Cell Press
Trends in Biochemical Sciences

Review

FUS and TDP-43 Phases in Health and Disease

Bede Portz,[1] Bo Lim Lee,[1] and James Shorter[1,*]

The distinct prion-like domains (PrLDs) of FUS and TDP-43, modulate phase transitions that result in condensates with a range of material states. These assemblies are implicated in both health and disease. In this review, we examine how sequence, structure, post-translational modifications, and RNA can affect the self-assembly of these RNA-binding proteins (RBPs). We discuss how our emerging understanding of FUS and TDP-43 liquid–liquid phase separation (LLPS) and aggregation, could be leveraged to design new therapies for neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and limbic-predominant age-related TDP-43 encephalopathy (LATE).

Highlights

Phase separation by proteins containing intrinsically-disordered regions (IDRs) underpins the biogenesis of functional membraneless organelles, as well as the formation of aggregated structures linked to neurodegenerative disease.

One class of IDR, termed a prion-like domain (PrLD), is frequently found in RNA-binding proteins, such as FUS and TDP-43, which form condensates with a range of material states.

FUS and TDP-43 form condensates in health and disease, and their phase separation is governed by distinct molecular grammar and regulation.

Aberrant phase separation is likely more diverse than liquid-to-solid transitions and may include inappropriate liquid phases.

Drugging condensates to treat disease is a promising strategy that may involve altering saturation concentrations of key scaffolds, or the specific partitioning of disruptive agents into discrete condensates.

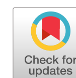
Biology on the Mesoscale

A revolution in understanding cellular organization is occurring on the mesoscale, between the largest protein complexes and membrane-encompassed organelles. In this vast space, an increasingly large number of cellular assemblies reside without precise stereospecific interfaces, defined stoichiometric ratios of components, or a delimiting membrane [1]. Such assemblies, dubbed condensates, include the nucleolus and various ribonucleoprotein (RNP) granules such as **stress granules** (**SGs)** (see Glossary), paraspeckles, DNA-damage foci, signalasomes, and the pyrenoid, among others. Not only are condensates widespread, some posit they are as old as life itself, possibly facilitating biochemistry in the RNA world [2].

Many of these condensates are thought to arise through the process of liquid–liquid phase separation (LLPS) or liquid–gel phase transitions, and can be described by a phase diagram [2–4] (Figure 1A). LLPS results in the switch-like demixing of biomolecules into concentrated liquid droplets. The resulting condensates can retain the properties of a liquid, readily exchanging components with the bulk solution, fusing with other droplets, and wetting surfaces upon collision [3,4]. The high local concentration of specific biomolecules within a condensate can facilitate further transitions in material state, resulting in gelatinous or solid assemblies, or nucleating the growth of fibrillar structures [5]. A central principle underlying LLPS is multivalency, the ability of one biomolecule to simultaneously interact with multiple other copies of itself (**homotypic phase separation**) or with multiple other biomolecules (**heterotypic phase separation**) [6]. In the case of proteins, multivalency is frequently, but not exclusively, facilitated by intrinsically-disordered regions (IDRs), which lack a stable 3D structure and whose structural heterogeneity allows interacting motifs to be displayed in multiple orientations [7]. The contribution of IDRs to LLPS, including those of FUS and TDP-43, have been the subject of multiple studies and reviews [8–10]. Often encoded in these domains are '**stickers**', residues or motifs that enable protein–protein or protein–nucleic acid interactions, interspersed by flexible '**spacers**' that modulate sticker interactions [11]. Here, we describe the role of a specific subclass of IDR, prion-like domains (PrLDs) (Box 1) in LLPS function and dysfunction, with specific focus on archetypal yet contrasting examples of PrLD proteins, FUS and TDP-43 [12–15].

[1]Department of Biochemistry and Biophysics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

*Correspondence:
jshorter@pennmedicine.upenn.edu
(J. Shorter).



 https://doi.org/10.1016/j.tibs.2020.12.005
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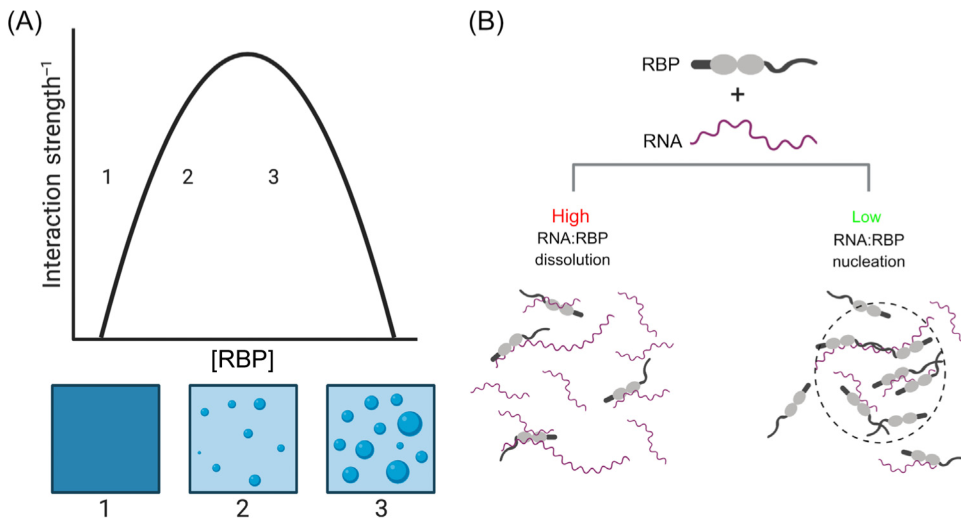

Figure 1. Model Phase Transitions. (A) A phase diagram describing homotypic phase separation. Crossing the phase boundary leads to a transition from a well-mixed solution (position 1) to a biphasic solution, with light and dense phases (position 2). Further increasing the concentration (position 3) increases the volume fraction of the dense phase, without altering the light or dense phase concentrations. (B) High ratios of RNA:RNA binding proteins (RBPs), including FUS and TDP-43, can prevent phase separation and aggregation [21,32]. Conversely, lower ratios of RNA to RBPs can lead to multiple copies of a protein bound to a single RNA, creating a high local protein concentration and facilitating intermolecular interactions between RBPs, thus nucleating a phase transition [20,139].

Functional Phase Separation

LLPS enables the switch-like condensation of biomolecules resulting in a dilute (light) phase and concentrated (dense) phase that can differ in concentration by >50-fold (Figure 1A) [16]. This switch-like behavior enables highly nonlinear changes in protein localization to arise from small changes in concentration or environmental conditions [17]. This nonlinearity could potentially poise biological systems to: (i) effect rapid and dramatic changes in the subcellular availability of particular molecules; or (ii) alter effective stoichiometry of interacting proteins via selective partitioning into condensates [17]. LLPS can be tuned by altering the interaction strength between molecules (e.g., via post-translational modification) [18,19] or by nucleating high local concentrations

Box 1. Prions versus Prion-like Domains

Prions are infectious proteins that underlie transmissible spongiform encephalopathies in mammals [119–121]. However, prions are also found in yeast, where they can confer beneficial, heritable phenotypes [119,122–126]. Proteins that form prions in yeast often harbor a prion domain, a distinctive low-complexity domain enriched in uncharged, polar residues (especially glutamine, asparagine, tyrosine, and serine) and glycine, which encodes the ability to form a prion [14,119]. PrLDs represent a specific class of disordered, low-complexity protein domains, with sequence composition reminiscent of canonical yeast prion domains [12,13,15,91,127]. In yeast, prions enable highly penetrant, protein-based epigenetic inheritance, which can be advantageous [12,119,123]. Prion proteins stochastically sample a self-templating '[*PRION*$^+$]' conformation, that is typically a stable amyloid state, which can alter gene expression and metabolic programs transgenerationally [12,119,123]. However, not all yeast prions are amyloids. Recently, [*SMAUG*$^+$] has been defined as a yeast prion that is embodied by a non-amyloid condensate [128]. Notably, condensates in human cells are rich in proteins with PrLDs, many of which undergo LLPS both *in vivo* and *in vitro*, adopting a range of material states [22,30,66,129]. Similar to the gene ontology of yeast prions, human PrLD proteins are enriched among RNA-binding proteins (RBPs), chromatin-modifying enzymes, and transcription factors; classes of proteins that can exert highly pleiotropic control of gene expression [12,14]. In another similarity to prions, there is mounting evidence to suggest that prion-like TDP-43 aggregation can spread from cell-to-cell in a seed-dependent manner in cultured cells and mouse brains, that mimics features of human disease [130–132]. Together, similarities in the sequence composition, ability to self-assemble into a range of material states, gene ontology, and aggregation propensity between yeast prion proteins and human PrLD-containing proteins, raises tantalizing possibilities that metastable control of gene expression may be driven by PrLD-mediated phase transitions to various material states in human cells [12,14,66,133,134].

Glossary

Heterotypic phase separation: a phase transition by two or more components, such as FUS and RNA, resulting in a dense phase composed of each component. Cellular condensates, such as SGs, are heterotypic.
Homotypic phase separation: a phase transition by a single component, such as purified FUS in an *in vitro* experiment. Homotypic phases are less likely to occur in cells.
Poly(ADP Ribose) (PAR): a polymer of two or more ADP-ribose units, which can regulate phase separation. PAR can seed FUS condensates at sites of DNA damage, and aids in TDP-43 recruitment to SGs.
Saturation concentration (C_{sat}): the concentration above which a solution will demix into dense and light phases. The effective C_{sat} of an RBP can be tuned by factors such as RNA, PAR, and post-translational modifications.
Spacers: the intervening residues in a protein, or nucleobases in a RNA, between the stickers. The length and flexibility of the spacers can affect the material properties of the condensate.
Stickers: amino acid residues or motifs in a protein, or nucleobases in a RNA, that engage in noncovalent interactions with other molecules to stabilize a condensate. The number and spacing of stickers can affect the material property of the condensate.
Stress granules (SGs): highly heterotypic condensates that form in the cytoplasm in response to stress. SGs are enriched in translationally arrested mRNAs and PrLD-containing RBPs, including FUS and TDP-43.

(e.g., by docking multiple molecules on a scaffold, such as RNA, or **poly(ADP-ribose)** (**PAR**)) [17,20–23] (Figure 1B). In this way, cells can quickly alter effective protein concentrations without the time and energy expenses inherent to protein synthesis or degradation. For regulatory proteins like FUS and TDP-43, this phase separation could facilitate stimulus responsive gene-expression programs. Conversely, aberrant phase separation could potentially result from alterations to the sensitivity, duration, reversibility, localization, or composition of dense phases.

FUS and TDP-43 as Case Studies

The PrLD-containing RNA-binding proteins (RBPs), FUS, and TDP-43, provide some of the most well-studied examples of RBPs that undergo LLPS. FUS and TDP-43 can form condensates with a range of material states implicated in both normal function and disease. Both RBPs are involved in RNA metabolism at multiple stages, including transcriptional regulation, pre-mRNA splicing, RNA processing, and RNA localization. Further, both are components of stress-induced RNP granules known as SGs and are found in cytoplasmic inclusions in degenerating neurons that are key pathological hallmarks of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) [13]. Cytoplasmic TDP-43 inclusions are also a key feature of limbic-predominant age-related TDP-43 encephalopathy (LATE), a newly recognized neurodegenerative disorder that mimics Alzheimer's disease [24]. FUS and TDP-43 have a modular architecture shared among a family of proteins known as heterogeneous nuclear ribonucleoproteins (hnRNPs) [13], including disordered PrLDs as well as folded RNA-recognition motifs (RRMs) and other RNA-binding domains (RBDs) such as the disordered arginine–glycine rich regions (RGG boxes) of FUS and the ordered N-terminal region of TDP-43 (Figures 2 and 3). This architecture is important; PrLDs can tune both the critical concentration required for phase separation as well as the

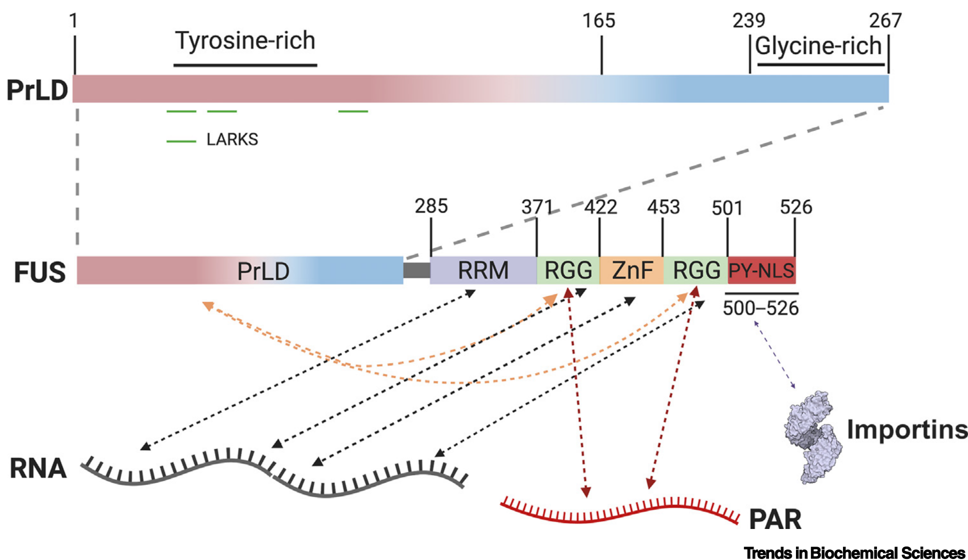

Figure 2. Interaction Interfaces Regulating FUS LLPS. Residues 1–239 of FUS harbor the prion-like domain (PrLD), which is followed by a glycine-rich region extending to residue 267, a RNA-recognition motif (RRM), two arginine-glycine rich regions (RGGs), a zinc finger (ZnF) domain, and a PY-nuclear localization signal (PY-NLS) [13]. Low complexity aromatic-rich kinked segments (LARKS) in the PrLD are capable of forming homotypic cross-β interactions [52–54]. Arginine residues in RGG domains and tyrosine residues in the PrLD can form intramolecular (orange arrows) and intermolecular interactions [13,46]. RRM, RGG, and ZnF domains, further mediate interactions between FUS and RNA molecules (black arrows) [13]. The RGG domains also interact with poly(ADP Ribose) (PAR) (red arrows) [55]. The PY-NLS interacts with importins which regulate FUS condensation (purple arrow) [39,48,57,58]. Collectively, these interactions govern FUS assembly via competition between inter- and intramolecular interactions tuned by post-translational modifications and scaffolds like RNA and PAR.

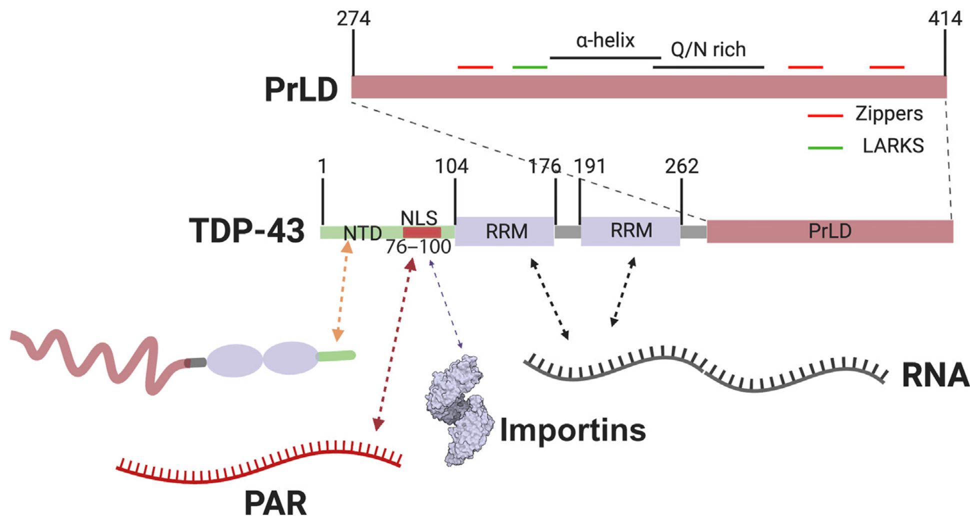

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Figure 3. Interaction Interfaces Regulating TDP-43 Liquid–Liquid Phase Separation (LLPS). TDP-43 consists of an N-terminal domain (NTD) that can form homotypic interactions (orange arrow) [18,76], and which contains a nuclear localization signal (NLS) harboring two poly(ADP Ribose) (PAR)-binding motifs (red arrow) [13,22]. The NLS also engages importins, which can regulate TDP-43 condensation (purple arrow) [39]. The NTD is followed by two RNA-recognition motif (RRM) domains and a prion-like domain (PrLD). The two RRM domains facilitate the interactions between TDP-43 and RNA molecules (black arrows), which are important for regulation of TDP-43 LLPS [32]. The PrLD itself contains various subdomains capable of forming intermolecular interactions with other copies of TDP-43 (top). These subdomains include a region with α-helical propensity, a Q/N-rich region, numerous Zippers, and low complexity aromatic-rich kinked segments (LARKS) [78,80,83].

material state of the resulting condensate, and the RBDs can interact with nucleic acids and other polymers that may serve as nucleators and regulators of condensation [20–22,25] (Figure 1B). Importantly, PrLDs enable both homotypic interactions and heterotypic interactions, giving rise to RNP granules with complex proteomes [26,27]. The relative ratios of codemixing proteins govern the collective **saturation concentration** (**C_{sat}**), 3D organization, and material state of the resulting condensate [28,29].

Evolution has likely tuned the C_{sat} of various RBPs to achieve regulated and functional condensation. For example, the respective C_{sat} for *in vitro* LLPS and estimated cellular concentrations of FUS and TDP-43, are very similar [22,30]. Evidence of more nuanced tuning of saturation conditions resulting from interactions with polymeric scaffolds, including PAR [22,31] and RNA [20,21,32,33], post-translational modifications [18,19], oligomerization state [34], and the speed at which scaffold complexes diffuse through the cell [34], all suggest ways in which FUS and TDP-43 could be poised to condense as conditions warrant. These additional layers of regulation can divorce C_{sat} from total cellular concentration of the protein, enabling local condensation or dissolution [34]. This divorce might have distinct consequences for function and dysfunction such as the trafficking of RNAs in RNP granules to regulate local translation [35]. Moreover, FUS and TDP-43 appear to be supersaturated in motor neurons (the selectively vulnerable neuron in ALS), which may make them even more prone to LLPS and pathological aggregation [36].

FUS and TDP-43 Partition into Heterotypic Condensates

C_{sat} is a straightforward concept when applied to homotypic condensates, including many of the test-tube condensates that facilitated a detailed understanding of phase behavior for PrLD proteins [30,37]. Cellular condensates, by contrast, contain a host of factors. For example, SGs contain both FUS and TDP-43 along with hundreds of proteins and diverse translationally-

arrested mRNAs [22,26,27,33,38,39]. As such, particular proteins may partition into a condensate exhibiting liquid-like properties (e.g., a spherical shape, the ability to fuse, and rapid recovery from photobleaching), while lacking a clearly defined C_{sat}. Such observations may be reconciled by the fact that the specific protein under study may not itself be the scaffold responsible for nucleating the condensate, but rather may be a client that preferentially partitions into a heterotypic phase [40]. Further, if two or more proteins 'co-scaffold' the same dense phase with one another, the C_{sat} for such condensates would be a function of the concentration of each protein and the stoichiometric ratios between the two. Recent work explores this concept via both theory and experiment [28,29]. The nucleolus forms via heterotypic LLPS in a manner that depends on the concentrations of and interactions between multiple protein and RNA components [29], and this behavior can be modeled for other condensates [28,29]. Together, these studies elegantly demonstrate the intuitive concept that heterotypic condensates arise from heterogeneous interactions [41,42]. Importantly, these studies dispel the notion that individual proteins that undergo LLPS in cells must display a fixed C_{sat} [43]. This concept is likely to be of central importance to understanding: (i) how different cell types with varied gene-expression programs form and regulate condensates; and (ii) how changes in the relative ratios of co-condensing proteins could sensitize certain cell types to aberrant phase transitions (Box 2) and disease.

Molecular Grammar of FUS LLPS

Both FUS and TDP-43 mislocalize to the cytoplasm and aggregate in certain forms of ALS and FTD, yet they do so in a mutually-exclusive manner [44]. Thus, ALS and FTD cases with FUS inclusions lack TDP-43 inclusions, and vice versa [45]. Relatedly, the 'molecular grammar' linking primary sequence to condensation has been dissected for both FUS and TDP-43, revealing distinct rules of engagement imparted by different sequence features (Figures 2 and 3). The FUS PrLD renders FUS intrinsically aggregation-prone and in isolation can undergo LLPS driven by tyrosine–tyrosine interactions, albeit at concentrations orders of magnitude higher than the full-length protein [21,46,47]. Notably, *in vitro* this synergy persists even *in trans*, when the PrLD and

Box 2. Defining Aberrant Phase Transitions Beyond Aggregation

Proposed roles for phase transitions as organizers and drivers of biochemical reactions, concentration buffers, and a means by which the cell can compute responses to changing environmental conditions, motivate us to consider how aberrant phase separation may manifest itself as a function of changes in C_{sat} that go beyond simple hastening of solidification [17]. Mutations that constitutively lower the C_{sat} of a phase-separating protein would reduce the light phase concentration of the protein, and correspondingly increase the proportion of total protein found in the dense phase. If the functional phase of the protein in a given cellular context were the light phase, the result could be loss-of-function. Conversely, if a given function took place in or was otherwise facilitated by the dense phase, this shift could result in a potentially adverse gain-of-function. A phase-separating protein with distinct functions in both the light and dense phase further expands the potential definition of aberrant LLPS, as distinct light and dense phase functionalities could become unbalanced as a result of mutations altering C_{sat}. Imbalanced function of proteins exerting highly pleiotropic control of gene expression, such as TDP-43 and FUS, could in turn elicit systemic dysfunction resulting from global changes in gene expression, a maladaptive version of prion behavior observed in yeast [128,135].

If a protein is only functioned in a given phase, biological decision-making could be altered through alterations in C_{sat}. If only the light phase were functional, environmentally-triggered LLPS would result in marked reductions in the available pool of functional protein via titration into the dense phase. This temporarily reduced functionality could facilitate a particular reversible response. Here, mutations increasing C_{sat} could prevent or delay the response, while mutations lowering C_{sat} could render cells environmentally hypersensitive, invoking costly transitions to gene regulatory programs. Two recent studies demonstrating increased enzymatic activity as a function of liquid–liquid and liquid-to-solid transitions, lend credence to the notion that alterations in C_{sat} could adversely impact cellular health [136,137]. In these examples, a reduction in C_{sat} could lead to increased condensation, with a corresponding gain-of-function. Conversely, the loss of liquid and solid phases in these instances could lead to a loss-of-function. With the exception of an extremely cooperative process of aggregation, where nearly all available material enters the aggregated phase, the definitions of aberrant phase transitions resulting from alterations in C_{sat} offered here are largely divorced from the material state of the dense phase. Thus, these definitions are fully compatible with the possibility not just of pathology via accelerated gelation or solidification, but the existence of toxic liquids [65,96].

RGGs are added as separate recombinant proteins [46]. The PrLD of FUS contains multiple tyrosines, which form π–π and cation–π interactions with arginine residues found outside the PrLD in RGGs [46,48]. In addition to enabling interdomain contacts within and between FUS molecules, RGG domains also function as RNA-interaction motifs with degenerate specificity [49,50]. RNA itself regulates FUS assembly, acting as a nucleator at lower RNA:FUS ratios, while antagonizing LLPS at higher RNA:FUS ratios [20,21,51].

Together, interdomain contacts within and between FUS molecules, and between FUS and RNA, create a multitude of possible points of regulation of FUS LLPS and the material state of the resulting condensates. RNA–RGG interactions could obscure RGG arginines from PrLD tyrosines, thus causing the PrLD to interact with the RBD of other FUS molecules not bound to RNA (Figure 2). Certain FUS:RNA ratios could drive multimerization of FUS along a single RNA (Figure 1B) [20]. Multiple copies of FUS bound to a single RNA via the RRM or zinc finger domains would create a high local concentration that could nucleate PrLD–PrLD interactions, such as those observed in FUS fragments that form kinked cross-β polymers [20,52]. Among such cross-β forming stretches are members of a class of motifs dubbed 'low complexity aromatic-rich kinked segments' (LARKS), which may assemble the core of some FUS fibril polymorphs [52–54]. Conversely, less dense FUS nucleation could disfavor proximity-driven PrLD interactions, instead favoring tyrosine–arginine driven RGG–PrLD interactions (Figure 2). Similar regulation could be achieved via RGG domain interactions with PAR, and PAR has been shown to nucleate FUS condensates at sites of DNA damage [55,56]. Likewise, the interspersal of other RBPs along a nucleating RNA or PAR and in the surrounding liquid may all compete with FUS–FUS interactions [21,46,51]. In these ways and others, the structure of bound RNA, and the relative density and spacing of motifs recognized by FUS and other RBPs, could alter the properties of the resulting condensates.

Regulation of FUS LLPS

The ability of FUS to make interdomain contacts *in cis* and *trans,* involving RGG domains that are also capable of interacting with RNA, suggests that FUS LLPS has multiple modes of potential regulation. Recently, multiple groups reported that the nuclear-import receptor, Karyopherin-β2 (Kapβ2), also chaperones FUS phase transitions, preventing [39,48,57–59] and even reversing [39,57,60] FUS LLPS, gelation, and fibrilization. Kapβ2 binds primarily to the PY-nuclear localization signal (PY-NLS) of FUS, which then enables extensive additional contacts across FUS, which effectively disrupts FUS–FUS interactions [39,57,58]. Post-translational modifications of FUS can also regulate both interdomain contacts and binding to Kapβ2, which in turn regulate FUS LLPS [58]. One such example is phosphorylation of the critical tyrosine of the FUS PY-NLS, which can alter the association of FUS and Kapβ2, and in turn regulate nucleocytoplasmic partitioning [61].

Related to the important role of RGG domains in FUS LLPS, post-translational modifications to arginine residues are also important regulators of FUS condensation. Arginine methylation of FUS reduces condensation and SG association [58]. Conversely, FUS hypomethylation, a molecular phenotype of FUS inclusions in FTD, drives FUS gelation to more stable cross-β structures [48]. In addition to arginine methylation, a number of other FUS post-translational modifications have been cataloged and implicated in tuning LLPS [62]. The role of RNA as a modulator of FUS LLPS may itself be tuned by tyrosine phosphorylation of the FUS RRM, which has been identified as a site of phosphorylation [62,63]. Aside from the RRM, phosphorylation of the PrLD of FUS has also been observed to antagonize phase separation and aggregation [19,64].

Molecular Grammar of TDP-43 LLPS

Like FUS, TDP-43 also undergoes LLPS *in vitro* at near physiological concentrations, though it may require the addition of a crowding agent such as dextran [18,22,32]. It is a component of

cellular condensates with a range of material states, including liquid-like SGs [26,27], various nuclear foci [65], and amyloid-like myogranules in muscle [66]. TDP-43 is also a component of nuclear RNP granules including paraspeckles [67] and nuclear stress bodies [68], which both contain specific, stress-induced, noncoding RNAs (ncRNAs). *In vitro*, full-length TDP-43 undergoes LLPS at physiological concentrations [22,32], whereas the C-terminal fragments harboring the PrLD tend to aggregate [22,69,70]. Similarly, cytoplasmic aggregates containing TDP-43, and TDP-43 fragments retaining the C-terminal PrLD, are a pathological hallmark of ALS and FTD [71], and the vast majority of TDP-43 mutations associated with ALS are found in the PrLD [13,72]. The PrLD is also required for TDP-43 recruitment to SGs [73], which are enriched in numerous disease-associated, aggregation-prone RBPs [26,27]. Notably, C-terminal fragments of TDP-43 lack the N-terminal PAR-binding motifs, which are important for both LLPS and SG recruitment [22].

It has been proposed that dysregulated TDP-43 LLPS may cause or promote TDP-43 aggregation, which can be toxic [32]. For this reason, the molecular determinants of TDP-43 LLPS are an area of intense study, and like FUS, multiple regions of TDP-43 beyond the PrLD are implicated in its self-assembly. However, likely owing to sequence differences in the PrLD, TDP-43 does not appear to obey the same molecular grammar rules for LLPS deduced for other PrLD-containing RBPs, including FUS [32,46] and hnRNAPA1 [74]. By contrast, TDP-43 lacks extensive RGG domains and thus has a lesser capacity for RGG–RNA interactions and intramolecular cation–π interactions between RGG domain arginines and tyrosines in the PrLD.

TDP-43 relies on multiple intermolecular contacts facilitated by distinct and nonmutually exclusive regions in order to condense. The structured N-terminal domain (NTD) of TDP-43 is capable of forming intermolecular interactions [18] and multiple reports suggest NTD dimerization is important for the role of TDP-43 in splicing regulation [75,76]. The role of TDP-43 LLPS in splicing regulation is beginning to be explored and recent work suggests TDP-43 is capable of regulating the splicing of at least some transcripts independent of phase separation [77], but in other cases splicing is enhanced by TDP-43 LLPS [78]. NTD–NTD interactions resulting in head-to-tail TDP-43 oligomers antagonize pathologic fibrillization of TDP-43 PrLDs [76] (Figure 3). Other NTD–NTD interactions, or nucleation along RNA polymers, may instead result in the close apposition of PrLDs, facilitating specific intermolecular interactions, including those that drive LLPS (Figure 3).

Within the TDP-43 PrLD is a conserved stretch of amino acids that has been shown to form a transient α-helix amidst an otherwise disordered region [79–81]. This region is important for LLPS in the context of the isolated PrLD [80]. Notably, the helical propensity of this region increases in the context of TDP-43–TDP-43 interactions. Mutations in the helix are associated with ALS and contribute to a liquid-to-solid transition *in vitro* [80]. Together, these observations implicate transient structure in TDP-43 LLPS, and suggest that cellular mechanisms capable of altering the TDP-43 structural ensemble could in turn alter its phase behavior. More recently, methionine residues in a region overlapping the conserved α-helical subdomain have been implicated in LLPS and gelation via cross-β polymerization, tuned by the oxidation state of the methionine residues [81]. Thus, the helical region may enable different interactions with a spectrum of stabilities, which may relate to the range of material states adopted by various TDP-43 condensates.

Mutagenesis of TDP-43 has revealed residues in addition to those in the α-helical region that are important for LLPS, which has enabled elucidation of general rules. For example, aromatic residues adjacent to glycines or serines contribute to TDP-43 LLPS *in vitro* [82], and the spacing

of hydrophobic 'sticker' residues interspersed by flexible linkers have also proven important [77]. This feature is likely applicable to other PrLD-containing systems [11]. The PrLD also harbors short stretches of amino acids that in isolation are capable of ordered oligomerization *in vitro,* including LARKS. The structure of the TDP-43 LARK oligomer has been determined using X-ray diffraction and microcrystal electron diffraction. These structures reveal more labile β-sheets than those adopted by classical cross-β forming prion proteins, as well as motifs forming more stable steric zippers [83]. The range of stabilities impacted by LARKS and steric zippers could in theory relate to the range of material states adopted by TDP-43 in cells. Notably, some ALS-linked mutations disrupt the formation of these kinked structures, motivating future studies aimed at determining the extent to which these structures form in the context of full-length TDP-43 [83]. Together, emerging evidence supports a model whereby different TDP-43–TDP-43 interactions spanning the structured NTD to the disordered C-terminal PrLD, collectively modulate the self-assembly of TDP-43.

Regulation of TDP-43 LLPS

The possibility of multiple intermolecular interactions across TDP-43 likely enables regulation. Phosphorylation of multiple residues in the PrLD is associated with TDP-43 aggregation in diseased neurons [84]. Conversely, phosphomimetic mutations in the folded N-terminal domain of TDP-43 disrupt LLPS and reduce the viscosity of droplets *in vitro* [18]. The extent to which TDP-43 phosphorylation in diseased neurons represents a pathologic mechanism or a failed effort by the cell to dissolve TDP-43 condensates remains to be elucidated. Experiments where TDP-43 is phosphorylated *in vitro*, prior to and after initiation of phase separation and aggregation, could aid in determining the contributions of PrLD phosphorylation to TDP-43 condensation. It is also possible that different phosphorylation sites on TDP-43 exert differential effects on condensation. TDP-43 is also acetylated on lysines in the RRMs [85]. Acetylation is associated with cell stress and leads to reduced RNA binding, which in turn may contribute to cytoplasmic localization, hyperphosphorylation, and insoluble inclusion formation [85,86]. As such, acetylation could be an upstream step in the development of TDP-43 pathology that may be targeted by drugs.

The oligomerization state of TDP-43 can regulate the onset of LLPS and aggregation. The N-terminal domain provides an oligomerization interface, which along with recruitment to RNA via the RRMs of TDP-43, could enable nucleation of TDP-43 condensation or aggregation via regulated or dysregulated complex formation [18]. To model the effects of TDP-43 nucleation on cell survival, Donnelly and colleagues employed a light-inducible oligomerization system that has been successfully used to study LLPS of the FUS IDR [34] and SG formation [87] by fusing the light-inducible oligomerization domain, Cry2, to full-length TDP-43 [32]. This approach yielded a construct that rapidly coalesced in response to light stimulation [32]. This 'opto-TDP-43' system recapitulates important features of ALS and FTD pathology, including the formation of cytoplasmic TDP-43 inclusions that kill neurons [32]. Notably, aggregation was inhibited by the addition of specific RNAs recognized by TDP-43, which the authors dubbed 'bait RNAs' [32] (Box 3), consistent with prior studies indicating that RNA buffers the phase separation of PrLD-containing proteins, including FUS [21,51].

More recent work has also implicated specific post-translational modifications in regulating the assembly of TDP-43, resulting in co-occurring liquid and liquid-crystalline TDP-43 phases [86], in contrast to the polymers proposed for myogranules or redox-sensitive RNA-transport granules [81]. In this work, RRM acetylation, known to reduce RNA binding [85], causes RNA-depleted TDP-43 condensates to form. However, these condensates are layered, containing a more ordered liquid-crystalline shell that encompasses a liquid core [86]. The shell forms from

Box 3. 'Bait RNAs' as a Potential New Therapeutic Modality

The role of RNAs in regulating PrLD-containing RBP phase separation, and recent success delivering short RNA drugs to the central nervous system (CNS) to target neurodegenerative disease, motivate efforts to design oligo therapeutics [138]. Donnelly and colleagues have shown that a short bait RNA that binds TDP-43 prevents aggregation when delivered to cultured cells [32]. Notably, both toxic gain-of-function of FUS and TDP-43 aggregates, and loss-of-function due to sequestration within aggregates, are potential mechanisms of pathology in ALS and FTD. For this reason, bait RNAs represent compelling therapeutic candidates. In contrast to antisense oligonucleotides that reduce protein expression, the bait RNA approach modulates protein solubility through reversible binding, not reductions in cellular protein concentration. Targeting one mRNA using antisense oligos can prevent the expression of multiple proteins. Thus, a potential challenge to a bait RNA development is the requirement that they be delivered in sufficient quantity, or be sufficiently potent, to abrogate aggregation. However, bait RNAs could function catalytically in collaboration with cellular factors including nuclear-import receptors, which have been shown to cause FUS to release RNA, enabling bait RNAs to be recycled for further rounds of activity [57,58].

acetylated TDP-43, or when a RNA-binding defective mutant TDP-43 is expressed, which recruits wild-type TDP-43 [86]. The liquidity of the core is maintained by various members of the Hsp70 family of ATP-dependent chaperones [86]. This work reveals the complexity of TDP-43 LLPS regulation, with post-translational modifications regulating RNA binding, which in turn regulates self-assembly that is further regulated by protein chaperones. In the cytoplasm, nuclear-import receptors that engage the TDP-43 NLS, Importin-α/β, can prevent and reverse TDP-43 aggregation [39,88,89]. The range of material states adopted by TDP-43- liquid, liquid-crystalline, labile cross-β, amyloid-fibrillar, and amorphous aggregates, represents the diversity of mesoscale assemblies PrLD proteins can adopt and motivates a reconsideration of an early paradigm at the intersection of the LLPS and neurodegeneration fields, (i.e., that solids are 'bad' for cells while liquids are 'good').

Good Liquids and Bad Solids?

In vitro, condensates of PrLD-containing RBPs mature over time into less dynamic gels, fibers, and aggregates, and this liquid-to-solid transition is hastened by ALS- and FTD-associated mutations [30,32,37,70,90,91]. A compelling paradigm has emerged that posits liquid-like condensates are functional, or at least nonpathogenic, and solid aggregates are a toxic endpoint of aberrant phase transitions. However, recent work challenges the exclusivity of this premise while providing preliminary evidence for pathogenic liquid phases (Box 2).

In human cells, including induced pluripotent stem cell (iPSC) derived neurons, chronic phase separation of TDP-43 in the cytoplasm is toxic, even without a transition to amorphous aggregates or fibrils [65]. High-throughput screens in simpler models suggest similar routes to toxicity. The single cell brewer's yeast, *Saccharomyces cerevisiae*, lacks direct orthologs of TDP-43 and FUS, but ectopic expression of these genes recapitulates many salient features of human pathology, including cytoplasmic mislocalization, aggregation, and cytotoxicity [47,70]. These features have been leveraged in screens identifying disease modifiers [47,92] and therapeutic protein disaggregases [93–95]. Deep mutational scanning of the PrLD of TDP-43 in yeast, revealed thousands of genetic variants that modified TDP-43 toxicity [96]. Curiously, mutations predicted to increase aggregation propensity yielded larger cytoplasmic aggregates but reduced toxicity. Conversely, some mutations predicted to disfavor aggregation, generated smaller more dynamic TDP-43 assemblies with fluorescence recovery after photobleaching (FRAP) kinetics consistent with liquidity, but actually increased toxicity [96]. A detailed assessment of the *in vitro* propensity of disease-linked TDP-43 variants to phase-separate into liquid versus solid assemblies, juxtaposed with follow-up studies in newly developed cell-based assays that report on toxicity [32,65], could shed light on the prevalence of toxic liquid phases of TDP-43.

SGs: Protective, Problematic, or Both?

Another important question, as it pertains to mechanisms of FUS and TDP-43 toxicity, involves the role of SGs [15,41,42,87,97,98]. The overlap between ALS- and FTD-associated proteins and their local enrichment in SGs, led to the logical hypothesis that SGs may be crucibles of cytoplasmic PrLD aggregation that is the hallmark of these diseases [15]. However, it has also emerged that toxic TDP-43 aggregation can arise independently of SGs, and the RNA and PAR environment in SGs may be protective [22,32,65,99–101]. However, misregulated SG dynamics may still contribute to toxic aggregation. One possibility is that incomplete SG dissolution after the cessation of stress, results in small aggregates capable of nucleating further aggregation outside the previously protective environment of the SG [22]. If such nuclei were below the diffraction limit, they could escape detection upon SG dissolution, only to reappear later after a critical size was reached [102]. Indeed, using a light-induced oligomerization domain fused to the SG nucleating protein, G3BP1, Taylor and colleagues found that repetitive or persistent SG formation directly leads to TDP-43 aggregates [87]. Notably, like the light-driven TDP-43 aggregates formed independently of SGs by Donnelly and colleagues, these condensates contained C-terminal TDP-43 fragments and TDP-43 phosphorylated at serines 409 and 410, both hallmarks of pathological TDP-43 inclusions found in ALS and FTD patients [32,84,87]. Collectively, these studies suggest there are likely to be multiple routes to TDP-43 proteinopathy, with or without SGs as an intermediate [22,100]. It will be important to determine if interventions targeting TDP-43, such as RNA oligonucleotide therapies [32] or protein disaggregases [39,93-95,103–105], can target multiple paths to TDP-43 dysfunction. Additionally, TDP-43 is a component of other RNP granules, including NEAT1-nucleated paraspeckles [67], nuclear stress bodies [68], and transport granules in neuronal cells [90,106], raising the possibility that misregulation of other RNP granules could be the culprit in nucleating toxic PrLD aggregation. Dissecting the relationship between RNP granules and neurodegeneration will require improved understanding of granule dissolution [107,108], or as a failsafe, degradation [109], in addition to ongoing efforts to understand nucleation and to catalog constituent RNAs and proteins.

Drugging LLPS

C_{sat} is partly a function of interaction strength which is itself a function of the solvent environment. A variety of compounds have been shown to dissolve membraneless organelles in cells and liquid droplets or hydrogels *in vitro*. The aliphatic alcohol, 1,6-hexanediol, was first observed to dissolve the nuclear pore, a membraneless organelle, and this observation was later extended to a multitude of RNP granules and *in vitro* condensates [110–112]. More recently, cellular concentrations of ATP, the crucial cellular metabolite, were shown to prevent protein phase separation *in vitro*, leading to the hypothesis that ATP acts as a 'hydrotrope' in cells to create a solvent favoring solubility [113]. Remarkably, the nontoxic and bioavailable molecules lipoic acid and lipoamide can modulate FUS LLPS *in vitro* and antagonize FUS LLPS in cells, which could in turn reduce FUS aggregation [114]. Likewise, tool compounds have emerged to combat TDP-43 LLPS and aggregation by binding interfaces important for self-assembly [115–117]. Compound screens have also revealed drugs that reduce SG assembly, including in motor neurons derived from iPSCs from patients harboring ALS-associated mutations [33]. One hit from this screen, mitoxantrone, was able to increase nuclear localization, and decrease SG accumulation, for a mutant form of TDP-43 lacking an NLS [33]. Together, these studies suggest that small-molecules could target PrLD LLPS, despite longstanding challenges inherent to drugging IDRs.

Our broadened definition of aberrant phase separation (Box 2) expands our imagination with respect to therapeutic interventions beyond dissolving condensates. Cells are revealing ways in which C_{sat} is modulated through alterations in the valency of polymeric scaffolds [20–22,56] and post-translational modifications [18,48,85,86], and the concerted 'co-scaffolding' of dense

phases [28,29,41]. Each suggests strategies for pharmacological intervention. In cases where condensation may alter function, partial modulation of enzymes that alter C_{sat} through post-translational modifications or polymeric scaffolds, could conceivably effect highly nonlinear changes in cell behavior. Instead of targeting the disordered PrLDs central to aberrant LLPS directly, focusing on upstream enzymatic modifiers of LLPS provides targets such as folded active sites, which are often effectively targeted by existing classes of drugs. Could targeting enzymes that tune C_{sat}, leverage small changes in enzyme output to exert highly nonlinear changes in phase behavior, while avoiding pleiotropic and off-target effects associated with more complete enzymatic inhibition? Recent work shows that some anticancer drugs selectively partition into condensates, in which their targets reside in a manner that appears independent from binding the drug target itself [118]. The extent to which small-molecule drugs may specifically partition into discrete condensates in cells, and how such partitioning impacts efficacy, are likely to be areas of considerable interest. A crucial question remains whether deleterious phases, including aggregates, can be reversed by small-molecule drugs, or whether proteins must be targeted prior to undergoing an aberrant phase transition. An orthogonal approach to drugging condensates involves mimicking their cellular regulation, including novel RNA therapeutic approaches (Box 3).

Concluding Remarks

FUS and TDP-43 are archetypes for PrLD-mediated self-assembly into condensates with a range of material properties. Further study of these proteins is likely to continue to uncover new biology that extends to other PrLD RBPs (see Outstanding Questions). Despite their similarities, they obey a distinct molecular grammar, are subject to distinct modes of regulation, and aggregate in distinct forms of disease. Thus, they suggest the complexity of cellular condensates that could be formed from the diverse collection of PrLD-containing proteins in the human proteome [12,14,15]. Together, they point towards the exploration of altered RNP granule dynamics as a biological framework to explore new therapeutic approaches for fatal neurodegenerative diseases. Systems-scale integration of proteomic, transcriptomic, imaging, genetic, and epidemiologic data will be required to inform hypotheses and guide drug-screening strategies to combat these diseases.

Outstanding Questions

FUS and TDP-43 can condense into assemblies with a range of material states. How does the position of a condensate, on a continuum of liquid to aggregate, relate to function in various cellular contexts?

Which phase or phases, light or dense, represent the functional phase for FUS and TDP-43 for a given process? Does the functional phase vary between cell types?

What role do altered SG dynamics play in neurodegenerative disease onset?

What proteins or pathways are involved in RNP granule dissolution or degradation?

Do sequence and structural features of specific RNAs result in the nucleation of functional and materially distinct condensates?

Does modulating phase separation represent a viable therapeutic strategy for fatal neurodegenerative diseases involving PrLD aggregation?

Acknowledgments

We thank Hana Odeh, Katie Copley, and Charlotte Fare for helpful feedback on the manuscript. B.P. is supported by the BrightFocus Foundation Postdoctoral Fellowship A2019612F. J.S. is supported by grants from Target ALS (USA), ALSA, the Office of the Assistant Secretary of Defense for Health Affairs (USA), through the Amyotrophic Lateral Sclerosis Research Program (W81XWH-20-1-0242), the G. Harold & Leila Y. Mathers Foundation (USA), and National Institutes of Health (NIH) (R01GM099836, R21AG061784, and R21AG065854). J.S. is a consultant for Dewpoint Therapeutics and Maze Therapeutics.

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