-3 binding partners and found that about one half of them may undergo LLPS spontaneously. We further analyzed the phase separation behavior of representative 14-3-3 binders and discussed how 14-3-3 proteins may be involved. By modulating the conformation and valence of interactions and recruiting other molecules, we speculate that 14-3-3 proteins can efficiently regulate the functions of their targets in the context of LLPS. Considering the critical roles of 14-3-3 proteins, there is an urgent need for investigating the involvement of 14-3-3 proteins in the phase separation process of their targets and the underling mechanisms.

Keywords Protein–protein interaction · Scaffold protein · Intrinsically disordered region · Condensate · Regulation

Abbreviations

IDRs	intrinsically disordered regions
PTMs	post-translational modifications
LLPS	liquid–liquid phase separation
PML	promyelocytic leukemia
hnRNPs	heterogeneous nuclear ribonucleoproteins
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
COVID-19	coronavirus disease 2019
YAP	Yes-associated protein

Introduction

The 14-3-3 proteins are ubiquitously expressed in various tissues and mediate many cellular signaling pathways. The name "14-3-3" was given because these proteins elute in the 14th fraction of bovine brain homogenate on the DEAE cellulose column and the 3.3 fraction in the subsequent starch gel electrophoresis [1]. They interact with numerous protein targets, including kinases, phosphatases, transmembrane receptors, and transcription factors [2, 3]. Through these protein–protein interactions, 14-3-3 proteins participate in cell cycle regulation, gene expression control, apoptosis, signal transduction, and many other vital biological processes [4–6]. Dysregulation of 14-3-3 protein expression has been observed in several cancers [7]. Furthermore, 14-3-3 proteins have chaperone activity which may play a role in neurodegenerative disease progression [8]. Since 14-3-3 proteins are potential therapeutic targets, many 14-3-3 modulators have been

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discovered/designed to target these specific interactions [9, 10].

The binding targets of 14-3-3 proteins usually carry specific motifs containing phosphorylated serine or threonine. Most of these motifs are embedded in intrinsically disordered regions (IDRs) [11, 12]. In contrast to traditional folded protein domains, IDRs do not fold into stable three dimensional structures in the free state. Their amino acid compositions significantly differ from those of folded proteins. Generally, IDRs lack bulky hydrophobic amino acids and are enriched in charged amino acids [13, 14]. While IDRs are unfolded, they perform critical functions. Structural disorder facilitates post-translational modifications (PTMs), association/dissociation kinetics, conformational changes, and so on [15, 16]. Consequently, numerous IDRs are involved in cellular regulation and signal transduction [17–19].

Recently, IDRs have become a theme of many research efforts because they are closely associated with liquid–liquid phase separation (LLPS), which underlies the formation of membraneless organelles and cellular condensates [20–25]. Increasing evidences suggest that many types of biomolecular condensates, such as stress granules, P granules, and nuclear speckles, are formed through LLPS [24, 26–29]. Enriching interacting components inside droplets has been shown to promote protein aggregation [30–32], enzyme reaction [33, 34], gene expression [35–37], and virus replication [38, 39]. LLPS is driven by multivalent interactions and can be readily regulated by effector molecules or PTMs [22, 29, 40].

To date, 14-3-3 proteins have not been reported to undergo LLPS alone or regulate LLPS of their binding partners. However, the high LLPS propensity of 14-3-3 binding partners and the bivalent nature of 14-3-3 proteins suggest that 14-3-3 proteins could be potentially involved in LLPS regulation. In this review, we first summarized the interacting modes of 14-3-3 and mechanism of LLPS, and then we proposed that 14-3-3 proteins could be recruited into the droplets and regulate the phase separation behavior of their binding partners.

Structure and Function of 14-3-3 Proteins

The 14-3-3 proteins are a family of highly conserved scaffold proteins. They are expressed in various types of tissues [41–45]. In mammals, seven 14-3-3 isoforms encoded by different genes have been identified: α/β, γ, ε, δ/ζ, η, θ, and σ, where α and δ are the phosphorylated forms of 14-3-3β and ζ, respectively [46–48]. The length of human 14-3-3 isoform varies from 245 residues to 255 residues. Each 14-3-3 protein sequence contains a divergent N terminus, a divergent C terminus, and a conserved core region. A phylogenetic tree generated from sequence alignment is shown in Fig. 1a. 14-3-3 proteins form stable homodimers or heterodimers with a cup-shaped structure (Fig. 1b). Each protomer consists of nine antiparallel α-helices where α1 and α2 of one protomer interact with α3 and α4 of the other protomer to form the dimer interface [49, 50].

14-3-3 proteins interact with their binding partners primarily through the amphipathic binding grooves constituted by α-helices α3, α5, α7, and α9 (Fig. 1b). Three distinct 14-3-3 binding motifs are identified: motif I [RXXp(S/T)XP/G], motif II [RX(F/Y)Xp(S/T)X(P/G)], and motif III [p(S/T)$X_{1\text{-}2}$-COOH], where p(S/T) is a phosphorylated serine or threonine, and X is any type of residue [51–55]. The three conserved basic residues Lys49, Arg56, and Arg127 within the amphipathic groove form ionic and hydrogen bonds with the phosphorylated residue (Fig. 1c) [49]. Although the sequences of the seven 14-3-3 isoforms are highly conserved, they bind to the same target with different affinities. Interestingly, their binding affinities obey a hierarchized profile with conserved relative K_D ratios [56].

It should be noted that not all interactions with 14-3-3 proteins require the above-mentioned phosphorylated motifs. Some non-phosphorylated peptides also bind to 14-3-3 proteins with high affinity [57, 58]. If a protein contains multiple motifs, they could occupy both amphipathic grooves within a 14-3-3 dimer simultaneously [59–62]. Besides the amphipathic grooves, the last two C-terminal α-helices are also shown to contribute to interactions with some partners [63–65].

So far, hundreds of 14-3-3 binding proteins have been identified [66–68]. Affinity-purification/mass spectrometry combined with domain-based cluster analysis suggested that 14-3-3 proteins can potentially engage around 0.6% of the human proteome [69]. Overall, the 14-3-3 binding proteins show great structural and functional diversity. A recent Gene Ontology analysis of 14-3-3 partners showed that 25% are non-receptor Ser/Thr protein kinases, 20% are DNA binding proteins, 12% are cell cycle proteins, 10% are RNA-binding proteins, and several other categories [12]. Therefore, 14-3-3 binding proteins are extensively involved in cell cycle regulation, apoptosis, autophagy, signal transduction, and gene expression control [4–6].

14-3-3 proteins regulate the function of their targets through several modes [70]: (i) A 14-3-3 dimer can act as a scaffold to bring two interacting proteins in close proximity, thus mediating the formation of protein complexes. For example, 14-3-3β mediates the interaction between Bcr and Raf [71] and 14-3-3ζ stabilizes interaction between α4 integrin and paxillin [72]. (ii) 14-3-3 proteins can occlude interactions between the target proteins and their effectors. Interaction between BAD and Bcl-2 promotes apoptosis. Datta et al. found that phosphorylation of BAD at Ser136 and Ser112 recruits 14-3-3 proteins, which disrupts the

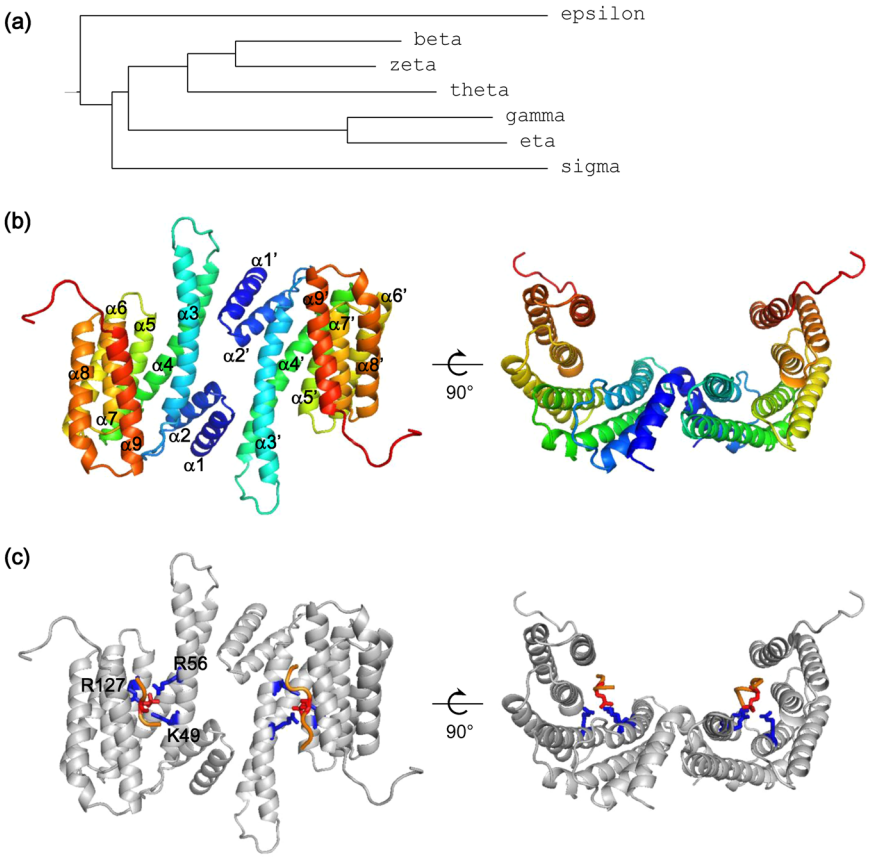

Fig. 1 Sequence and structure analysis of 14-3-3 proteins. **a** A phylogenetic tree generated from sequence alignment of seven human 14-3-3 isoforms. **b** Two perpendicular views of 14-3-3σ dimer. Each protomer is shown as cartoon in rainbow color. The nine antiparallel α-helices in one protomer is labeled from α1 to α9. **c** Interactions between the pSer214 residue of tau peptide and the conserved basic residues Lys49, Arg56, and Arg127 within the amphipathic groove of 14-3-3σ. The tau peptide is shown as an orange tube, where the pSer214 residue is shown as red sticks. Residues Lys49, Arg56, and Arg127 of 14-3-3σ are shown as blue sticks. Crystal structure of the 14-3-3σ/tau complex (Protein Data Bank ID 4FL5 [60]) is used in **b** and **c** for illustration

binding of BAD to Bcl-2 and thereby promotes cell survival [73]. (iii) 14-3-3 proteins can induce conformational changes on the target proteins, altering their activity. A well-studied example is the serotonin N-acetyltransferase, an enzyme that controls the daily rhythm in melatonin synthesis. 14-3-3 proteins increase the activity of serotonin N-acetyltransferase by modulating the structure of the substrate-binding sites [74, 75]. Sometimes, different modes may work synergetically to modulate the activity of the 14-3-3 binding partners. For example, the binding site of 14-3-3ζ on the kinase domain of ASK1 is in close proximity to the kinase active site. Therefore, 14-3-3ζ may inhibit the activity of ASK1 by blocking its accessibility and/or affecting its conformation [76].

By participating in numerous signaling pathways, dysregulation of 14-3-3 proteins is involved in many diseases [7, 8]. Expression levels of the majority of 14-3-3 isoforms are elevated in many tumors, such as meningioma [77], astrocytoma [78], glioblastoma [79], lung cancer [80, 81], breast cancer [82, 83], and hepatocellular carcinoma [84, 85]. Because 14-3-3σ is a tumor suppressor, its expression in tumors is negatively regulated [77, 86]. By interacting with GSK-3β, LRRK2, or FOXO3a, or co-localizing with α-synuclein in the Lewy bodies or tau in the neurofibrillary tangles, 14-3-3 proteins are associated with Alzheimer's disease and Parkinson's disease [87–93]. Furthermore, 14-3-3 proteins are also associated with other neurological disorders, such as lissencephaly, schizophrenia, bipolar disorder, Williams syndrome, and Down syndrome [94, 95].

Liquid–Liquid Phase Separation: Function and Mechanism

Many proteins are able to undergo LLPS when the concentration exceeds a threshold value, in particular under crowded conditions. LLPS usually results in formation of liquid droplets in which proteins are enriched. The protein concentration inside the droplets can be tens of folds higher than that of the surrounding dilute phase [96, 97]. The phase separation behavior of a protein can be readily affected by several environmental conditions, such as salt concentration, pH, and temperature [98, 99].

LLPS can be driven by multivalent interactions [22, 29]. Tandem repeats of binding element are multivalent, which is well examplified by the nephrin–NCK–N-WASP system [100]. Multivalence can also be achieved by oligomerization, as in NPM1 or SynGAP [101, 102]. Furthermore, IDRs form dynamic, multivalent interactions, such as cation–π

interactions, electrostatic interactions, and hydrophobic interactions [103–105]. The interaction mode between multivalent molecules can be described by the stickers-and-spacers model [22, 106], in which stickers are groups of residues forming attractive interactions, while spacers connect the stickers and influence their interactions.

As indicated by the stickers-and-spacers model, LLPS will be altered if the strength of interaction, the valence of molecules, the distribution of stickers, or the length of spacers is changed. PTM is a universal way to regulate LLPS [22, 40, 107, 108]. PTM can induce conformational changes or alter electrostatic interactions and hydrophobic interactions, thereby promoting or suppressing LLPS. Phosphorylation [109–113], methylation [98, 111, 114–116], acetylation [117, 118], polyubiquitination [119, 120], and PARylation [121, 122], have been shown to alter LLPS of many proteins.

Proteins within a droplet can be generally divided into two classes [123]. The first class is scaffolds or drivers, which are multivalent and essential for LLPS. The second class is clients or regulators. Clients and regulators are dispensable for LLPS and are recruited into the droplets via interactions with the scaffolds or drivers. In promyelocytic leukemia (PML) nuclear bodies, the PML protein represents the scaffold, while SUMO-1, Daxx, and Sp100 are the clients and dynamically exchanged at the PML nuclear bodies [124, 125]. It has been shown that partition of clients into droplets varies with the scaffolds concentration or valency [123]. Recently, Orti et al. systematically analyzed the features of drivers, clients, and regulators, and observed some difference among them in disorder content, sequence composition, and prevalence of PTMs [126].

Accumulated evidences suggest that LLPS underlies the formation of various membraneless compartments, such as stress granules, P granules, nuclear speckles, and nucleoli [24, 26–29]. Thus, LLPS plays critical roles in many cellular processes, including assembly of mitotic spindle [127], cellular stress response [128, 129], gene expression [35–37], and RNA splicing [130]. Furthermore, LLPS is also associated with pathological processes, such as protein aggregation and virus replication [30–32, 38, 39]. Consequently, misregulation of LLPS may lead to the occurrence of diseases [31, 97, 131–133].

14-3-3 Proteins are Potential Regulators of Phase Separation

As discussed above, 14-3-3 proteins participate in numerous signaling pathways and regulate various biological processes, where LLPS may occur. However, the involvement of 14-3-3 proteins in LLPS has not been investigated. Since LLPS of 14-3-3 proteins has not been reported, we first

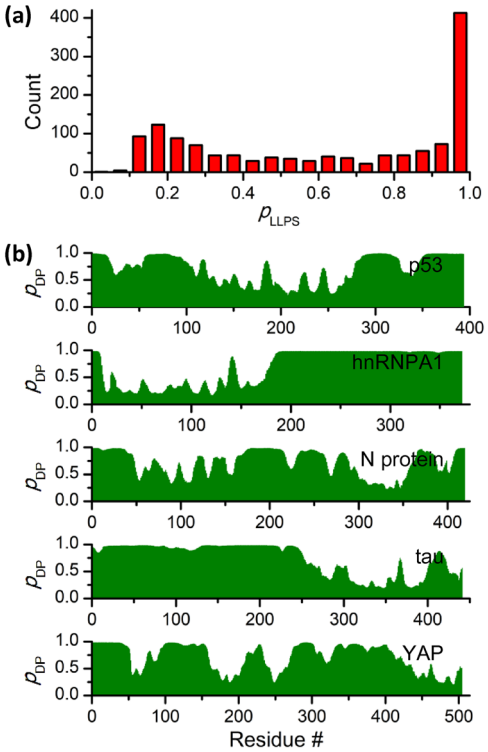

Fig. 2 LLPS propensity prediction of 14-3-3 binding partners using FuzDrop [134]. **a** Statistical analysis of LLPS probability (p_{LLPS}) for 14-3-3 binding partners. According to FuzDrop, proteins with p_{LLPS} greater than 0.61 likely form droplets spontaneously. p_{LLPS} values for all proteins are provided in Supplementary Table S1. **b** FuzDrop predicted droplet-promoting propensity profiles (p_{DP}) of p53, hnRNPA1, SARS-CoV-2 nucleocapsid protein, tau, and YAP. Residues with $p_{DP} > 0.60$ are likely to mediate LLPS

predicted their LLPS propensities using the FuzDrop server [134]. FuzDrop prediction gives the probability of LLPS (p_{LLPS}) for human 14-3-3β, γ, ε, ζ, η, θ, and σ are 0.2884, 0.1581, 0.1586, 0.2169, 0.1364, 0.1318, and 0.2124, respectively, suggesting that all seven 14-3-3 isoforms will not phase separate spontaneously. We further analyzed the LLPS propensities for the binding partners of human 14-3-3 proteins. 1370 14-3-3 binding partners were collected from the IntAct database [135] and then subjected to FuzDrop prediction (Table S1). Surprisingly, more than one half of the 14-3-3 binders show p_{LLPS} values larger than 0.6 (Fig. 2a), suggesting that a large number of the 14-3-3 binders could undergo phase separation. A search of literature showed that LLPS of some 14-3-3 binding partners has been characterized experimentally (Table 1). The FuzDrop prediction profiles of five representative 14-3-3 binders were shown in Fig. 2b. Given the high LLPS propensities of 14-3-3 binding partners and the above discussion on the modes through which 14-3-3 proteins regulate their targets, we speculate that 14-3-3 proteins could enhanced or suppressed LLPS of their binding partners (Fig. 3a). Some 14-3-3 binding partners contain multiple

Table 1 Experimental characterization of phase separation of 14-3-3 binding partners

Protein	UniProt ID	Function	Phase separation properties	References
APC	P25054	Cellular signaling	LLPS in vitro; LLPS with Axin in cells.	[206] [207]
CEP152	O94986	Scaffold protein	LLPS with Cep63 in vitro and in cells.	[208]
CHAF1A	Q13111	DNA binding protein	LLPS in cells	[209]
DACT1	Q9NYF0	Cellular signaling	LLPS in cells	[210]
DCP1A	Q9NPI6	mRNA decapping factor	LLPS in cells.	[211]
DDX3X	O00571	RNA helicase	LLPS in vitro and in cells.	[118]
EDC3	Q96F86	Scaffold protein	LLPS in vitro.	[212–215]
ERC1	Q8IUD2	Scaffold protein	LLPS in cells.	[216]
GIT1	Q9Y2X7	GTPase activator	LLPS in vitro and in cells.	[217]
H1.2	P16403	DNA binding protein	LLPS in vitro	[218]
hnRNPA1	P09651	RNA-binding protein	LLPS in vitro and in cells.	[32, 176, 177, 179]
hnRNPA2	P22626	RNA-binding protein	LLPS in vitro, in cells, and in *C. elegans*.	[116, 219–222]
hnRNPD	Q14103	RNA-binding protein	LLPS in cells.	[222]
HSF1	Q00613	Transcription factor	LLPS in cells.	[223, 224]
HTT	P42858	microtubule-mediated transport	LLPS in cells.	[225]
LATS1	O95835	Cellular signaling	LLPS in cells	[226]
MPRIP	Q6WCQ1	actin filament binding protein	LLPS in cells.	[227]
NELFE	P18615	Transcriptional regulation	LLPS with NELFA in vitro and in cells.	[228]
NPM1	P06748	Nucleolar chaperone	LLPS in vitro and in cells.	[101, 229–231]
p53	P04637	Transcription factor	LLPS in vitro and in cells.	[35, 164–168]
PCNT	O95613	Scaffold protein	LLPS in cells	[232]
PLK4	O00444	Serine/threonine-protein kinase	LLPS in vitro and in cells.	[233, 234]
RAD52	P43351	DNA repair protein	LLPS in vitro and in cells.	[235, 236]
SARS-CoV-2 nucleocapsid protein	P0DTC9	RNA-binding protein	LLPS in vitro and in cells.	[38, 39, 189–199, 237, 238]
SOX-2	P48431	Transcription factor	LLPS in vitro.	[239]
SRRM2	Q9UQ35	Splicing factor	LLPS with SON in cells.	[240]
SynGAP	F6SEU4	Cellular signaling	LLPS with PSD-95 in vitro and in cells	[102, 241, 242]
tau	P10636	Microtubule-associated protein	LLPS in vitro and in cells.	[30, 96, 97, 99, 104, 105, 154–157, 159, 160, 179, 243–251]
TFE3	P19532	Transcription factor	LLPS in cells.	[252]
TFEB	P19484	Transcription factor	LLPS in vitro and in cells.	[253]
UBQLN4	Q9NRR5	Cellular signaling	LLPS in vitro.	[254]
USP42	Q9H9J4	Deubiquitinating enzyme	LLPS in vitro and in cells.	[255]
YAP	P46937	Transcription factor	LLPS in vitro and in cells.	[203, 204]

14-3-3 binding sites which may be occupied by a 14-3-3 dimer. Upon 14-3-3 proteins binding, significant conformational changes may occur within the 14-3-3 binding partners. Therefore, LLPS of the 14-3-3 binding partners may be modulated (Mode I in Fig. 3b). 14-3-3 proteins could also act as cross-linkers by binding to multiple partners simultaneously, thus increasing the valence of interaction for LLPS and promoting phase separation (Mode II in Fig. 3b). PTMs are key factors regulating LLPS. 14-3-3 proteins could modulate LLPS of their binding partners by facilitating the occurrence of PTMs by recruiting enzymes (Mode III in Fig. 3b). In the following text, we summarized

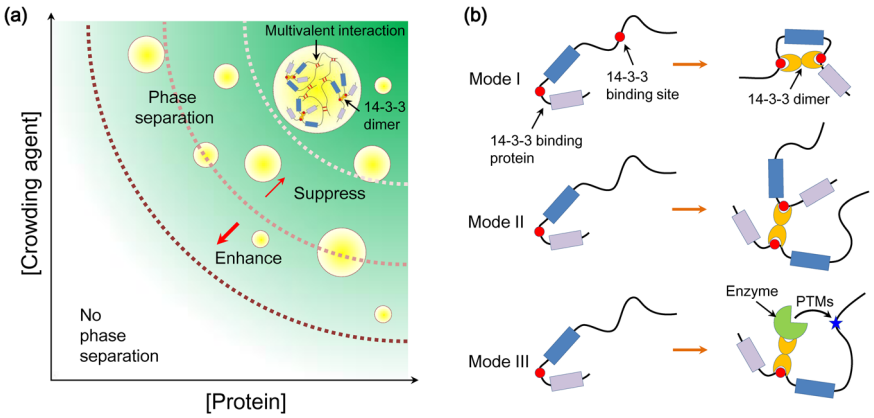

Fig. 3 Potential influence of 14-3-3 proteins on the LLPS propensity of 14-3-3 binding partners. **a** A phase diagram illustrating that LLPS of 14-3-3 binding partners may be regulated by 14-3-3 proteins. Yellow circles indicate the droplets formed. Multivalent interactions driving LLPS and recruitment of 14-3-3 into the droplets are indicated. When 14-3-3 proteins promote LLPS of their partners, the phase boundary is shifted towards lower protein concentration and lower crowder concentration. On the contrary, if 14-3-3 proteins suppress LLPS of their partners, the phase boundary is shifted towards higher protein concentration and higher crowder concentration. **b** Schematic illustration of modes through which 14-3-3 proteins may regulate the LLPS propensities of their targets. In mode I, a 14-3-3 dimer binds to its target and induces conformational change. In mode II, a 14-3-3 dimer binds to multiple targets, thus increasing the valence of interaction for LLPS. In mode III, a 14-3-3 dimer bridges its target with the modifying enzyme, thus facilitating the occurrence of PTMs

the phase separation properties of five representative 14-3-3 binders and discussed how 14-3-3 proteins could play a role.

Microtubule-Associated Protein Tau

Tau is a microtubule-associated protein mainly distributed in axons. Its primary function is to regulate the assembly and spatial organization of microtubule [136, 137]. Tau also plays a role in cellular signaling and chromosome stability [138, 139]. Because hyperphosphorylation and abnormal aggregation of tau have been observed in a number of neurodegenerative diseases, tau is considered as an important target for neurodegenerative disease treatment [140–143].

The interactions between 14-3-3 and tau have been recognized for a long time and may contribute to tau aggregation [144–146]. Crystal structures and binding affinity characterization show that tau segments embracing phosphorylated residues pSer214 and pSer324 bind to the amphipathic grooves of 14-3-3 [60]. However, amphipathic groove-specific binding ligands are not able to completely inhibit binding of tau to 14-3-3 [147]. Moreover, Stefanoska et al. showed that an N-terminal 11-amino acid-long motif (residues 18–28) modulates interactions between tau and 14-3-3 [148].

Recently, it was found that LLPS is an intrinsic property of tau and may be involved in its function and aggregation [149–153]. For example, tau droplets can concentrate tubulin and nucleate microtubule bundles as well as regulate the activity of microtubule-severing enzymes and the movement of molecular motors [154, 155]. LLPS also promotes tau aggregation or formation of toxic oligomers [30, 96, 97, 99, 156, 157].

LLPS of tau is mainly driven by electrostatic interactions between the negatively charged N-terminal and the C-terminal domains and the positively charged proline-rich domain and the microtubule-binding domain [104], whereas hydrophobic interactions are also involved [97, 105]. Many factors promoting tau aggregation have been found to promote tau phase separation [105, 153], and several proteins, including EFhd2 [158], PDI [159], Hsp22 [160], and TIA1 [157], have been found to regulate the LLPS of tau. Considering the extensive interactions between tau and 14-3-3 proteins, we speculate that the conformational changes on tau induced by 14-3-3 may influence the phase separation of tau (mode I in Fig. 3a). Furthermore, 14-3-3 proteins promote tau phosphorylation [161], which may also regulate tau LLPS (mode III in Fig. 3a).

Tumor Suppressor p53

The tumor suppressor p53 protein is a transcription factor. Its inactivation is found in many cancer types. 14-3-3 proteins regulate the transcriptional activity, stability, and cellular localization of p53 [6]. So far, three 14-3-3 binding sites located in the disordered C-terminal domain of p53 have been characterized experimentally [6].

Early studies have demonstrated that p53 is uptaken into membrane-less cellular bodies, such as Cajal bodies and promyelocytic leukemia protein bodies [162, 163]. Droplets of p53 were first reported by Boija et at. when they mixed

p53 with transcriptional coactivator MED1 [35]. Later, anomalous liquid condensates of p53 were observed by Safari et al. when they explored the aggregation process of p53 at near-physiological conditions and in crowded environments [164]. p53 also forms droplets when overexpressed in yeast [165]. While experiments using recombinant p53 suggest that electrostatic interactions between the negatively charged N-terminal domain and the positively charged C-terminal domain play a key role in mediating p53 droplet formation [166], some mutations are found to markedly promote p53 condensation in cancer cells [167, 168]. Though the function of p53 condensates remains elusive [169], formation of p53 droplets may be related to p53 aggregation [164, 165, 168, 170].

Oligomerization is a key factor in LLPS as it generates multi valences and enhances intermolecular interactions [109, 132, 171]. Interactions with 14-3-3 proteins are found to enhance the tetramerization of p53 [172]. In this context, 14-3-3 proteins are expected to enhance the LLPS propensity of p53 and have an effect on the subsequent functioning process (mode II in Fig. 3a).

Heterogeneous Nuclear Ribonucleoprotein hnRNPA1

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a family of RNA-binding proteins that regulate diverse biological processes. hnRNPA1 is concentrated in the nucleus and involved in mRNA processing. During stress, hnRNPA1 is sequestered in the cytoplasm and forms stress granules with other RNA-binding proteins and mRNA [173]. A 14-3-3 binding motif is located on the N-terminus, where phosphorylation on Ser4/6 is required for hnRNPA1/14-3-3 interactions [174]. 14-3-3 can mediate re-entry of hnRNPA1 into the nucleus or cooperate with hnRNPA1 to control splicing of genes under stress conditions [174, 175].

hnRNPA1 consists of two folded RNA recognition motifs and a low complexity domain. While LLPS of hnRNPA1 is mainly driven by the low complexity domain via aromatic-aromatic and aromatic-arginine interactions, the folded RNA recognition motifs modify the phase behavior through intramolecular electrostatic interactions between the low complexity domain and the RNA recognition motifs [32, 176]. LLPS of hnRNPA1 has been suggested to contribute to the assembly of stress granules and the formation of amyloid-like fibers [32, 177, 178]. Since conditions that favor LLPS also enhance hnRNPA1 aggregation, it is suggested that LLPS is on pathway to its aggregation [179].

14-3-3 proteins have been shown to regulate the component of stress granules. MK2-induced phosphorylation of tristetraprolin at Ser52 and Ser178 promotes formation of tristetraprolin/14-3-3 complexes, resulting in exclusion of tristetraprolin from arsenite-induced stress granules [180]. Moreover, 14-3-3 proteins were able to regulate the distribution of hMex-3B in P-bodies and stress granules [181]. Importantly, both tristetraprolin and hMex-3B are predicted to undergo phase separation spontaneous with p_{LLPS} values of 0.9986 and 0.9988, respectively. Modulating the component of stress granules by 14-3-3 or building up physical cross-linking between hnRNPA1 may influence the material properties or functions of stress granules (mode II in Fig. 3a).

SARS-CoV-2 Nucleocapsid Protein

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the worldwide pandemic of the coronavirus disease 2019 (COVID-19) [182]. The SARS-CoV-2 genome encodes about 30 proteins, among which the nucleocapsid (N) protein is the most abundant viral protein in infected cells [183–185]. N protein is a multifunctional protein. It packages viral RNA into virions and plays a critical role in virus transcription and assembly [186].

14-3-3 proteins have been reported to regulate the shuttling of SARS-CoV N protein between the nucleus and the cytoplasm [187]. Recently, Tugaeva et al. characterized the interactions between N protein and 14-3-3 proteins [188]. They found that N protein interacts with all seven human 14-3-3 isoforms with K_d values in low micromolar range. The main binding site for N protein to bind 14-3-3 is phosphorylated Ser197, which is located in the central IDR.

The phase separation propensity of N protein has been subjected to extensive studies. N protein undergoes LLPS with RNA where the N-terminal RNA-binding domain, the central IDR, and C-terminal dimerization domain play an essential role [39, 189–194]. LLPS of N protein is modulated by phosphorylation at the serine/arginine-rich region by CDK1, GSK-3β, or SRPK1 [38, 193, 195]. The N protein/RNA condensates recruit RNA polymerase complex of SARS-CoV-2, which may provide a mechanism for efficient transcription of viral RNA [38]. N protein also phase separates with G3BP or hnRNPA2 into stress granules [190, 193, 196, 197]. Furthermore, LLPS of N protein may be associated with the dysfunctional inflammatory responses and antiviral immunity [194, 198]. Consequently, modulating the phase separation propensity of N protein could be a potential treatment for COVID-19 [39, 199].

Though the influence of 14-3-3 on LLPS of N protein has not been investigated so far, binding of 14-3-3 to N protein is expected to modify the conformation of the central IDR or introduce intermolecular crossing-linking, which could play a role in modulating the phase separation propensity of N protein (mode I and mode II in Fig. 3a). Recruitment of 14-3-3 into N protein condensates may also

regulate the signaling pathways involved in immune response.

Transcriptional Coactivator YAP

The Yes-associated protein (YAP) is a key transcriptional coactivator of the Hippo pathway [200]. Unphosphorylated YAP is located in the nucleus and interacts with the TEAD transcription factors [201]. Upon association with TEAD, YAP induces expression of genes involved in anti-apoptotic processes [202]. On the contrary, when YAP is phosphorylated by LATS1-2, it binds to 14-3-3 proteins and remains in the cytosol [202]. Therefore, 14-3-3 proteins play a role in regulating the Hippo pathway.

To date, there are two studies showing that YAP undergoes phase separation. In one study, Yu et al. found that interferon-γ promotes phase separation of YAP after anti-PD-1 therapy in tumor cells [203]. YAP droplets induced by interferon-γ are localized in the nucleus and recruit TAZ, TEAD4, EP300, and MED1, forming a transcriptional hub for gene expression. Therefore, disrupting LLPS of YAP suppresses tumor cell growth [203]. In the other study, Cai et al. investigated the phase separation behavior of YAP under hyperosmotic stress [204]. In hyperosmotically stressed cells, YAP condensates are observed in both cytoplasm and nucleus. Importantly, the components of cytoplasmic and nuclear YAP condensates are different. Cytoplasmic YAP condensates concentrate kinase, including LATS1 and NLK, whereas nuclear YAP condensates contain transcription factor and transcription regulator, such as TEAD1 and TAZ. While both studies confirm the essential role of C-terminal IDR of YAP in LLPS, formation of YAP condensates is regulated by Ser127 and Ser128 phosphorylation [204]. The Ser127 phosphorylation creates a binding site for 14-3-3 proteins and promotes formation of cytoplasmic YAP condensates, whereas phosphorylation on Ser128 abolishes YAP/14-3-3 binding and suppresses LLPS of YAP in the cytoplasm [204, 205]. Therefore, 14-3-3 proteins may play a critical role in regulating the formation of cytoplasmic YAP condensates which sequester YAP from the nucleus (mode I and II in Fig. 3a).

Conclusions and Perspectives

In the past ten years, our understanding on LLPS has achieved rapid development. Increasing studies show that LLPS plays a fundamental role in formation of membraneless organelles and cellular condensates, and dysregulation of LLPS is closely related to human diseases. 14-3-3 proteins interact with various targets and form an interacting hub for many cellular signaling pathways. While numerous investigations have been performed on 14-3-3 proteins, its involvement in LLPS is overlooked. To reveal the potential correlation between 14-3-3 proteins and LLPS, in this review, we summarized the LLPS propensity of 14-3-3 binding partners and found that about one half of them may undergo LLPS spontaneously. We further analyzed the phase separation behavior of representative 14-3-3 binders and discussed how 14-3-3 protein may be involved. By modulating the conformation and valence of interactions and recruiting other molecules, we speculate that 14-3-3 proteins can efficiently regulate the functions of their targets in the context of LLPS. Considering the critical roles of 14-3-3 proteins, there is an urgent need for investigating the involvement of 14-3-3 proteins in the phase separation process of their targets and the underlying mechanisms.

Data Availability

All data are included in the article and its supplementary information files.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no competing interests.

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