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Review

Phase Separation and Neurodegenerative Diseases: A Disturbance in the Force

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SUMMARY

Protein aggregation is the main hallmark of neurodegenerative diseases. Many proteins found in pathological inclusions are known to undergo liquid-liquid phase separation, a reversible process of molecular self-assembly. Emerging evidence supports the hypothesis that aberrant phase separation behavior may serve as a trigger of protein aggregation in neurodegeneration, and efforts to understand and control the underlying mechanisms are underway. Here, we review similarities and differences among four main proteins, α-synuclein, FUS, tau, and TDP-43, which are found aggregated in different diseases and were independently shown to phase separate. We discuss future directions in the field that will help shed light on the molecular mechanisms of aggregation and neurodegeneration.

THE FORCE AWAKENS: AN INTRODUCTION INTO THE FIELD OF LLPS

In a neuronal system far, far away… Protein aggregates are villains of the neurodegeneration Empire, raiding brains and killing neurons. Evading the dreaded aggregation, the phase separation rebellion is a reversible process of molecular self-assembly fighting for free movement and exchange of molecules, allowing neurons to live long and prosper. However, members of the phase separation rebellion, such as α-synuclein, FUS, tau, and TDP-43 are found in these pathological aggregates. Turmoil has engulfed the scientific republic when a growing number of voices hypothesized that some members of the rebellion may succumb to aberrant phase separation behavior and serve the dark aggregation process of the Empire…

The discovery of the broad biological role of liquid-liquid phase separation (LLPS; Table 1) as a true Force connecting most aspects of cellular biology, raised many voices and controversies in the scientific "republic" over the past decade. LLPS is the reversible unmixing of two macromolecular solutions, creating two separate phases in constant exchange with one another: the dilute phase and, contained within it, the condensed phase (Alberti et al., 2019; Boeynaems et al., 2018). Phase transition is driven by the minimization of the global free energy of the macromolecular solution, which is achieved by maximizing weak inter- and intramolecular interactions between the constituting macromolecules. This phenomenon only occurs at a certain solubilization limit, known as the saturation concentration, which is characteristic for each phase-separating system (Alberti et al., 2019). Whether a system undergoes LLPS depends not only on the molecular identity and solution concentration of the biopolymer (Table 1) but also on diverse environmental variables, such as temperature (Cinar et al., 2019), salt type and concentration (Wolf et al., 2014), co-solutes (Brangwynne et al., 2009; Hayes et al., 2018), and pH (Adame-Arana et al., 2019; Ruff et al., 2018). However, the ability to undergo LLPS has been suggested to be a common property of all biopolymers under specific conditions, regardless of their sequence (Alberti et al., 2019; Banani et al., 2017).

In recent years, LLPS has been proposed to explain the formation and dynamic behavior of a variety of cellular membraneless compartments, formed by proteins binding to nucleic acids or other polypeptides acting like polymeric scaffolds (Table 1) (Banani et al., 2016). Some of these structures, like the multiple subcompartments within the nucleus, are common to all cells. The nuclear speckles, paraspeckles, nucleoli, or Cajal bodies are examples of such nuclear structures, some of which were described as early as the 19th century (Cajal, 1903; Wagner, 1835). However, LLPS-driven membraneless compartments are not exclusively nuclear. The processing bodies (P-bodies) (Sheth and Parker, 2003) or the stress granules (SGs) (Kedersha et al., 1999), composed by pools of translation-stalled mRNAs, are formed in the cytoplasm. Highly specialized condensates are actually specific to some cell types. For example, neurons present membraneless compartments that are unique to the synapses (Wu et al., 2019a; Zeng et al., 2018) and axonal compartments (Liao et al., 2019).

Most of these functional substructures share certain features such as a membraneless spherical shape defined by surface tension, a dynamic behavior, and an assembly mechanism (Alberti et al., 2019; Brangwynne et al., 2009, 2011), while they differ in composition, subcellular localization, and function (Decker and Parker, 2012). They are usually referred to as droplets, speckles, granules, bodies, compartments, foci, densities, puncta, or clusters. However, due to their ability to highly concentrate and condense specific assemblies of biomolecules in discrete cellular sites, an accepted overarching term is "biomolecular condensate" (Table 1) (Banani et al., 2017; Choi et al., 2020). Biomolecular condensates can influence a myriad of biochemical concepts including reaction kinetics (Kuznetsova et al., 2015;

Table 1. Glossary

Biomolecular condensate	Membraneless cellular compartment concentrating a certain set of multivalent biopolymers, formed through LLPS (Banani et al., 2017; Brangwynne et al., 2009, 2011). Despite presenting different localization, composition, and functionality, they share similar morphology, dynamics, and assembly processes (Banani et al., 2017).
Biopolymer	Natural macromolecules composed of repeated monomeric units linked by covalent bonds produced by living organisms from cellular matter. There are three classes of biopolymers as defined by their monomeric unit and structure: polypeptides, polynucleotides, and polysaccharides (Choi et al., 2020).
Complex coacervation	Process involving interactions among oppositely charged polyions, which leads to phase separation (Aumiller and Keating, 2016; Overbeek and Voorn, 1957; Pak et al., 2016). In biological systems, complex coacervation is known to occur between proteins with highly opposed net charges (Bhopatkar et al., 2020; Pak et al., 2016) or positively charged proteins and nucleic acids (Aumiller and Keating, 2016; Banerjee et al., 2017; Lin et al., 2019; Zhang et al., 2017). Phase separation of the oppositely charged polyions is mainly driven by electrostatic interactions that lower the net charge and result in a polymer-rich dense phase (Veis, 2011). Nevertheless, hydrophobic and dipole interactions, as well as nucleic acid conformation, also play a role in this mechanism (Timilsena et al., 2019), by generating additional interactions.
IDR	Polypeptide segment that is unlikely to adopt a defined, fixed 3D structural conformation, as opposed to structured domains. As such, they are characterized by protein-folding energy landscapes lacking the well-defined, funnel-shaped energy minimum of folded domains. They instead present a hilly plateau, resulting from their varied set of possible conformations. IDRs generally lack bulky hydrophobic amino acids that allow building well-organized hydrophobic cores, and their functions, if described, thus arise through different interactions and dynamics (Uversky, 2013). IDRs are involved in various interactions with partners of all types in many biological processes like molecular recognition, cell-cycle control, and signaling (Piovesan et al., 2017; Vucetic et al., 2007; Wright and Dyson, 2015). They are highly abundant in the proteome and found in all domains of life (Uversky, 2010; Xue et al., 2012).
LLPS	Reversible process by which a solution of biopolymers (proteins or nucleic acids) separates into two coexisting phases: a condensate phase with liquid-like properties, and a dilute phase. LLPS is mediated by weak intermolecular interactions in conditions where biopolymer-biopolymer and solvent-solvent interactions are energetically favored over biopolymer-solvent interactions. Noncovalent interactions act like physical crosslinks building a biopolymer network with varied material properties ranging from liquid to solid behavior (Alberti et al., 2019; Boeynaems et al., 2018; Choi et al., 2020).
LCR	Polypeptide segments with a biased amino acid frequency distribution as compared with the amino acid proportions found in the proteome, and mostly predicted to structurally be IDRs (Mier et al., 2020).
PrLD	Subcategory of LCRs enriched in glycines and uncharged polar amino acids (Ser, Tyr, Gln, and Asn) and showing sequence similarities to yeast prion proteins (Alberti et al., 2009). PrLDs are often found in aggregation-prone RBPs associated with neurodegenerative disorders, such as TDP-43 and FUS (Alberti et al., 2009; King et al., 2012).
Neurodegenerative diseases	Incurable, age-dependent diseases presenting irreversible and progressive loss of selective neuronal populations. Neurodegenerative diseases are diverse in pathobiology, causing a diversity of impairments including memory loss, cognitive changes, and loss of mobility, speech, or breathing (Gitler et al., 2017). Neurodegenerative diseases are classified based on clinical presentation, anatomic distribution, or molecular abnormalities. Despite their diversity, they are believed to share key pathological processes leading to neuronal dysfunction and death (Dugger and Dickson, 2017).
Nuclear import receptors	Specialized proteins that bind the NLS of cargo proteins in the cytoplasm in order to transport them into the nucleus through the nuclear pore complex (NPC).
Nuclear pore complex	Group of proteins spanning the double nuclear membrane thereby forming channels (pores) that enable communication between the nucleoplasm and the cytoplasm, mostly focused in the transport of biopolymers like proteins and RNA. In recent years, impaired nucleocytoplasmic active transport and passive diffusion due to alterations in the NPC and the nuclear membrane integrity has emerged as a key feature of a wide range of neurodegenerative diseases, such as ALS, FTD, or Huntington's disease (Kim and Taylor, 2017; Li and Lagier-Tourenne, 2018).
Oligomerization	Specific, reversible, noncovalent intermolecular interactions of monomeric subunits resulting in a macromolecular complex. As opposed to polymers (such as actin and tubulin), oligomers have a defined number of monomers per assembly. Oligomers differ from biomolecular condensates in size, stoichiometric restriction, and temporal dynamics (Banani et al., 2017). In contrast to pathological oligomerization, which refers to the association of misfolded species forming precursors of large pathological assemblies, physiological oligomerization involves well-folded native domains (Mizuno and Kawahara, 2013) that are often important for protein functionality. Pathological oligomers can also differ from each other in size, β sheet content and exposed hydrophobicity (Bengoa-Vergniory et al., 2017). The distinct roles of functional and pathological oligomers in disease are often muddled and better distinction of the species is required going forward. This necessitates better definition of the structural and functional features of the oligomers, which is often missing.
Pathological assemblies or aggregates	The terms "aggregates" and "pathological assemblies" are often used interchangeably in the literature. Pathological proteinaceous assemblies are hallmarks of protein conformation diseases (or proteinopathies), which include neurodegenerative diseases (Westermark et al., 2002). Pathological assemblies are formed by a still unclear molecular

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Table 1. *Continued*

	mechanism that involves the self-assembly of misfolded protein species, which assemble into insoluble protein aggregates that may be amorphous, filamentous, or amyloid like (Dobson, 2017). The different disease-associated assemblies contain, predominantly, but not exclusively, a specific protein, which is unique to the disorder or a subtype thereof (Grundke-Iqbal et al., 1986a; Neumann et al., 2006, 2011; Spillantini et al., 1997), and may present an abnormal pattern of posttranslational modifications (Tables S2–S5) and other alterations, such as disulfide bridges (Cohen et al., 2012; Furukawa et al., 2006, 2011). Several neurodegenerative-disease-associated proteins were shown to self-assemble into soluble oligomers that may, in some cases, be intermediate species of larger aggregates (Cremades et al., 2017; French et al., 2019; Lashuel et al., 2002; Mann et al., 2019). The different types of assemblies, fibrils, oligomers, and aggregates are thought to arise from different mechanisms and to result from distinctive triggers (Walker, 2016). However, how and under which conditions different pathological assemblies are formed remains to be elucidated.
Scaffolds and clients	Scaffolds are believed to be the constitutive components of multicomponent biomolecular condensates, essential for condensate assembly, and central for the composition of biomolecular condensates (Decker and Parker, 2012; Kedersha et al., 1999; Yang et al., 2020). In contrast, the so-called "clients" are the transiently recruited factors that bind to scaffolds in order to integrate into the biomolecular condensate (Ishov et al., 1999; Banani et al., 2016). Recent studies, however, have challenged this view, by showing that the stoichiometry of specific interaction nodes on biopolymers rather than the molecules per se are central to the role in condensate assembly and composition of multicomponent condensates (Guillén-Boixet et al., 2020; Sanders et al., 2020; Yang et al., 2020).
Stickers and spacers	Stickers are defined as biopolymer elements that preferentially interact with each other rather than with the solvent or with the so-called spacers (non-sticker biopolymer regions) (Choi et al., 2020). Stickers form reversible physical crosslinks through noncovalent intra- or intermolecular interactions, giving biomolecular condensates their network-like architecture (Rubinstein and Dobrynin, 1997). Formation of such biomolecular condensates is driven mainly by two types of multivalent peptide structure interactions: modular proteins connected by multiple linkers and their binding motifs or weak multivalent interaction in IDR-containing proteins with folded domains (Li et al., 2012; Nott et al., 2015; Smith et al., 2016; Su et al., 2016; Wang et al., 2018b). In the case of nucleic acids, stickers are represented by structured regions or short sequence motifs, in some cases even individual nucleotides, that can bind to protein segments or other nucleic acid sequences (Banani et al., 2017; Choi et al., 2020).
Valency	Number of identical or non-identical molecular elements involved in specific inter- and intramolecular interactions (Kiessling and Lamanna, 2003). Biopolymers involved in LLPS are generally multivalent molecules, harboring a high repeat number of stickers that enhance weak interactions and change the proximity of biopolymers in cells (Choi et al., 2020). Valency and the affinity between them are key parameters for PS as both high valency and affinity promote assembly at a lower c_{sat} and decrease the dynamic rearrangement in the dense phase (Li et al., 2012).

Li et al., 2012), specificity of biological processes (Decker and Parker, 2012; O'Connell et al., 2012; Prouteau and Loewith, 2018), buffering (Eldar and Elowitz, 2010), adaptive filtration (Schmidt and Görlich, 2016), and mechanical force generation (Bergeron-Sandoval et al., 2018). These physicochemical properties suggest a wide range of possible cellular functionalities for biomolecular condensates because they enable a tight spatiotemporal control of biochemical reactions, such as the essential cellular functions of cell differentiation (Liu et al., 2020; Quiroz et al., 2020), cytoskeletal regulation (Case et al., 2019; Hernández-Vega et al., 2017), and metabolic control (Prouteau and Loewith, 2018). Because they lack membranes, these potential functions are switched on and off by the formation and disassembly of the condensates in reaction to small environmental changes like temperature or pH changes (Lin et al., 2015; Molliex et al., 2015; Nott et al., 2015). Their membraneless nature additionally allows for a rapid movement of molecules between phases, without the delay from transportation over a barrier, enabling fast chemical equilibrium between subcompartments of different properties and their assembly and disassembly occurs independently from any energy input (Griffin et al., 2011). Due to this quick responsiveness, biomolecular condensates are regarded as a faster version of cellular regulation when compared with transcriptional and translational control (Banani et al., 2017; Riback et al., 2017).

Ultimately, LLPS is widely considered a key physiological process that allows for the dynamic biogenesis of at least a subset of essential membraneless organelles. While repeat RNA presents phase separation properties (Jain and Vale, 2017) that may be relevant in neurodegenerative disorders, this review will focus on polypeptide LLPS. In recent years, key discoveries on biomolecular condensates have linked the process of LLPS to protein aggregation in neurodegenerative diseases, improving both our biophysical knowledge of LLPS and our understanding of the pathology. Indeed, an increasing set of proteins that can physiologically undergo LLPS are found in pathological aggregates (Table 1). Protein aggregates are pathognomonic for several human disorders, most notably neurodegenerative diseases (Table 1), including amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), Alzheimer's disease (AD), and Parkinson's disease (PD). This has led to the hypothesis that LLPS may favor the formation of aggregates (reviewed in Li et al., 2013; Polymenidou and Cleveland, 2011), and a flurry of studies has focused on understanding the behavior of neurodegenerative-disease-associated proteins within biomolecular condensates both *in vitro* and in cells. In this review, we discuss the current understanding of LLPS behavior of four such proteins: α-synuclein, FUS, TDP-43, and tau (Figure 1). Despite apparent structural and functional differences (Figure 2), we describe shared properties that could underlie their ability to

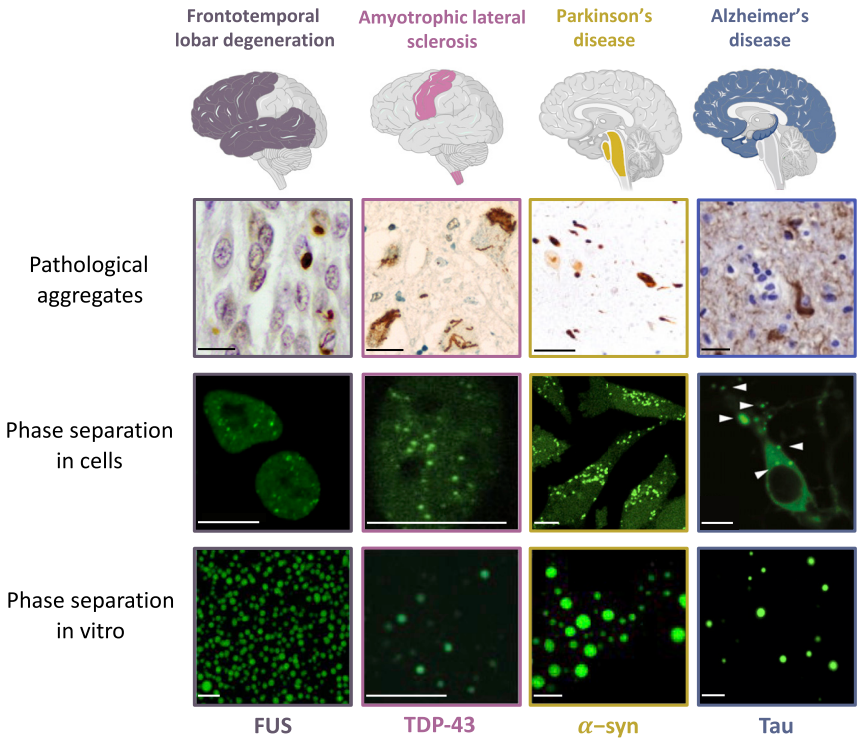

Figure 1. Proteins Associated with Neurodegenerative Diseases Show LLPS Behavior

Schematic representation of brain areas containing pathological aggregates of FTD-associated FUS (purple), ALS-associated TDP-43 (pink), PD-associated α-synuclein (yellow), and AD associated tau (blue). Second row: representative immunohistochemistry images of pathological aggregates found in postmortem brain samples from: FUS and TDP-43 (Mackenzie et al., 2010), α-synuclein (Mamais et al., 2013), and tau (Gibbons et al., 2018). Scale bar represents 20 μm. Third and fourth row: phase-separated protein in cells and *in vitro*, respectively. Images adapted from: FUS (Maharana et al., 2018; Qamar et al., 2018), TDP-43 (Gasset-Rosa et al., 2019), α-synuclein (Ray et al., 2020), and tau (Boyko et al., 2019; Wegmann et al., 2018). Scale bar represents 10 μm.

undergo LLPS and, under cellular or genetic modifications, transition to pathologic aggregation. While the definitive answer as to whether such a transition occurs in patient brains is missing, understanding the mechanisms involved may unravel key molecular steps leading to pathology and allow the development of new therapeutic approaches for these devastating and still incurable diseases.

THE PHANTOM MENACE: α-SYN, FUS, TAU, AND TDP-43 AS KEY PLAYERS IN NEURODEGENERATION AND IN PHASE SEPARATION BIOLOGY

First discovered 25 years ago as a transcription repressor of the human immunodeficiency virus genome, TAR DNA-binding protein 43 (TDP-43) is a multidomain nuclear protein with broad functions in RNA metabolism, from transcription and splicing to translation regulation (Afroz et al., 2019). It consists of a structured N-terminal domain (NTD) involved in physiological self-oligomerization (Table 1) (Afroz et al., 2017; Wang et al., 2018a), followed by two RNA-recognition motifs (RRMs) that bind UG/TG-rich sequences (Buratti et al., 2001; Lukavsky et al., 2013; Polymenidou et al., 2011) and a C-terminal low-complexity region (LCR) (Table 1) (Figure 2) that interacts with other factors (Buratti et al., 2001). In 2006, TDP-43 was found as the main component of the cytoplasmic, ubiquitin-positive protein inclusions marking the affected neurons of ALS and FTD patients (Neumann et al., 2006) (Table S1). In these pathological deposits, TDP-43 undergoes a number of posttranslational modifications (PTMs), including polyubiquitination, hyperphosphorylation, and proteolytic cleavage (Neumann et al., 2006). The latter releases its highly aggregation-prone LCR, which has the ability to phase separate (Babinchak et al., 2019; Li et al., 2018b). The LCR of TDP-43 is also a prion-like domain (PrLD; Table 1) due to sequence similarities with yeast prions (King et al., 2012), proteins that switch between an intrinsically unfolded and an aggregated state that imposes its conformation to its unfolded counterpart (Alberti et al., 2009) and are thereby conceptually similar to mammalian prions (Aguzzi and Polymenidou, 2004; Polymenidou and Cleveland, 2011). Due to the predominant TDP-43 pathology, ALS and FTD are also referred to as TDP-43 proteinopathies, which are now known to extend to other neurodegenerative diseases, including a fraction of PD and AD cases (Lagier-Tourenne et al., 2010) and a recently recognized, unusual subtype of AD, called limbic-predominant age-related TDP-43 encephalopathy or LATE (Nelson et al., 2019).

Another neurodegenerative-disease-associated protein sharing many functional and structural similarities to TDP-43 is the likewise nuclear protein fused in sarcoma (FUS). FUS was discovered in 1993 due to abnormal fusions of its promoter and the initial part of the gene with transcription factors, resulting in aberrant transcriptional activation in human myxoid liposarcoma. For this reason, FUS is also known as translocated in liposarcoma or TLS (Lagier-Tourenne et al., 2010). Over 15 years later, mutations in FUS were shown to cause ALS (Kwiatkowski et al., 2009; Vance et al., 2009), and its incorporation into protein inclusions in the nervous system of a subset of sporadic FTD patients without mutations was reported shortly after (Neumann et al., 2011) (Table S1). Much like TDP-43, FUS plays an important role in diverse processes of the RNA metabolism, including transcriptional and splicing regulation and RNA transport (Lagier-Tourenne et al., 2010; Sahadevan et al., 2020), but it is additionally involved in DNA damage repair (Wang et al., 2008). FUS is composed of a long N-terminal PrLD sectionally rich in glutamine, glycine, serine, and tyrosine (QGSY) and arginine/glycine (RGG), which comprises the first half of the protein and is involved in the self-oligomerization required to bind RNA (Yang et al., 2014, 2015) (Figure 2). The PrLD of FUS shows the highest similarity to yeast prions from all human RNA-binding proteins (RBPs) (King et al., 2012) and has been among the first identified

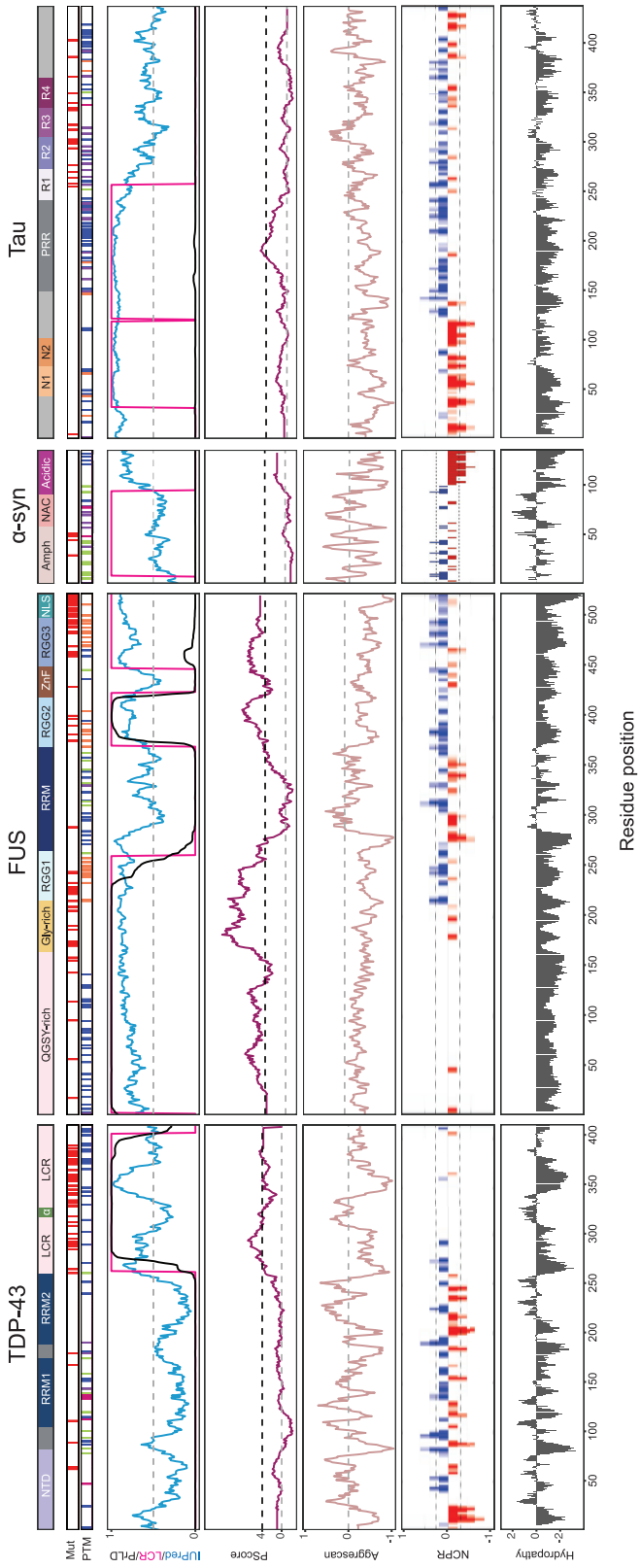

Figure 2. Patterns and Features of Amino Acid Sequences of TDP-43, FUS, α-Synuclein, and Tau

Top row: domain organization. NTD (N-terminal domain), RRM (RNA-recognition motif), LCR (low-complexity region), QGSY-rich (rich in glutamine, glycine, serine, and tyrosine), RGG (arginine/glycine-rich), ZnF (zinc finger), NLS (nuclear localization signal), NAC (non-Aβ component of AD plaque), N1-2 (polypeptide sequences encoded by exons 2 and 3), PRR (proline-rich regions), and R1–4 (microtubule-binding domains encoded by exons 9–12). Second row: mutations linked to neurodegenerative diseases, also listed in Tables S2–S5. Third row: position of PTMs, also found in Tables S2–S5, are indicated as follows phosphorylation (blue), ubiquitination (green), acetylation (purple), methylation (maroon), and SUMOylation (pink). Fourth row: predictions for intrinsically disordered regions (using IUPred2A; Mészáros et al., 2018), LCRs (using an algorithm based on amino acid segmentation called SEG; Wootton and Federhen, 1993), and PrLD (with prion-like amino acid composition, PLAAC; Lancaster et al., 2014). Fifth row: phase separating properties were assessed with a propensity Score (pscore) using a phase separation predictor (Vernon et al., 2018). Sixth row: prediction of aggregation hotspot (using AGGRESCAN; Conchillo-Solé et al., 2007). Seventh row: net charge per residue (NCPR) blue depicting positive and red negative charges (using CIDER v1.7; Holehouse et al., 2017). Last row: the hydrophobicity index was calculated with the hydrophobic Kyte & Doolittle scale on Expasy (Gasteiger et al., 2005).

and best characterized domains in the phase separation field (Kato et al., 2012; Molliex et al., 2015; Murthy et al., 2019; Wang et al., 2018b). Unlike TDP-43, FUS only presents a single RRM-binding RNA stem loops (Loughlin et al., 2019), but further binding of GUGGU-rich motifs (Lagier-Tourenne et al., 2012) can occur through its zinc-finger domain (ZnF) (Loughlin et al., 2019). FUS also presents two additional, shorter LCRs, of which one also classifies as a PrLD while the one next to its C-terminal nonclassical nuclear localization signal (NLS) does not (Figure 2).

Despite sharing important features such as functionality, structural elements, cellular localization, and mislocalization in disease, FUS and TDP-43 also differ in many aspects, like specific roles in the cell metabolism or their mechanism of nuclear import (Ling et al., 2013). Importantly, both FUS (Lagier-Tourenne et al., 2012) and TDP-43 (Ayala et al., 2011; Polymenidou et al., 2011) autoregulate their protein levels via binding their own RNA and TDP-43 also cross-regulates FUS in a similar manner (Polymenidou et al., 2011). In fact, TDP-43 and FUS proteinopathies are mutually exclusive (Mackenzie et al., 2010), illustrating the complex etiology behind neurodegenerative disorders.

One of the first proteins discovered to aggregate as a hallmark of any neurodegenerative disease was tau (Grundke-Iqbal et al., 1986a; Wolozin et al., 1986), which forms cytoplasmic aggregates known as neurofibrillary tangles in the affected neurons of AD patients. A group of six isoforms encoded by the gene *MAPT* (microtubule-associated protein tau), tau proteins are expressed mainly in the central nervous system (CNS) (Trojanowski et al., 1989). More than a decade later, with the discovery of mutations in the *MAPT* gene in FTD patients (Hutton et al., 1998), tau was recognized as a key pathological player in diseases beyond AD. Indeed, approximately half of all FTD patients show a neuropathological profile of a primary tauopathy (Ling et al., 2013). Tau isoforms in the CNS differ in the presence or absence of specific exons: N1-2 (exons 2 and 3) and the microtubule-binding R1 to R4 (exons 9–12) (Andreadis, 2005). Unlike FUS and TDP-43, tau are cytoplasmic proteins, without any RNA-binding domains, that bind axonic microtubules in order to regulate its assembly (Cleveland et al., 1977) and associated transport, as well as neurite outgrowth (Caceres and Kosik, 1990).

Structurally and functionally very different to TDP-43, FUS, and tau, α-synuclein forms pathological aggregates in a diverse group of diseases known as Lewy body diseases, which include

PD, PD with dementia, and dementia with Lewy bodies (McKeith et al., 1996) (Table S1). Discovered over 100 years ago, Lewy bodies, the pathognomonic hallmarks of these diseases, are complex structures containing α-synuclein aggregates (Spillantini et al., 1997), as well as lipid membranes and sometimes even broken organelles (Shahmoradian et al., 2019). Pathological species of α-synuclein present structural polymorphism and include large fibrils (Guerrero-Ferreira et al., 2020; Spillantini et al., 1997), as well as a variety of prefibrillar oligomeric species, which differ in molecular weight, morphology, and structural composition from large fibrillar assemblies and from each other (Cremades et al., 2017; Lashuel et al., 2002). While some of these species are on-pathway toward fibrillar aggregates (Cremades et al., 2012; Pieri et al., 2016), others have been associated with an "off-fibrillar pathway," leading to amorphous aggregates (Lorenzen et al., 2014). Oligomeric α-synuclein species are known to cause a variety of toxic effects like mitochondrial dysfunction (Parihar et al., 2009), membrane permeabilization (Colla et al., 2012; van Rooijen et al., 2010), imbalance in protein homeostasis (Lindersson et al., 2004; Xilouri et al., 2013), and synaptotoxicity (Choi et al., 2013). On the other hand, fibrillar α-synuclein has been associated with seeding of soluble α-synuclein into aggregates (Bousset et al., 2013), compromised integrity and function of cytosolic organelles (Flavin et al., 2017), and chronic inflammation (Gustot et al., 2015; Peralta Ramos et al., 2019). While both fibrillar and oligomeric α-synuclein are implicated in neurodegeneration, only fibrillar aggregates, either derived from animal models or patients (Luk et al., 2012a; Masuda-Suzukake et al., 2013; Recasens et al., 2014) or generated *in vitro* (Luk et al., 2012b; Peelaerts et al., 2015), were shown to propagate by sequestration of soluble α-synuclein and trigger lesions reminiscent of PD *in vivo*. The exact role of oligomeric and fibrillar species in the pathogenesis of PD is still under investigation.

α-synuclein is composed of an amphipathic N-terminal region, rich in lysines, that is implicated in membrane interactions and may form α-helical structures (Bartels et al., 2011; Burré et al., 2013). The acidic C-terminal domain is involved in its debated nuclear localization and in diverse interactions (Lautenschläger et al., 2018; Yin et al., 2011) and form aggregates at low pH (Hoyer et al., 2002). Connecting the two ends, a highly hydrophobic motif termed non-amyloid-β component of AD amyloid plaques (NACs), amyloidogenic and rich in hydrophobic amino acids (Figure 2), is known for its aggregation and β sheet formation (Bisaglia et al., 2009). Under native conditions, α-synuclein has been proposed to lack a stable structure and to alter its conformation in a context-dependent manner (Uversky and Fink, 2002), for instance when it is in contact with membranes (Bodner et al., 2009). While the physiological function(s) of α-synuclein remain(s) unknown, its localization to presynaptic terminals suggests a role in regulating synaptic homeostasis, including neurotransmitter release and synaptic function and plasticity (Lashuel et al., 2013).

Despite the different structural, functional, and localization features of the proteins, all four of them have been shown to undergo LLPS under physiological conditions *in vitro* and in cells (Molliex et al., 2015; Nott et al., 2015; Ray et al., 2020; Wegmann et al., 2018). Next, we detail how these distinct features enable the phase separation of α-synuclein, FUS, tau, and TDP-43, by comparing and contrasting their protein-intrinsic phase separation determinants.

THE RISE OF THE LLPS: STRUCTURAL DETERMINANTS OF α-SYN, FUS, TAU, AND TDP-43

Biomolecular condensates are formed via LLPS of multivalent molecules, containing various sticker elements (Table 1) in their primary sequence that dominate intra- and/or intermolecular interactions (Choi et al., 2020). Formation of biomolecular condensates can be mainly driven by two types of arrangements: proteins with multiple linker-connected modules and pockets that bind to other molecules (Choi et al., 2020; Li et al., 2012) or weak multivalent interactions in proteins with folded domains and intrinsically disordered regions (IDRs; Table 1) (Choi et al., 2020; Nott et al., 2015). IDRs are most frequently involved in weak intermolecular interactions that favor LLPS (Conicella et al., 2016; Maharana et al., 2018; Murthy et al., 2019; Schmidt et al., 2019), yet, surprisingly, they were recently shown to also mediate ultra-high-affinity ones, while fully retaining their disorder (Borgia et al., 2018). IDRs are found in many proteins involved in biomolecular condensates, including TDP-43, FUS, α-synuclein, and tau (Figure 2). The amino acid sequence of an IDR determines its phase behavior and directly correlates with the response to different LLPS stimuli and its properties in the dense phase of a given system (Banani et al., 2017). The physicochemical and conformational properties of IDR-containing proteins are defined by the number, the patterning (location and distribution), and the identity of hydrophobic, charged, and polar residues, which represent adhesive elements in IDRs and promote π-π (enabled by aromatic rings), π-charge, charge-charge, and dipole weak interactions (Vernon et al., 2018). However, the protein and the system determine the exact role of specific amino acids or combinations thereof, as well as the interaction types and strength (Alberti et al., 2019). These types of sequence-encoded interactions are highly dynamic as expected for the nature of phase-separated liquids (Banani et al., 2017). However, the weak interactions contributing to LLPS behavior are not restricted to the amino acid or nucleotide identity per se. For a subtype of phase-separating IDRs, transient formation of evolutionary conserved secondary structures, such as the α-helix in the LCR of TDP-43, can promote biomolecular condensation (Conicella et al., 2016; Lin et al., 2019).

Many IDRs enriched in biomolecular condensates are limited in their amino acid complexity (Kato et al., 2012) and are termed as IDRs with low-complexity regions (IDR-LCRs) (Table 1). One of the most common types of IDR-LCRs are PrLD (Alberti et al., 2009). In recent years, a key role of LCRs and PrLD in LLPS has emerged (Gasset-Rosa et al., 2019; Molliex et al., 2015; Patel et al., 2015), primarily implicating RBPs associated with neurodegeneration, including TDP-43 and FUS (King et al., 2012), and potentially connecting functional phase separation with pathological aggregation (Figure 2; Table S1).

IDRs as Drivers of LLPS

TDP-43 (Conicella et al., 2016) and FUS (Murray et al., 2017) have predicted IDRs of PrLD nature, which are essential for reversible and irreversible self-association linked to LLPS (Figure 2). The LCRs of both proteins are largely devoid of charged residues, but enriched in hydrophobic residues, which results in the LLPS of FUS and TDP-43 being largely driven by aromatic residues involved in π-interactions (Figure 3) (Qamar et al., 2018;

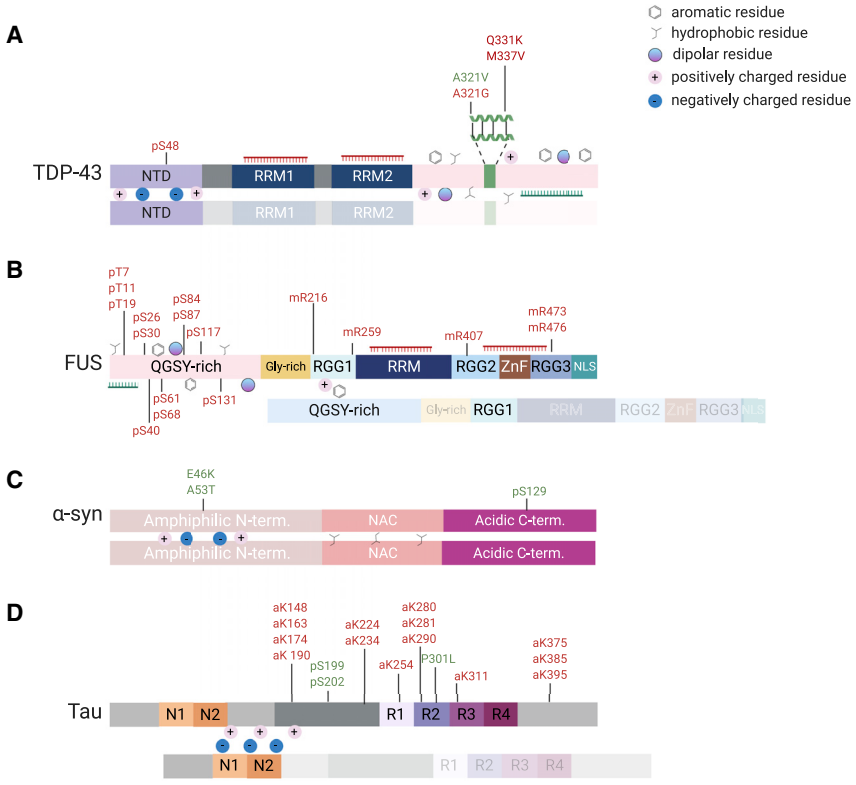

Figure 3. Protein-Intrinsic Modulators of LLPS in Neurodegenerative-Disease-Associated Proteins

The schematic summarizes primary sequence elements that decrease (red) or enhance (green) LLPS compared with unmodified TDP-43, FUS, α-synuclein (α-syn), or tau, as indicated.

(A) LLPS of TDP-43 is mediated by aromatic, dipolar, and positively charged (arginine) residues in the LCR (Schmidt et al., 2019), as well as a transiently formed α-helix (Conicella et al., 2016). Most ALS-associated mutations in the helix decrease LLPS (Conicella et al., 2016), with a rare exception of A321V, which enhances it (Conicella et al., 2016). NTD-mediated oligomerization (Afroz et al., 2017; Wang et al., 2018a) contributes to LLPS of TDP-43 and an oligomerization-disrupting mimic of phosphorylation at S48 (S48, Wang et al. 2018a) decreases it. Nonspecific RNA binding to the LCR increases LLPS (Conicella et al., 2016), while sequence-specific binding via the RRMs (Mann et al., 2019) decreases it.

(B) Interactions between arginines (positively charged) in one RGG motif and tyrosines (aromatic) in the LCR of FUS are implicated in condensate formation (Qamar et al., 2018). Phosphorylation (Monahan et al., 2017) and methylation (Qamar et al., 2018) on multiple positions on the FUS sequence decrease LLPS. Similar to TDP-43, nonspecific RNA binding to the LCR of FUS (Burke et al., 2015; Monahan et al., 2017) increases LLPS, while sequence-specific binding by the RRM (Mann et al., 2019), the ZnF or RRG of FUS (Schwartz et al., 2013) decrease the process.

(C) α-synuclein LLPS is driven by electrostatic interactions in the amphiphilic N-terminal domain and hydrophobic interactions between the NAC domains (Ray et al., 2020). PD-associated mutations (E46K, A53T) as well as a phosphorylation in S129 increase LLPS (Ray et al., 2020).

(D) Tau LLPS is driven by electrostatic interactions between the negatively charged N-terminal and positively charged C-terminal regions of the protein (Boyko et al., 2019; Wegmann et al., 2018). Phosphorylation (S199, S202) and a mutation in the R2 domain (P301L) have been shown to increase LLPS while acetylation in different domains are correlated with a decrease in LLPS (Ferreon et al., 2018). Different types of amino acids important for LLPS are found depicted schematically in the right upper corner. NTD: N-terminal domain, RRM: RNA-recognition motif, LCR: low-complexity region, QGSY-rich: rich in glutamine, glycine, serine, and tyrosine, RGG, arginine/glycine-rich; ZnF, zinc finger; NLS, nuclear localization signal; NAC, non-Aβ component of AD plaque; N1–2, polypeptide sequences encoded by exons 2 and 3; PRR, proline-rich regions; R1–4, microtubule-binding domains encoded by exons 9–12.

Schmidt et al., 2019). In particular, TDP-43 LLPS is driven by π-π intermolecular interactions of regularly spaced aromatic residues in its LCR (Li et al., 2018b; Schmidt et al., 2019), as well as a cooperative, transient α-helix (Conicella et al., 2016). Moreover, arginine and hydrophobic LCR residues were shown to tune the biomolecular condensate properties of TDP-43 (Figure 3) (Schmidt et al., 2019). On the other hand, FUS LLPS is driven by hydrogen bonds, π-interactions, and hydrophobic interactions within its LCR, while glutamine residues also participate in contacts favoring LLPS (Figure 3) (Murthy et al., 2019). Furthermore, cation-π interactions between the arginine in the C-terminal structured RGG motif and the tyrosine in the LCR have been shown to be important for LLPS (Figure 3) (Qamar et al., 2018). In contrast to TDP-43 (Conicella et al., 2016), no transient structural elements have yet been reported within the LCR of FUS and the protein seems to retain its conformational heterogeneity in the condensed phase (Figure 3) (Murthy et al., 2019). Contrary to FUS and TDP-43, α-synuclein seems to be a primarily disordered protein with an LCR, which is not, however, of PrLD nature (Figure 2). The implication of α-synuclein in biomolecular condensates has been long discussed, but the first evidence of α-synuclein phase separation in cells has just been reported (Hardenberg et al., 2020; Ray et al., 2020). LLPS seems to be driven by intermolecular electrostatic interactions in the N-terminal domain and hydrophobic interactions in the NAC domains (Figure 3) (Ray et al., 2020).

Unlike FUS (Maharana et al., 2018) and TDP-43 (Gasset-Rosa et al., 2019), physiological tau LLPS is yet to be described in cells without overexpression (Tan et al., 2019; Wegmann et al., 2018). The functional relevance of physiological tau LLPS is just beginning to emerge, as tau condensates along axons were shown to spatially regulate microtubule functions (Tan et al., 2019). Similar to α-synuclein, tau contains an IDR, which is an LCR but not a PrLD (Figure 2). However, LLPS of tau is thought to be driven by electrostatic interactions between the negatively charged N-terminal and positively charged C-terminal region of the protein (Boyko et al., 2019; Wegmann et al., 2018) (Figures 2 and 3).

Self-Oligomerization Domains and LLPS

While the LCR of TDP-43 and FUS is sufficient for phase separation *in vitro* at high concentrations (Burke et al., 2015; Conicella et al., 2016), the full-length protein has been shown to undergo LLPS *in vitro* and in cells at physiological concentrations and conditions (Burke et al., 2015; Qamar et al., 2018; Schmidt et al., 2019; Wang et al., 2018a), suggesting that additional structural elements may contribute to the LLPS behavior.

Interestingly, both cellular FUS (Yang et al., 2015) and TDP-43 (Afroz et al., 2017) have been proposed to self-oligomerize in the nucleus through their N-terminal domains, thereby enabling their nuclear functions. Indeed, nuclear oligomerization of TDP-43 is essential for regulation of alternative splicing of its nuclear RNA targets (Afroz et al., 2017), while the self-assembly of FUS is necessary for transcription regulation via chromatin binding (Yang et al., 2015). TDP-43 oligomerization is mediated by the fully folded N-terminal domain (Afroz et al., 2017), while FUS oligomerization is associated with its N-terminal QGSY-rich intrinsically disordered domain, which is similar to the LCR of TDP-43 (Figure 2), suggesting two very different mechanisms of self-association. TDP-43 oligomerization is crucial for LLPS *in vitro* and in cellular systems (Figure 3) (Wang et al., 2018a), but the relative contribution of the N-terminal domain and the C-terminal LCR in the physiological phase separation of the full-length protein remains unclear. FUS oligomerization and LLPS are driven by the same domain (Kato et al., 2012; Yang et al., 2015), but the underlying mechanism connecting the two processes, if any, is yet to be deciphered.

As for FUS and TDP-43, tau has been described to physiologically oligomerize (Wegmann et al., 2016) and phase separate (Wegmann et al., 2018) in dynamic compartments within cells. However, the connection between these two events, if any, remains elusive. In contrast, α-synuclein oligomerization has been mostly associated with toxic effects (Parihar et al., 2009; Colla et al., 2012; van Rooijen et al., 2010; Lindersson et al., 2004; Xilouri et al., 2013; Choi et al., 2013), and its involvement in LLPS and its potential functional role in these processes have yet to be discovered.

In contrast to physiological oligomerization, which remains dynamic and plays functional roles, oligomerization of neurodegenerative-disease-linked proteins is frequently associated with toxic intermediate species of pathological aggregates. Such oligomers have been described for α-synuclein (Pieri et al., 2016; Sahin et al., 2018), FUS (Sun et al., 2011), tau (Lasagna-Reeves et al., 2011), TDP-43 (Fang et al., 2014; French et al., 2019), and several others. If and how these toxic oligomers are involved in phase separation or transition and whether condensates favor oligomer formation remains to be discovered.

The distinction between physiological and pathological oligomers (Table 1) is often elusive, and it lies on the structural characteristics of the assemblies, which are not always defined. Our understanding of the role of oligomerization in functional and/or toxic pathways is contingent on this very important distinction. For example, while toxic oligomers might be viable targets for clearance to prevent aggregation, physiological oligomers were shown to antagonize aggregation of TDP-43 in cells (Afroz et al., 2017), suggesting that their stabilization might be an effective approach to reverse aggregation. A conceptually similar phenomenon has been described for transthyretin, a physiologically tetrameric protein, which forms toxic amyloid fibrils in patients with transthyretin amyloid cardiomyopathy. Small molecules that stabilize the physiological transthyretin tetramers were shown to inverse its aggregation (Hammarström et al., 2003) and were developed into an effective therapy, which slows progression and increases survival of patients with transthyretin amyloid cardiomyopathy (Maurer et al., 2018). This successful example illustrates that careful structural and functional characterization of oligomeric species of disease-associated proteins may open new avenues for therapeutic approaches.

The Role of RNA-Binding Domains in LLPS

RNA binding, specific or unspecific, has been shown to modulate the phase separation behavior of many proteins (Brangwynne et al., 2009), including FUS and TDP-43 (Figure 3). While constructs harboring the RRMs of TDP-43 and the RRM, RGG or ZnF of FUS showed an increase in protein solubility preventing LLPS when target RNAs are present *in vitro* and in cells (Mann et al., 2019; Schwartz et al., 2013), unspecific interactions of RNA with the LCR seems to enhance LLPS, most probably by saturation of protein-RNA interactions, for both proteins *in vitro* (Figure 3) (Burke et al., 2015; Conicella et al., 2016; Maharana et al., 2018). In contrast to TDP-43 and FUS, neither α-synuclein nor tau harbor any known RNA-binding domains. Consequently, a potential role of RNA binding of α-synuclein or tau in LLPS is largely unexplored, yet recent complex coacervation (Table 1) (Timilsena et al., 2019) studies showed enhanced LLPS of tau in the presence of small RNAs *in vitro* and in cells, especially tRNAs (Lin et al., 2019), suggesting that RNA species may play surprising roles in LLPS despite lack of classical RNA-protein interactions.

While the molecular mechanism underlying LLPS of FUS, TDP-43, α-synuclein, and tau differs in inter- and intramolecular interactions, oligomerization and RNA dependency, all four proteins harbor IDR-LCRs and they all phase separate *in vitro* and in cells (Figure 2). Due to their different sequence properties, each illustrates different phase behavior in reaction to stimuli and understanding those idiosyncrasies may be an important piece of the puzzle in the pathobiology of neurodegenerative diseases and may potentially elucidate the selectivity of aggregation of each protein in its associated disease.

RETURN OF THE JEDI AND THE EMPIRE STRIKES BACK: BALANCING PHYSIOLOGICAL AND ABERRANT PHASE TRANSITIONS

While the amino acid composition determines the LLPS behavior of a particular protein, additional layers of regulation affect the formation of biomolecular condensates (Brangwynne et al., 2009). A number of cellular regulators modify LLPS in physiological conditions, affecting both scaffolds and clients (Table 1). Importantly, most of the cellular modulators of LLPS that we discuss in this section, such as RNA or ATP, are heterogeneously distributed within cells, regulating the precise localization of condensates formed by certain proteins in specific subcellular compartments. Deviations in the tight physiological regulation of the components of biomolecular condensates as well as changes in the amino acid sequence, such as mutations and posttranslational modifications, might cause changes in their assembly and dynamics. The loss of dynamic nature of biomolecular condensates, a process termed maturation, may trigger the formation of immobile protein aggregates (Table 1), which are structurally distinct from the dynamic phase-separated protein condensates (Hofweber et al., 2018; Molliex et al., 2015; Patel et al., 2015; Qamar et al., 2018; Yoshizawa et al., 2018). Indeed, *in vitro* maturation of liquid droplets leading to aggregate formation over time has been shown for α-synuclein (Ray et al., 2020), FUS (Patel et al., 2015), tau (Wegmann et al., 2018), and TDP-43

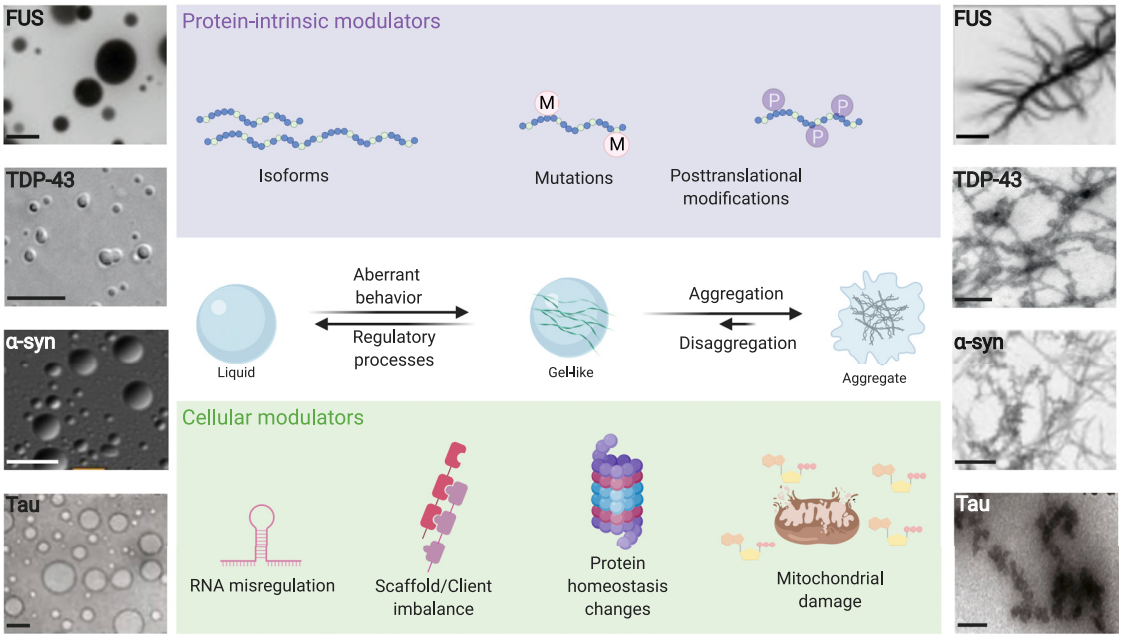

Figure 4. Maturation Model and Regulatory Mechanisms of Biomolecular Condensates

The transition from reversible dynamic LLPS to an irreversible state, a process termed maturation, has been shown for neurodegenerative-disease-associated proteins, including TDP-43 (Schmidt et al., 2019), FUS (Patel et al., 2015), a-synuclein (Ray et al., 2020), and tau (Wegmann et al., 2018). While they can form reversible bimolecular condensates (left lane), over time a transition to irreversible structures of fibrillar solid aggregates occurs (right lane). These processes are regulated by different protein-intrinsic modulators (purple box) and cellular modulators (green box) that are associated with neurodegenerative disorders. Disease-associated mutations (Patel et al., 2015) and posttranslational modifications (Ray et al., 2020) have been shown to enhance the transition from dynamic LLPS-mediated condensates to more static ones. Changes in valency due to different protein isoforms are associated with changes in saturation concentration (Ambadipudi et al., 2017) potentially leading to maturation. Changes in cellular LLPS modulators, which are also associated with neurodegenerative diseases like RNA misregulation (Gasset-Rosa et al., 2019; Kato et al., 2012), scaffold/client imbalance (Maharana et al., 2018), protein homeostasis changes (Sin and Nollen, 2015), and mitochondrial damage (Johri and Beal, 2012) could explain a transition from physiology to pathology. Left: *In vitro* LLPS of FUS (Patel et al., 2015), TDP-43 (Wang et al., 2018a), α-synuclein (Ray et al., 2020), and tau (Wegmann et al., 2018). Scale bar represents 10 μm. Right row: into insoluble aggregates by *in vitro* maturation of FUS (Patel et al., 2015), TDP-43 (Johnson et al., 2009), α-synuclein (Ray et al., 2020), and tau (Wegmann et al., 2018). Scale bar represents 200 μm.

(Conicella et al., 2016), all of which appear to be of fibrillar nature (Figure 4). A growing list of proteins physiologically undergoing LLPS for diverse cellular functions, which include α-syn, FUS, tau, and TDP-43, are found aggregated in affected neurons of patients with neurodegenerative diseases, which led to the hypothesis that protein aggregates may originate from an aberrant LLPS behavior (Figure 1; Table S1) (Li et al., 2013; Polymenidou and Cleveland, 2011). Consequently, a central question over the past decade has been if and how LLPS drives protein aggregation. While the mechanism(s) of this transition remain far from resolved, the field has made great progress in deciphering several key steps and drivers in the process.

It is important to note that, currently, the role of these pathognomonic protein aggregates in the pathogenesis of neurodegenerative diseases is still under debate. While some studies point at protein aggregates as the main culprits of neurotoxicity, others describe them as a cellular protective response. Whether the toxicity arises from non-physiological oligomers, larger protein aggregates or unrelated mechanisms remains elusive to date (Ross and Poirier, 2005; Winklhofer et al., 2008).

Protein Concentration as a Driver of LLPS and Aggregation

Formation of biomolecular condensates is influenced by protein expression, degradation, and localization, which indirectly can also modulate molecular crowding and electrostatic and hydrophobic interactions (Banani et al., 2017; Molliex et al., 2015), driving to aberrant condensates and fibrillar solids over time. Aging can affect the proteostasis network, altering protein expression and degradation (Klaips et al., 2018) and leading to increased protein concentrations that trigger LLPS (Gasset-Rosa et al., 2019; Molliex et al., 2015). While there are many hypotheses on how the aggregation process can occur in condensates (reviewed in Banani et al., 2017), one possibility is the formation of filamentous β-rich structures. IDRs, just like any other polypeptide sequences (Dobson, 2017), have the ability to form amyloid-like fibrils by β strand interactions, a phenomenon potentially enhanced in LLPS due to their high local protein concentration within the condensed phase and their tendency for a stretched conformation (Choi et al., 2020). In the condensed phase, the growth rate and nucleation of amyloid-like fibrils are probably increased, facilitating and stabilizing lateral contacts, which is supported by electron microscopy. Moreover, X-ray diffraction recordings of *in vitro* mature condensates (Burke et al., 2015; Han et al., 2012; Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015) show amyloid-like filaments similar to those found in pathological assemblies (Table 1) in neurodegenerative diseases (Fitzpatrick and Saibil, 2019; Laferrière et al., 2019; Strohäker et al., 2019).

Disease-Associated Mutations and Protein Isoforms Affect LLPS and Aggregation Behavior

Because LLPS is promoted by maximizing the interaction potential encoded in the primary sequence of biopolymers, any changes in sequence will alter phase behavior. Disease-associated mutations in proteins undergoing LLPS are the prime example of a phase behavior modifier, as they have been shown to either inhibit (Conicella et al., 2016) or enhance (Conicella et al., 2020) phase separation *in vitro*, as well as to accelerate the maturation process (Molliex et al., 2015; Murakami et al., 2015; Patel et al., 2015). For example, mutations in the PrLD of FUS (Patel et al., 2015), TDP-43 (Conicella et al., 2016; French et al., 2019), and in α-synuclein (Ray et al., 2020) either disrupt LLPS or accelerate the aberrant transition from LLPS to aggregates *in vitro* and in cells, due to changes in their biophysical properties (Figure 2).

The effect of different mutations on the phase separation behavior of proteins is not yet fully understood. However, it is possible that mutations that lower the saturation concentration could trap the protein in a condensed system, favoring β sheet formation and aggregation over time (Ray et al., 2020). Similarly, mutations that increase the maturation propensity may enhance the formation of β sheets and aggregation. On the other hand, mutations increasing the saturation concentration, potentially preventing LLPS under physiological conditions, would indicate an aggregation pathway independent from the phase separation process or even suggest that biomolecular condensates may be protective against protein aggregation. Additionally, disease-associated mutations may alter the binding to cellular LLPS modulators. ALS-linked FUS mutations indirectly suppress its physiological LLPS behavior and promote aggregation by preventing the interaction with its nuclear import receptor Transportin-1, which presents a chaperone/disaggregase activity *in vitro* and in cells (Dormann et al., 2010; Guo et al., 2018; Hofweber et al., 2018; Qamar et al., 2018). Furthermore, different protein isoforms can alter phase behavior by changing the valency of the biopolymer (Figure 2). For tau, isoform switching altering the number of repeat domains changes the ability for demixing and transition to amyloid-like aggregates (Ambadipudi et al., 2017).

Posttranslational Modifications as Molecular Switches for Interactions Triggering LLPS

PTMs can serve as on/off switches of LLPS that are characterized by fast and reversible spatiotemporal precision (Tables S2–S5). PTMs can alter the protein's ability to bind other factors and are often found in IDR-containing proteins prone to phase separate. Given that misregulation of PTMs potentially leads to aberrant phase behavior and many pathological protein assemblies, such as TDP-43, FUS, tau, and α-synuclein, showing specific PTM signatures (Tables S2–S5), there is a conceivable interplay between an aberrant PTM profile, phase separation behavior, and aggregation (Figure 2) (Ambadipudi et al., 2017; Grundke-Iqbal et al., 1986b).

The most abundant PTM in cells, phosphorylation, has been implicated in the modulation of LLPS. A diverse set of kinases, from serine/threonine or tyrosine specific (Amaya et al., 2018; Shattuck et al., 2019) to dual-specificity kinases (Rai et al., 2018; Wippich et al., 2013), have been shown to be involved in the assembly and disassembly of some membraneless organelles, such as SGs, by LLPS. For example, phosphorylation events by dual-specificity kinase 3 (DYRK3) were shown to regulate the disassembly of membraneless organelles, including SGs (Wippich et al., 2013) and nuclear speckles (Rai et al., 2018). Introduction of a negative charge can modulate LLPS directly by altering the involved interactions (such as π-charge and charge-charge interactions), or indirectly by interfering with processes that affect LLPS, like oligomerization (Wang et al., 2018a). For example, in the case of TDP-43, phosphorylation of Ser48, which lies within the NTD, has been shown to occur physiologically in cells, and to disrupt oligomerization and LLPS of TDP-43 *in vitro* (Wang et al., 2018a). FUS has also been reported to be physiologically phosphorylated in cells upon DNA damage, but in this case in several Ser/Thr-Tyr residues from its N-terminal LCR-PrLD (Deng et al., 2014; Monahan et al., 2017), which was subsequently shown to disrupt phase separation *in vitro* (Monahan et al., 2017).

Tau phosphorylation has been extensively studied in tauopathies, where it has been shown to play an essential role in physiology and transition to disease. Tau phosphorylation occurs physiologically to regulate some of its functions, including microtubule binding, axonal transport, and neurite outgrowth (Johnson and Stoothoff, 2004), and fetal phosphorylated tau occurs physiologically during development (Brion et al., 1993). Even if phosphorylation is not essential for phase separation, since non-phosphorylated tau can effectively undergo LLPS *in vitro* under physiologically relevant conditions, specific phosphorylation of tau favors tau LLPS and can drive the maturation to filamentous aggregates with amyloid-like structures *in vitro* (Wegmann et al., 2018; Yoshida and Ihara, 1993), connecting phosphorylated tau LLPS and aggregation. In fact, there seems to be a phosphorylation pattern that is linked to AD (Despres et al., 2017). While the role of TDP-43 phosphorylation in its aggregation process is still debated (Brady et al., 2011; Liachko et al., 2010; Nonaka et al., 2016), the phosphorylation pattern of tau has been shown to correlate with the progression of tau aggregation and the severity of disease in AD patients (Augustinack et al., 2002).

In contrast to TDP-43, FUS, and tau, the regulatory effects of PTMs on α-synuclein function and LLPS are still poorly understood. However, a recent study indicated that a specific phosphorylation event in the acidic tail of α-synuclein, which is known to be involved in PD pathogenesis (Figure 3), drives the maturation of condensed α-synuclein to amyloid-like fibrils (Ray et al., 2020).

In contrast to phosphorylation, arginine methylation does not alter a protein's net charge but can transform the charge distribution and steric effects (Evich et al., 2016). Physiologically relevant arginine methylation has been extensively reported to be a suppressor of LLPS, not only for neurodegenerative-disease-related proteins like FUS or hnRNP A2 (Ryan et al., 2018) but also unrelated factors like DDX4 (Nott et al., 2015). Interestingly for FUS, methylation in the RGG3 domain, residing next to its NLS, is a physiological regulator of its nucleocytoplasmic localization (Dormann et al., 2012; Tradewell et al., 2012). FUS methylation can suppress not only its LLPS *in vitro* and in cells (Hofweber et al., 2018; Qamar et al., 2018) but also its incorporation into SG and interferes with transportin-1 binding, which presents chaperone activity (Hofweber et al., 2018; Qamar et al.,

2018). Remarkably, FUS methylation is lost in FTD-FUS—but not ALS-FUS—aggregates (Dormann et al., 2012), suggesting a direct link between PTMs, cytoplasmic mislocalization, and aberrant transition of LLPS condensates.

Compared with phosphorylation, less is known about the emerging field of protein acetylation in general (Choudhary et al., 2014). Acetylation removes the positive charge of the lysine residue, which potentially affects the protein's interaction with other proteins or, most relevant for LLPS, nucleic acids (Choudhary et al., 2014). In fact, TDP-43 has been shown to be acetylated in several residues within its RRMs in cells, thereby altering the specificity and affinity of its RNA binding (Cohen et al., 2015; Morato et al., 2020; Wang et al., 2017; Yu et al., 2020). Some of these modifications occur in the vicinity of a salt bridge connecting the two RRMs and stabilizing their binding to nucleic acids (Flores et al., 2019; Lukavsky et al., 2013), thereby disrupting RNA binding of TDP-43, and in turn, promoting LLPS. These findings suggest a role of acetylation in modulating phase transitions that could lead to aggregation. However, direct evidence of this transition remains to be shown.

Interestingly, acetylation can also happen in the NLS, shifting TDP-43 to a more cytoplasmic localization (Morato et al., 2020), which could also indirectly affect physiological LLPS by altering its availability to interact with nucleic acids or other LLPS modulators, like ATP (Dang et al., 2020) (see below). A potential transition from physiological LLPS to aggregation is supported by the fact that this acetyl modification is present in TDP-43 pathological aggregates (Cohen et al., 2015). Whether there are any cellular conditions favoring sustained TDP-43 acetylation that may trigger aggregation requires further investigation.

Tau can become physiologically acetylated, but the functional role of this modification remains unclear. Lysine acetylation has been shown to disfavor tau LLPS *in vitro* (Ferreon et al., 2018).

While further work is necessary to fully understand the relevance of acetylation and other PTMs in tau LLPS, given that electrostatic, rather than hydrophobic interactions drive tau LLPS (Boyko et al., 2019), it is conceivable that a tight interplay between different PTMs modulate tau phase behavior. In fact, acetylation can modulate other PTMs, like phosphorylation, in a variety of proteins (Choudhary et al., 2014). Recently, it has been shown that acetylation of Lys321 suppresses tau aggregation *in vitro* and prevents phosphorylation of the nearby Ser324 (Carlomagno et al., 2017), a marker of aggregated tau in tauopathy mouse models and patients with AD. Moreover, tau acetylation competes with ubiquitin for lysine availability, thereby preventing its degradation by the proteasome, and favors aggregation of phosphorylated tau (Min et al., 2010). Tau acetylation was also shown to occur early in disease (Min et al., 2015; Sohn et al., 2016), which highlights the potentially central role of acetylation in aberrant phase transitions. Since acetylation can also affect subcellular localization (Choudhary et al., 2014), which, in turn, determines protein interactions with non-homogeneously distributed factors like nucleic acids, we hypothesize that acetylation may play a more important, direct or indirect role in the modulation of LLPS than currently appreciated.

More studies on the regulation and the role of these PTMs in physiology and pathology are necessary to fully understand the whole picture. It is possible that the competition of several PTMs for the same modifiable residues, as is the case of methylation, acetylation, ubiquitination, and sumoylation for lysines (Tables S2–S5), plays a yet unexplored significance in regulating aberrant phase transitions. The presence of some PTMs might trigger these abnormal transitions by preventing the physiologically regulating PTMs in the same residue(s) that either suppress or maintain LLPS under physiological conditions.

RNA as a Key Modulator of LLPS

Multicomponent biomolecular condensates often contain nucleic acids, particularly RNA (Brangwynne et al., 2009), which can influence their formation in two major, non-exclusive, ways (Drino and Schaefer, 2018). First, RNAs can constitute the scaffold of a biomolecular condensate, thus, becoming the so-called "architectural RNAs" (arcRNAs) (Chujo et al., 2016). RNA secondary structure plays a vital role in this process, by determining the complex composition of condensates in cells through the formation of specific RNA-RNA and RNA-RBP interactions (Langdon et al., 2018; Polymenidou et al., 2011). This process can occur during arcRNAs transcription, such as is the case of NEAT1 and paraspeckles (Clemson et al., 2009), or during successive steps. This can be achieved through the RNA-binding domains harbored by many proteins undergoing LLPS (Lin et al., 2015) and allows a precise and dynamic regulation via modulation of RNA transcription, since LLPS only occurs in the presence of the arcRNA. Examples of this case may be TDP-43 and FUS, shell components of paraspeckles, to which they are recruited by direct binding to NEAT1 (Lagier-Tourenne et al., 2012; Modic et al., 2019; West et al., 2016). Moreover, both TDP-43 and FUS mostly undergo physiological LLPS in the nucleus (Gasset-Rosa et al., 2019; Maharana et al., 2018; Reber et al., 2019), where they bind their specific pre-mRNA targets on primarily intronic sites (Lagier-Tourenne et al., 2012; Polymenidou et al., 2011).

Second, RNA can influence the properties of established biomolecular condensates regardless of the ability of the phase-separated proteins to bind RNA. In particular, high RNA/protein ratios have been recently shown to keep the nuclear solubility of FUS in cells and *in vitro* (Maharana et al., 2018), independently of sequence specificity or RNA binding. How high RNA concentrations can achieve FUS solubilization remains to be described, but secondary structure may play an important role (Langdon et al., 2018). Taken together, the regulation of nuclear LLPS of FUS and TDP-43 appears to be a complex mechanism depending on the binding of RNA targets and protein interactors, as well as the abundance of non-target RNAs within the different nuclear subcompartments. Changes in either of these processes could therefore lead to an aberrant phase behavior.

Indeed, LLPS may alter the RNA-binding properties of RBPs (shown for FUS; Reber et al., 2019), and the maturation process of these condensates stabilizes protein interactions (Lin et al., 2015; Maharana et al., 2018; Molliex et al., 2015) and changes protein conformation in a way that might be incompatible with RNA binding. Therefore, RNA availability may regulate phase separation of multicomponent systems, and dysregulation of certain RNA species could lead to aberrant phase behavior (Berry et al., 2015; Kedersha et al., 2013). Currently, there are two main schools of thought on how RNA impacts protein aggregation and toxicity. Several studies have found that RNA binding mediates protein aggregation and neurotoxicity, supported by the fact that mutations disrupting RNA-binding of FUS and

TDP-43 reduces aggregation and toxicity in cells (Flores et al., 2019; Voigt et al., 2010), yeast (Johnson et al., 2009; Sun et al., 2011), and fly models (Daigle et al., 2013; Voigt et al., 2010). However, recent evidence suggests that aggregation may happen through an RNA-independent pathway, and the RNA binding by RBPs through their RRMs can prevent its aggregation (Armakola et al., 2012; French et al., 2019; Gasset-Rosa et al., 2019; Mann et al., 2019).

Given their high RNA content, SGs, which have long been considered the origin of aggregate formation, are one example where an imbalance in RNA could lead to aberrant phase transition. Interestingly, mutations in many RBPs involved in SG formation, such as hnRNP A1, hnRNP A2B1, ATXN2, and TIA1, and not only TDP-43 or FUS, have been shown to cause or increase the risk of ALS/FTD (Elden et al., 2010; Kim et al., 2013; Mackenzie et al., 2017). Persistent SG formation by diverse stressors or a defect in their disassembly could favor the aberrant transition of LLPS-mediated TDP-43 condensates into aggregates (Zhang et al., 2019), as aggregated TDP-43 has been shown to co-localize with SG markers in ALS and FTLD-U postmortem brain (Liu-Yesucevitz et al., 2010). However, recent studies have shown that protein aggregation can happen independently from SG formation and maturation (Fang et al., 2019; Gasset-Rosa et al., 2019; Mann et al., 2019). Ample evidence supporting the role of RNA in both mediating and suppressing protein aggregation and neurotoxicity suggest that there may be two different, independent pathways by which aggregates originate from biomolecular condensates. This might explain the fact that aggregates of disease-associated RBPs like FUS and TDP-43 are not always identical in structure and co-aggregated proteins (Laferrière et al., 2019; Neumann et al., 2011, 2012). Whether aggregates originate from different biomolecular condensates, LLPS-independent pathways, or both, needs further investigation.

In neurodegenerative diseases, RNA metabolism is severely affected, altering a broad ensemble of functions such as RNA degradation (Weskamp and Barmada, 2018), RNA editing (Lorenzini et al., 2018), and cytoplasmic RNA export (Boehringer and Bowser, 2018). Interestingly, the growing field of the epitranscriptome is revealing the potential role of RNA modifications, such as methylation that can regulate RNA-protein interactions (Liu et al., 2015), in neurodegenerative diseases, such as AD (Han et al., 2020). A pathological synergistic mechanism could be behind the neurotoxicity-related RNA metabolism defects: alterations in RNA metabolism due to changes in the cellular environment can lead to a change in the equilibrium of phase-separated systems and cytoplasmic aggregation of RBPs, which, in turn, cannot perform their RNA-associated functions and enhance cellular stress and neurodegeneration.

The Role of Protein-Protein Interactions in LLPS and Aggregation

Small changes in scaffolds and client valencies (Table 1) can result in major alterations in client recruitment, thereby modifying the properties of biomolecular condensates (Sanders et al., 2020; Su et al., 2016) and potentially leading to aberrant behavior. Similarly, LLPS can also be aberrantly influenced by the altered interaction of the phase-separated proteins with other proteins under non-physiological circumstances. Granulins, the proteolytic product of progranulin (encoded by the gene *GRN*), can selectively modulate the LLPS of the TDP-43 LCR *in vitro* through direct interaction (Bhopatkar et al., 2020) and are known to contribute to the TDP-43-induced cytotoxicity and thus to disease progression (Salazar et al., 2015). Notably, disease-associated mutations in other genes that are not encoding for the aggregated proteins can also culminate in the formation of these species. For example, mutations in *TARDBP* only account for a small number of familial cases of TDP-43 proteinopathies (Mackenzie et al., 2010). In fact, mutations in several other genes, like *GRN* itself (Baker et al., 2006; Cruts et al., 2006) or *VCP* (Gitcho et al., 2009), also result in the aggregation of TDP-43, highlighting the tight cellular regulation of these proteins and how other factors can influence their LLPS.

Subcellular Localization as a Determinant of Interacting Factors Influences LLPS

Subcellular localization plays a key role in this transition, as it can further determine other factors influencing LLPS such as available protein or RNA interactors. For example, cytoplasmic FUS, mislocalized due to ALS-linked mutations disrupting its NLS (Kuang et al., 2017; Kwiatkowski et al., 2009), might still bind its specific targets but in an environment with lower overall RNA concentration (Hock et al., 2018; Maharana et al., 2018; Sahadevan et al., 2020), which could lead to sustained LLPS over time. Interestingly, FUS aggregation in ALS is always caused by a genetic mutation and accumulates in aggregates, where it is thought to be the main component, while FUS co-aggregates with other proteins of its family (EWSR and TAF-15) and its nuclear transporter, transportin-1, in FTD cases (Neumann et al., 2011, 2012), suggesting that different molecular events trigger FUS aggregation in the two diseases.

Strikingly, nucleocytoplasmic transport has been shown to be defective in many neurodegenerative diseases (Li and Lagier-Tourenne, 2018), which suggests a central role of an altered nuclear pore complex (Table 1) in disrupting the equilibrium between the nucleus and the cytoplasm in these disorders (Kim and Taylor, 2017). *In vitro*, nuclear import receptors (Table 1) can act as molecular chaperones and reverse aberrant phase transitions of disease-linked RBPs with prion-like domains such as TDP-43, hnRNP A1, hnRNP A2, and the FET protein family, including FUS, EWSR1, and TAF15, by binding to regions that directly contribute to LLPS (Guo et al., 2018; Hofweber et al., 2018; Qamar et al., 2018; Yoshizawa et al., 2018). In yeast and mammalian cells, nuclear import receptors can antagonize RBP phase transitions and toxicity (Guo et al., 2018; Hofweber et al., 2018; Qamar et al., 2018; Yoshizawa et al., 2018).

ATP and Small Molecules Regulate LLPS

In recent years, it has been suggested that LLPS behavior and material properties of biomolecular condensates are regulated by small molecules like ATP (Brangwynne et al., 2011; Jain et al., 2016). ATP can affect phase separation as an energy source (Parry et al., 2014), as substrate for ATPases regulating the dynamics of biomolecular condensates (Jain et al., 2016), and as a hydrotrope (Patel et al., 2017), thereby altering protein solubility. ATP concentrations differ in certain cellular subcompartments, like mitochondria (Imamura et al., 2009), or in certain conditions, such as genetic disorders or cellular stressors (Mendelsohn et al., 2018), which can work as localized switches of LLPS.

Recent studies have shown that the physiological LLPS of FUS is regulated by ATP binding *in vitro*. While small ATP concentrations favored FUS organization in droplets, higher ones dissolved LLPS, due to the bivalent binding behavior of ATP at low concentrations and its hydrotropic features in high concentrations (Kang et al., 2018; Patel et al., 2017). Similarly, TDP-43 properties can be modulated by ATP *in vitro*. ATP can bind the NTD of TDP-43 and favor functional oligomerization (Wang et al., 2019), which is important for LLPS (Wang et al., 2018a). More recently, the RRMs of TDP-43 have been shown to bind ATP at physiologically relevant concentrations, a binding that stabilizes their thermodynamic stability and potentially prevents transition to aggregation (Dang et al., 2020; Patel et al., 2017). However, ALS-associated TDP-43 mutation D169G can prevent its binding to ATP *in vitro* (Dang and Song, 2020), suggesting that changes in the charge or hydrophobicity may be a driving force for aggregation.

ATP is required for the dynamics of some membraneless compartments formed by LLPS such as SGs, which form in the cytoplasm in response to a variety of cellular stressors (Hock et al., 2018; Kedersha et al., 2013; Molliex et al., 2015; Zhang et al., 2019). Thus, modulation of cellular ATPases can actually alter their assembly and disassembly (Jain et al., 2016), and depletion of ATP has been shown to increase their viscosity and decrease their dynamics (Brangwynne et al., 2011). Interestingly, SG proteins in general are enriched in features that favor protein LLPS, such as IDRs, increased valency (Table 1), multiple domains, RNA-binding motifs, and increased potential for PTMs (Kuechler et al., 2020) (Figure 2). FUS (Andersson et al., 2008), tau (Brunello et al., 2016), and TDP-43 (Colombrita et al., 2009), as well as other key proteins involved neurodegenerative diseases, such as hnRNP A1 (Guil et al., 2006), can be incorporated into SGs. In fact, many of the aforementioned LLPS modifiers for these proteins, such as FUS methylation (Hofweber et al., 2018), also determine the inclusion of these proteins into SGs, potentially playing a modulating role in transition to aggregation.

Interestingly, mitochondrial dysfunction is implicated in aging and neurodegenerative diseases (Wu et al., 2019b). Defects in mitochondrial respiration could lead to an overall reduction of ATP levels or impairment of ATP-dependent processes, which might maintain the liquid state of biomolecular condensates (Parry et al., 2014; Patel et al., 2017). In general, cellular stress plays a significant role in droplet demixing and transition to aggregation. For example, PD-associated stressors have been shown to enhance maturation of α-synuclein biomolecular condensates (Ray et al., 2020). Under stress conditions, TDP-43 undergoes transient stress-induced de-mixing, leading to cytoplasmic aggregation, nuclear clearance, and cellular death, in a SG-independent pathway (Gasset-Rosa et al., 2019; Mann et al., 2019). Excitingly, small compounds have been shown to interrupt the cytoplasmic accumulation process and prevent aggregation in the cells, limiting the toxic effect of cytoplasmic aggregation (Fang et al., 2019).

ATTACK OF THE CLONES: LLPS-DERIVED AGGREGATES AS SEEDS FOR PRION-LIKE TEMPLATING AND STRAIN DIVERSITY

Cell-to-cell propagation of protein aggregates and spreading through interconnected brain regions is an underlying mechanism of neurodegenerative diseases (Polymenidou and Cleveland, 2011; Vaquer-Alicea and Diamond, 2019) and has been shown for many of the proteins undergoing LLPS, such as α-synuclein (Cavaliere et al., 2017; Luk et al., 2012a), tau (de Calignon et al., 2012; Wu et al., 2016), and, recently, also TDP-43 (Porta et al., 2018). While the role of biomolecular condensates in seeding of protein aggregates is unknown, the crowded environment of the condensed phase may well accommodate both the formation and templating of pathological assemblies. A recent study showed that introducing either FUS or TDP-43 fibrils could indeed trigger TDP-43 aggregation within endogenous droplets (Gasset-Rosa et al., 2019), suggesting a synergistic effect of exogenous aggregates and cytoplasmic liquid droplets in the formation of immobile structures. These results support the notion that LLPS is at the transition between proper protein function and risk of aggregation, yet they imply mechanisms beyond the classical prion-like templating (Polymenidou and Cleveland, 2011), since non-homologous "seeds" (FUS and TDP-43) trigger TDP-43 aggregation.

Structural diversity of pathological assemblies underlies disease heterogeneity of prion strains (Sigurdson et al., 2019), a notion that was expanded to tau (Dujardin et al., 2020; Sharma et al., 2018), α-synuclein (Strohäker et al., 2019), and, recently, also TDP-43 (Laferrière et al., 2019). The role of LLPS in the emergence of pathological assemblies with distinct structures and differential propagation mechanisms, remains unknown. In view of the different neuropathological profiles characterizing patients with subtypes of tauopathies, α-synucleinopathies, TDP-43-, and FUS proteinopathies, an attractive hypothesis may be that all the modifiers of LLPS and its transition to aggregation described above, may contribute to the inception of structurally distinct aggregates. This could be illustrated within the two subtypes of FTLD-TDP, type A, characterized by compact oval or crescent-shaped cytoplasmic TDP-43 aggregates and short dystrophic neurites, predominantly in layer II of the neocortex, while type C pathology mainly features TDP-43 pathological assemblies along tortuous neurites predominantly in the superficial cortical laminae (Kawakami et al., 2019). Patients within these two subtypes show distinct clinical manifestations, genetic associations, and, most importantly, rates of progression, with FTLD-TDP-A patients experiencing a significantly faster disease progression (Laferrière et al., 2019). It is conceivable that these distinct TDP-43 pathological structures arise from initially physiological LLPS-derived condensates, that becomes aberrant within the specific subcellular microenvironment, i.e., cytoplasmic and adjacent to the nucleus in type A versus neuritic/axonal compartments in type C. This would lead to distinct interactions with other proteins, RNAs, and organelles, which are specifically localized in the respective locations, and these interactions could, in turn, dictate their differential seeding and toxic properties (Laferrière et al., 2019). Future studies are needed to elucidate the determinants of the differential features of neurodegenerative disease subtypes in FTLD and beyond.

A NEW HOPE: MOVING FORWARD TO UNDERSTAND THE LINK BETWEEN LLPS AND PROTEIN AGGREGATION

In normal aging as in neurodegenerative diseases, proteostasis network failure, increase of protein insolubility, and amyloid-

like aggregation is part of the normal cellular senescence process (David et al., 2010; Klaips et al., 2018). Interestingly, aggregation-prone proteins undergoing LLPS are found to form β sheet structures, which results in increased toxicity and contribute to the functional decline by affecting a significant amount of protein regulating cellular and organism lifespan (David et al., 2010; Wang et al., 2014). However, a direct link between LLPS and protein aggregation is still missing. Most LLPS studies until now are based on *in vitro* systems, or protein overexpression in cellular models coupled with harsh stress conditions (Hergesheimer et al., 2019). While informative, these approaches are prone to misinterpretation, since overexpression alters protein stoichiometry, which may result in scaffold-client and proteasome imbalance and proteostasis stress (Figure 4), a phenomenon observed in aging organisms and correlated with increased protein aggregation (Klaips et al., 2018; Walther et al., 2015). Despite significant progress in our understanding of these processes over the past years, the definitive answer on how these mechanisms really occur physiologically, and how they contribute to the transition to pathology is still missing. In this last section we present new technologies that have been underutilized in this quest and present some of the most promising therapeutic approaches that have resulted from the study of LLPS in human disease.

Biophysical Assays to Understand Dynamics and Atomic Structures of LLPS-Derived Condensates and Pathological Assemblies

To fully understand by which process a biopolymer undergoes LLPS, which residues and therefore interaction types are important, and what happens in cases of protein-intrinsic modulators, high resolution structural information is needed. However, because most protein domains involved in LLPS are IDRs, the study of their dynamic and structural properties is challenging. In recent years, many new biophysical approaches have been optimized to understand the role of these regions in LLPS (Babinchak et al., 2019; Conicella et al., 2016; Murthy et al., 2019). For example, Raman spectroscopy has been used to study changes in secondary structural elements of biomolecular condensates and the change induced by modulators (Murthy et al., 2019). On the other hand, nuclear magnetic resonance (NMR) spectroscopy has enabled the studying of dynamics of phase-separating proteins in their dilute and condensed phase (Conicella et al., 2016, 2020; Murthy et al., 2019). In contrast to Raman spectroscopy, NMR enables the identification of key residues for intermolecular interactions (Conicella et al., 2016, 2020), as well as site-specific transient secondary structures important for LLPS (Conicella et al., 2016, 2020). Dynamics, distance distribution, and differences between conformational ensembles in both phases can be studied using single-molecule Förster resonance energy transfer (smFRET) (Nasir et al., 2019) and electron paramagnetic resonance (EPR) spectroscopy (Babinchak et al., 2019). Interestingly, smFRET can be used to study conformational heterogeneity and dynamics of biopolymer in cells (König et al., 2015) enabling biophysical and structural measurements in cellular environments.

In recent years, several studies explored the atomic structures of fragments from proteins with IDRs, connected to phase separation and neurodegeneration. Analyses of recombinant TDP-43 by X-ray diffraction (Guenther et al., 2018a), or cryoelectron microscopy (cryo-EM) (Cao et al., 2019) illustrated its structural versatility, supporting the potential existence of different pathological TDP-43 strains in patient brains (Laferrière et al., 2019). Indeed, parts of its LCD (Guenther et al., 2018b) or its RRM2 (Guenther et al., 2018a) can form either steric zippers or reversible amyloid-like structures, termed low-complexity aromatic-rich kinked segments or LARKS. LARKS are formed by peptides derived from several IDR-containing proteins (Hughes et al., 2018) and may therefore be critical for LLPS, albeit their role in the context of the full-length protein, the biomolecular condensate and the complex cellular environment remains unclear. Other TDP-43 LCD segments form reversible or irreversible amyloid fibrils (Cao et al., 2019), and at least one ALS-associated mutation converts the former to the latter, supporting a pathogenic mechanism triggered by irreversible fibrils. A switch between reversible to irreversible fibrils has also been reported during muscle regeneration, when TDP-43 forms reversible amyloid-like structures, termed myogranules, which have a functional role in sequestering mRNA encoding sarcomeric proteins (Vogler et al., 2018) but are increased in inclusion body myopathies, muscle diseases characterized by cytoplasmic accumulations of RBPs, including TDP-43 (Picchiarelli and Dupuis, 2020).

Cryo-EM studies on α-synuclein (Li et al., 2018a) and tau (Fitzpatrick et al., 2017; Lippens and Gigant, 2019) support their polymorphic nature, in line with the neuropathological heterogeneity reported in patients. The next challenge in this field is to relate these findings with the complex structures found in cells, and, most importantly, in the human brain.

Atomic resolution structures of these entities are essential not only for understanding their heterogeneity in disease but also for engineering small molecules that selectively bind the pathological forms, for personalized diagnosis (for instance with positron emission tomography or PET imaging) and therapy.

New Imaging Techniques to Go Deeper in Our Observations

Biophysical, biochemical, and structural approaches, like those described above, are critical for understanding both the physiological role of LLPS and the molecular interactions that lead to the conversion of physiological to pathological assemblies. However, in order to acquire essential four-dimensional information within the cellular environment, imaging technologies and cellular and animal models are necessary. Most previous studies used conventional imaging techniques, including light and confocal microscopy, which, due to the Abbe law, are limited by their ability to physically separate two molecules close to 250 nm from each other (Zalevsky, 2013). Better characterization of biomolecular condensates and membraneless organelles requires the ability to access near single-molecule resolution, using not only fixed but also live samples, by super-resolution microscopy (SRM). The use of SRM would allow higher spatial resolution and direct quantitative imaging (Huang et al., 2010), two important factors toward improving our understanding of LLPS and aggregation mechanisms. Additionally, live-cell imaging coupled with SRM can be used to record in real-time events at a more resolutive scale (Chen et al., 2018; Wang et al., 2014). Recently, the use of SRM uncovered an unknown role of the nucleolus (Frottin et al., 2019), direct observation of amyloid

fibrils growth and aggregation (Pinotsi et al., 2014), and for three-dimensional live imaging of the interactions between cytoskeleton and mitochondria (Chen et al., 2018). The use of SRM in neurodegenerative disease research so far has uncovered novel mechanisms participating to the disease (De Rossi et al., 2020; Yuan et al., 2016).

Improving Our Experimental Models for the Study of LLPS in Disease

Most "*in vivo*" experiments exploring LLPS have been performed in the *Xenopus laevis* oocyte model (Brangwynne et al., 2011; Hayes et al., 2018). Despite being a powerful tool for live imaging, this model only allows for single-cell analysis. The second limitation of numerous studies is the use of artificial models to answer physiological questions and the limited use of *in vivo* models. It is worth mentioning that most of the published work has been performed in cell lines, even when exploring properties of neuronal proteins, with few exceptions (Markmiller et al., 2018; Wegmann et al., 2018). Moving forward, it would be important to use more relevant models such as primary or human neuronal cell cultures to explore specific mechanisms at play in neurodegenerative diseases. For example, the use of *C. elegans* models for these specific questions is a considerable asset (Alexander et al., 2014). *C. elegans* allows for direct observation of liquid droplets by SRM (Wang et al., 2014). Similarly, *Dario renio* and *Drosophila melanogaster* models have been used to model pathological changes associated with tau (Chapuis et al., 2013; Hassan-Abdi et al., 2019), α-synuclein (Prabhudesai et al., 2016), TDP-43 (Anderson et al., 2018; Asakawa et al., 2020), or FUS (Matsumoto et al., 2018; Steyaert et al., 2018). These models offer the advantage of *in vivo* imaging at the subcellular resolution and with relatively low cost compared with larger animal models. Moreover, these models allow for exploring protein aggregation mechanisms in living organisms (David et al., 2010; Lobos-Ruiz et al., 2018; Tsuboyama et al., 2020) and are powerful for high-throughput screening of therapeutic compounds for neurodegenerative diseases (Chen et al., 2015; François-Moutal et al., 2019; Lobos-Ruiz et al., 2018).

Emerging Therapeutics Targeting LLPS-Related Mechanisms

Targeting LLPS to treat neurodegenerative diseases is a tempting idea, since this approach could block the transition to aggregation while maintaining cellular physiological functions (Mullard, 2019). This is particularly true for TDP-43 proteinopathies, which result from a combination of direct toxicity by TDP-43 aggregates and loss of normal nuclear function. This combination excludes many therapeutic strategies that are being pursued for other neurodegenerative diseases, including gene silencing by antisense oligonucleotides (ASO) or related technologies, which would be catastrophic in this case as TDP-43 is essential for neuronal survival. An exciting alternative is ASO treatment against Ataxin 2, another RBP undergoing phase separation, which has proven effective in preventing TDP-43 aggregation in a mouse model of ALS (Becker et al., 2017). Unlike TDP-43, loss of Ataxin-2 protein is well tolerated in mice (Kiehl et al., 2006), and this therapeutic strategy may be moving toward clinical trials in the coming years. Another promising approach might be to restore the equilibrium to maintain the LLPS state and block the transition to aggregation. One possibility is to target the enzymes responsible for the PTMs driving this transition. For example, inhibition of CK-1δ responsible for TDP-43 phosphorylation (Martínez-González et al., 2020), inhibition of TDP-43 SUMOylation by anacardic acid (Maurel et al., 2020), or increase TDP-43 acetylation by activation of HSF1 (Wang et al., 2017) revert its aggregation, while maintaining its nuclear physiological localization in models for ALS. Similarly, inhibition of PARP-1/2, responsible for polyADP-ribosylation of TDP-43, by small molecules blocks the formation of cytoplasmic aggregates (McGurk et al., 2018). Another attractive strategy for positively interfering with TDP-43 LLPS might be to stabilize the nuclear physiological oligomers to counteract its pathologic cytoplasmic aggregation (Afroz et al., 2017), while preserving its normal nuclear function. While therapeutic compounds preserving physiological LLPS and preventing condensate maturation of α-synuclein and tau have not been developed yet, these emerging approaches described above for TDP-43 will surely inspire future directions for α-synucleinopathies and taupathies.

In general, small molecules interfering in the aggregation process is a promising approach, and the use of *in vivo* high-throughput models such as *Drosophila melanogaster* or *C. elegans* is powerful for testing new compounds, as was shown in models for FUS proteinopathies (Wheeler et al., 2019). Another approach would be to restore the equilibrium between scaffolds and clients, necessary for proper LLPS. However, this requires a better understanding of LLPS, the proteins involved, its physiological role(s), and its regulation mechanisms, using relevant models and techniques.

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

A.Z. performed the computational analyses shown in Figure 2 and prepared all figures, with the help of M.P.B.; M.P.B. prepared all the supplementary tables, with the help of A.Z.; A.Z., M.P.B., P.D.R., and M.P. reviewed the figures and wrote the manuscript.

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