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Aberrant Phase Transitions: Side Effects and Novel Therapeutic Strategies in Human Disease

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Phase separation is a physiological process occurring spontaneously when single-phase molecular complexes separate in two phases, a concentrated phase and a more diluted one. Eukaryotic cells employ phase transition strategies to promote the formation of intracellular territories not delimited by membranes with increased local RNA concentration, such as nucleolus, paraspeckles, P granules, Cajal bodies, P-bodies, and stress granules. These organelles contain both proteins and coding and non-coding RNAs and play important roles in different steps of the regulation of gene expression and in cellular signaling. Recently, it has been shown that most human RNA-binding proteins (RBPs) contain at least one low-complexity domain, called prion-like domain (PrLD), because proteins harboring them display aggregation properties like prion proteins. PrLDs support RBP function and contribute to liquid–liquid phase transitions that drive ribonucleoprotein granule assembly, but also render RBPs prone to misfolding by promoting the formation of pathological aggregates that lead to toxicity in specific cell types. Protein–protein and protein-RNA interactions within the separated phase can enhance the transition of RBPs into solid aberrant aggregates, thus causing diseases. In this review, we highlight the role of phase transition in human disease such as amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and in cancer. Moreover, we discuss novel therapeutic strategies focused to control phase transitions by preventing the conversion into aberrant aggregates. In this regard, the stimulation of chaperone machinery to disassemble membrane-less organelles, the induction of pathways that could inhibit aberrant phase separation, and the development of antisense oligonucleotides (ASOs) to knockdown RNAs could be evaluated as novel therapeutic strategies for the treatment of those human diseases characterized by aberrant phase transition aggregates.

Keywords: RNA-binding proteins, phase separation, RNA therapeutics, neurodegenerative disease, low-complexity domain

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INTRODUCTION

Eukaryotic cells are characterized by morphologically distinct compartments displaying multiple roles in biological processes. The complementary use of light- and electron-microscopic imaging techniques has allowed to shape eukaryotic subdomains highlighting the presence of membrane-less organelles (MLOs), including paraspeckles, nuclear speckles, Cajal bodies,

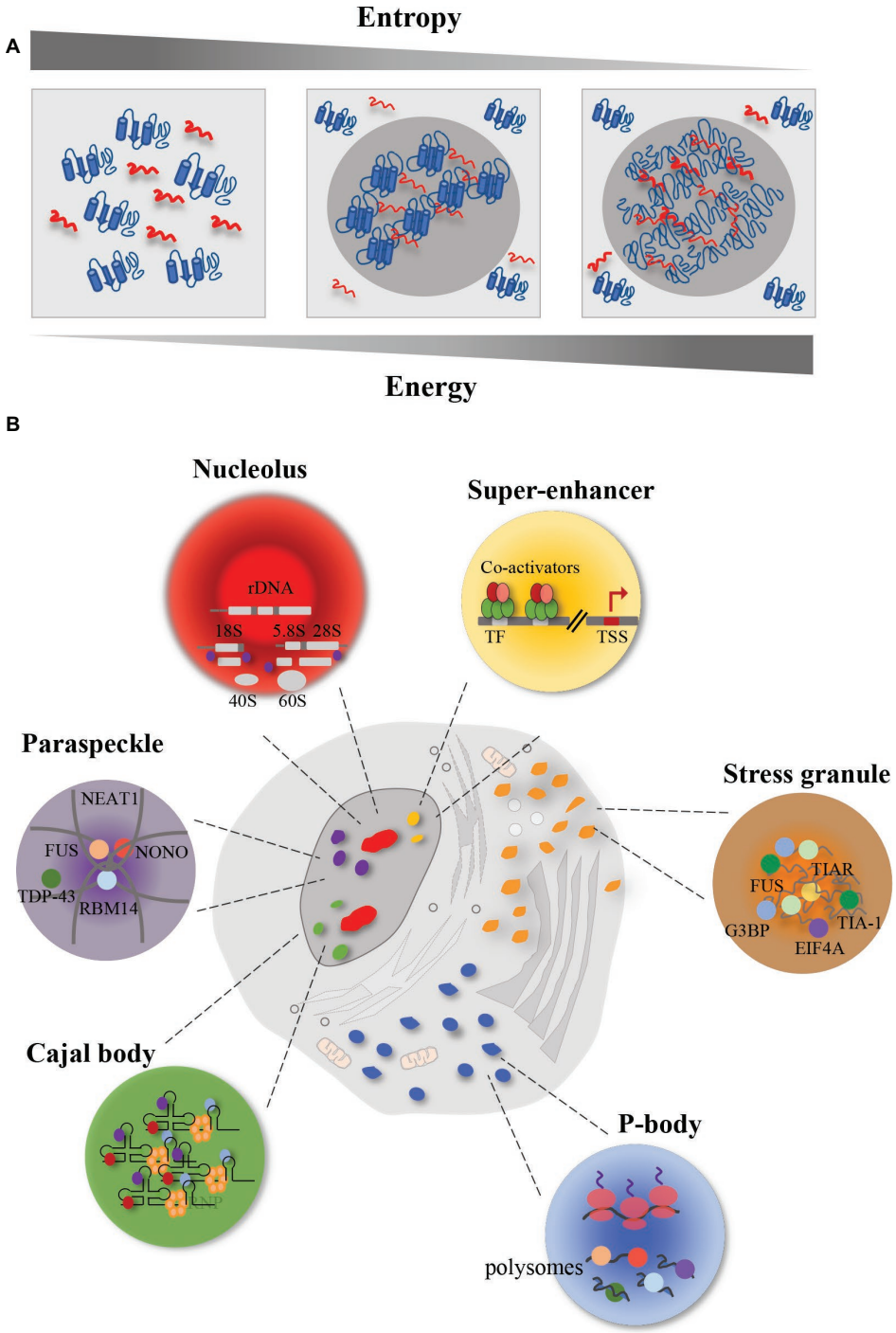


FIGURE 1 | Schematic representation of membrane-less organelles (MLOs). Eukaryotic cells contain different MLOs formed by liquid–liquid phase separation. **(A)** Representation of liquid–liquid phase separation. In order to promote interaction between macromolecules and to obtain a chemical equilibrium, high energy is required with a correspondent reduction of entropy. In this condition, proteins (in blue) and RNA (in red) are prone to undergo liquid–liquid phase separation. In particular, the increase in energy and the reduction in entropy lead to a decrease in macromolecule solubility and an increase in protein-RNA interactions. The result is a higher concentrated phase, that promotes the formation of membrane-less organelles (MLOs). **(B)** Representation of membrane-less organelles (MLOs). The MLOs form sub-compartments both in the nucleus and in the cytoplasm. Nucleus is characterized by the presence of nucleoli (in red) that are involved in the ribosome biogenesis; paraspeckles (in purple), whose formation is promoted by the lncRNA *NEAT1*, that interacts with several RBPs, including FUS, in the *core,* and TDP-43 in the shell; Cajal bodies (in green) that are involved in the snRNP biogenesis; and super-enhancers (in yellow) that are clusters of master transcription factors and transcriptional co-activators involved in gene expression. In the cytoplasm, eukaryotic cells harbor P-bodies (in blue), involved in the control of mRNA translation and mRNA storage, and stress granules (in orange) whose formation, upon stress condition, requires the interaction between RNA (in grey) and proteins.

stress granules (SGs), and processing bodies (P-bodies), in addition to the classical membrane-enclosed organelles (such as nuclei, mitochondria, endoplasmic reticulum, and Golgi apparatus) (Matera, 1999). These MLO compartments shape similarly, with analogous build up characteristics, but they differ in composition and sub-cellular localization. Indeed, the MLOs form sub-compartments both in the nucleus and in the cytosol (**Figure 1**), and contain nucleic acids and proteins necessary to accomplish their function, thus providing a spatiotemporal control of biological activities (Shin and Brangwynne, 2017). For this reason, these compartments must remain separated from cytoplasm and nucleus (Banani et al., 2017). The multiple components that concentrate within these subdomains render them a suitable interface for various cellular processes, such as transcription, RNA processing, mRNA transport, RNP assembly, ribosome biogenesis, translational repression, mRNA degradation, and intracellular signaling (Banani et al., 2017).

Several efforts have been devoted to understand the process of MLO formation and how phase separation is involved in promoting their assembly (Hyman et al., 2014). The relevance of these MLOs is demonstrated by the fact that changes in their organization are associated with disease phenotypes (Aguzzi and Altmeyer, 2016). Growing evidences suggest that these organelles are involved in the pathogenesis of neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (Murakami et al., 2015; Rhoads et al., 2018), as well as in cancer (Aguzzi and Altmeyer, 2016; Bouchard et al., 2018).

This review explores individual MLOs, with emphasis on how they contribute to biological functions and how their dysregulation promotes the development of human disease. In this regard, we describe possible therapeutic strategies to monitor the correct formation of these compartments, and therapeutic approaches to selectively destroy aberrant MLOs.

PHASE SEPARATION: MECHANISM, COMPARTMENTS, AND BIOLOGICAL FUNCTIONS

Phase separation is a physiological process by which macromolecules separate in a dense phase and in a dilute phase within cells, thus allowing the formation of distinct chemical environments (Shin and Brangwynne, 2017). In order to promote interaction between macromolecules, rather than between macromolecules and solvent, and to obtain a chemical equilibrium among the compartments just formed, high energy is required (Johansson et al., 1998), with a correspondent reduction of entropy (**Figure 1A**). The result is a higher concentrated phase, where the proteins and DNA/RNA cluster ~ 10–100-fold more (Li et al., 2012), and a lower concentrated phase (Banani et al., 2017). In the phase separation process, macromolecule solubility is decreased (Alberti, 2017). Liquid–liquid phase separation (LLPS) occurs spontaneously in eukaryotic cells when a critical concentration or temperature threshold is exceeded, thus forming sub-compartments in a reversible fashion (Hyman et al., 2014; Shin and Brangwynne, 2017). The assembled MLOs must remain separated by the surrounding environment, both in the nucleus and in the cytoplasm. These organelles in turn support the transport of molecules in and out themselves, thus allowing chemical reactions inward (Hyman et al., 2014).

To obtain liquid–liquid demixing, several conditions are required, such as electrostatic and hydrophobic interactions, achieved by the presence of low-complexity sequences, and intrinsically disordered protein regions (IDRs) (Nott et al., 2015), that are involved in protein–protein (Lee et al., 2015), and protein-RNA interactions (Allain et al., 2000). RNA can initiate by itself phase separation *via* protein-RNA (Molliex et al., 2015) and RNA–RNA interactions (Jain and Vale, 2017; Van Treeck et al., 2018) and serves as a molecular seed that triggers liquid demixing (Molliex et al., 2015).

Eukaryotic cells harbor MLOs both in the nucleus and in the cytoplasm. The first MLO identified was the **P granule** of *Caenorhabditis elegans* embryos, involved in cytoplasmic polar partitioning (Strome and Wood, 1983). P granules have been defined as a liquid-like compartment. Indeed, they display a spherical morphology due to surface tension. They can fuse together with other P granules and be deformed by flows, then rapidly rearranging (Brangwynne et al., 2009). P granules contain mRNAs and RNA helicases and play a key role in the post-transcriptional regulation of mRNA in the germ cells (Voronina et al., 2011).

In the cytoplasm, eukaryotic cells can form processing bodies (P-bodies) and stress granules (**Figure 1B**). **P-bodies** are cytoplasmic ribonucleoprotein (RNP) granules (Franks and Lykke-Andersen, 2008). Fluorescence microscopy has shown that proteins and RNAs shuttle between cytoplasm and P-bodies and fuse, showing liquid-like properties (Kedersha et al., 2005). These RNP granules play a role in post-transcriptional regulation, by controlling mRNA translation and degradation (Parker and Sheth, 2007). Indeed, their assembly depends on the loading of mRNAs into polysomes. When mRNAs are associated with ribosomes, P-bodies decrease in abundance and size (Sheth and Parker, 2003; Teixeira et al., 2005); whereas when mRNAs dissociate from ribosomes, as a result of translation inhibition, P-bodies increase in dimensions (Teixeira et al., 2005; Koritzinsky et al., 2006). Indeed, mRNA decay can occur also in absence of P-bodies (Eulalio et al., 2007), thus assuming that P-bodies act by segregating mRNAs rather than degrading them (Parker and Sheth, 2007).

In addition to P-bodies, cytoplasm of eukaryotic cells harbors other types of RNA granules, such as **stress granules (SGs)** (Collier and Schlesinger, 1986; Arrigo et al., 1988). Fluorescence microscopy (Jain et al., 2016) and electron-dense regions' micrographs (Souquere et al., 2009) revealed that SGs have a highly concentrated *core* made up by proteins and mRNA, and a surrounding structure which is less dense and more dynamic (Protter and Parker, 2016). Phase separation is extremely sensitive to changes in chemical conditions, thus playing an important role in stress adaptation. In fact, stress is a transient phenomenon and SGs are transient structures rapidly disassembling upon removal of the stress condition.

For instance, after removal of the adverse condition, SGs disassemble, protein synthesis is reactivated after the translation inhibition induced by the stress, and RBPs can either go back to the nucleus or remain in the cytoplasm to carry out their functions (Gilks et al., 2004; Panas et al., 2016).

The formation of these bodies occurs when stress conditions block translation initiation, by phosphorylating eIF2α or inactivating eIF4A (Mokas et al., 2009). In general, this block induces sudden increase in non-polysomal mRNAs, masked by translating ribosome, that is free to interact with RBPs, such as TIA-1,TIA-R, and G3BP (Kedersha et al., 1999; Tourrière et al., 2003). These proteins interact with other proteins through specific domains, thus promoting SG formation (Protter and Parker, 2016). Phase transition can be promoted and largely affected by post-translational modifications of SG-associated protein, such as methylation, phosphorylation, and glycosylation, that alter protein–protein interaction (Tourrière et al., 2003; Ohn et al., 2008; Nott et al., 2015).

In general, SGs help cells to respond to adverse conditions, such as oxidative stress, heat shock, and DNA damage (Kedersha and Anderson, 2007; White and Lloyd, 2012). Upon DNA damage, Moutaoufik and colleagues observed that UV-induced SGs were smaller and less numerous than the SGs induced by other stressors, such as arsenite or heat treatment (Moutaoufik et al., 2014). The cellular response to UVC irradiation involves several steps that allow cells to identify the damage and to repair the DNA. These processes include the surveillance of genome integrity, the recognition of damaged DNA, the activation of the DNA repair program, including cell signaling events and cell cycle arrest, thus allowing cells to repair the DNA before resuming proliferation (Polo and Jackson, 2011). Indeed, it has been shown that upon UV-induced DNA damage, SG assembly occurs in a cell cycle-dependent fashion (Pothof et al., 2009), with cyclin A-positive S phase cells and γH2AX-positive cells negative for SG-specific staining (Pothof et al., 2009). These studies demonstrate that SG formation occurs only in a time window of G2-M transition or after exit from mitosis. In fact, as cells prepare for cell division, most MLOs disassemble and then start to reassemble during the late stages of cytokinesis (Andrade et al., 1993; Hernandez-Verdun et al., 2002; Fox et al., 2005; Aizer et al., 2013). In particular, the kinase activity of DYRK3 plays an essential role during mitosis to prevent the formation of aberrant LLPS condensates composed by nuclear and cytoplasmic proteins and RNA, by keeping the condensation threshold of its substrates high (Rai et al., 2018).

During stress, fluctuations in cytosolic pH can promote widespread condensate formation and a core group of nucleating RBPs is sufficient to initiate formation of the stress granule. For instance, in budding yeast, cells can enter into a quiescent state upon removal of nutrients that causes a shift in the cytosolic pH from 7.4 down to ~6.0. This increase in proton concentration triggers a phase transition; as soon as conditions improve, yeast fluidize the cytoplasm by using proton pumps and neutralizing the pH, thus restoring normal conditions (Munder et al., 2016).

Collectively, phase separation offers a suitable architecture to regulate and compartmentalize biochemical processes inside cells.

Several MLOs form within the cell nucleus (**Figure 1B**). A typical example of MLO with liquid-like properties is the **nucleolus** that forms around ribosomal DNA loci in the cell nucleus (Shaw and Jordan, 1995; Brangwynne et al., 2011). Nucleoli are RNA-protein compartments displaying a key role in the ribosome biogenesis (Andersen et al., 2002). Taking advantage of the electron microscopy, it has been shown that this process occurs in three distinct sub-regions of the nucleolus, formed as a result of LLPS (Boisvert et al., 2007). The transcription of rDNA starts in the fibrillar centers sub-region, that is enriched in RNA polymerase I (RNAPI). Then, this process continues in the dense fibrillar components sub-region, where also processing and modification of pre-rRNA transcripts occur. The assembly of the ribosome is accomplished in the granular components, enriched in proteins (Boisvert et al., 2007). This organization into sub-compartments resembling a multi-layer structure has been found also in other liquid-like MLOs in the cell nucleus, including paraspeckles (Feric et al., 2016).

Paraspeckles are nuclear bodies involved in the control of gene expression and DNA repair, and characterized by the presence of RBPs, including the *Drosophila* behavior/human splicing (DBHS) family of splicing proteins (the paraspeckle protein 1 PSPC1, RBM14, and NONO), FUS and TDP-43 proteins (Fox et al., 2002). It has been shown that the formation of paraspeckles is driven by RNA, in particular by the long non-coding RNA (lncRNA) *NEAT1* (Souquere et al., 2010). Knockdown of *NEAT1* leads to the disintegration of paraspeckles (Clemson et al., 2009). Indeed, paraspeckles form their *core* around the central part of *NEAT1*, whereas the surrounding structures (the shell and the patch) form around its 5′ and 3′ ends (Souquere et al., 2010). Interestingly, it has been shown that FUS localizes in the *core*, whereas TDP-43 concentrates in the shell (Hennig et al., 2015). This different localization reflects distinct roles displayed by the two RBPs in paraspeckles formation. In *Fus*−/− mice, *Neat1* accumulated at its transcription sites but did not form the *core*-shell structure; moreover, it was found diffused throughout the nucleoplasm (West et al., 2016). Interestingly, in *Fus*−/− mice, the *core* group proteins SFPQ, NONO, and PSPC1 accumulated at the *Neat1* transcription sites, indicating that FUS protein was not essential for their association with *Neat1*. On the contrary, the patch protein BRG1 and RBM14 were not enriched at *Neat1* transcription sites, indicating an essential role for FUS in stabilizing the interaction of these proteins with nascent *Neat1* transcripts (West et al., 2016). On the other hand, TDP-43 downregulation induced the accumulation of *NEAT1* transcripts and the formation of paraspeckles (Shelkovnikova et al., 2018). This accumulation was probably due to the protective role displayed by paraspeckles against defective miRNA pathway, caused by TDP-43 depletion (Shelkovnikova et al., 2018).

Electron microscopy has allowed the identification of other MLOs in the nucleus, such as the **Cajal body** (Gall et al., 1999). Cajal bodies show a coiled structure and form on active snRNA loci (Frey and Matera, 2001). They share the same properties of other MLOs (Handwerger et al., 2003; Kato et al., 2012). Their assembly is initiated by small nuclear RNAs (snRNAs), including small nucleolar RNAs (snoRNAs) and

small Cajal body-specific RNAs (scaRNAs), that interact with RBPs, which in turn recruit other proteins (Machyna et al., 2013). An essential aggregation factor of these structures is the protein coilin, which through its multi-modular domains gathers RBPs and RNAs, leading to the formation of Cajal bodies (Machyna et al., 2014). These bodies maintain structural integrity during interphase (Carmo-Fonseca et al., 1993), and are implicated in the small nuclear ribonucleoprotein (snRNP) biogenesis, spliceosome formation, telomere maturation, and maintenance (Machyna et al., 2013).

In addition to MLOs, a phase separation model has been recently proposed to explain basic mechanisms of the transcriptional regulation, such as **super-enhancers** (Hnisz et al., 2017; Sabari et al., 2018). Super-enhancers are clusters of transcriptional enhancers assembled by simultaneous binding of master transcription factors, transcriptional co-activators, RNAPII and RNA, that drive the expression of genes involved in defining cell identity (Whyte et al., 2013). Many molecules bound at enhancer-regions can undergo reversible chemical modifications (e.g., acetylation, phosphorylation, methylation) at multiple sites. Upon such modifications, these molecules change their interactome, thus promoting changes in the overall charge and in the affinities of the interacting molecules, and obtaining a high-density assembly of biomolecules at active sites (Hnisz et al., 2017; Sabari et al., 2018). In this way, super-enhancers are susceptible to perturbation and their activity is fine-tuned by internal and external cues. During the process of tumor pathogenesis, chromosomal translocation or overexpression of oncogenic transcription factors favors the formation of super-enhancer at sites of oncogenes (Hanahan and Weinberg, 2011), thus driving aberrant gene expression programs. Phase separation can also be promoted by proteins involved in the proteasome-degradation pathway (Li et al., 2014). In the case of the tumor suppressor SPOP (speckle-type POZ protein), involved in ubiquitination and proteasomal degradation of substrates (Li et al., 2014), target proteins drive SPOP-mediated separation process, and when cancer-associated mutations of *SPOP* gene occur, substrate binding and phase separation are displaced (Bouchard et al., 2018). SPOP localizes in various nuclear bodies including speckles and DNA-damage *loci* (Nagai et al., 1997; Marzahn et al., 2016). Cancer mutations in SPOP negatively regulate the LLPS process between SPOP and substrates and prevent their ubiquitination, leading to upregulation of these proteins and impaired proteostasis (Bouchard et al., 2018).

Thus, phase transitions in MLOs and at super-enhancers allow the accomplishment of gene expression programs both in healthy and diseased cellular states.

PHASE SEPARATION PROMOTED BY LOW-COMPLEXITY RNA-BINDING PROTEINS

A well-defined protein structure is essential to accomplish protein functions within the cell. However, many protein portions lack a well-defined structure still remaining functional. These segments are indicated as intrinsically disordered regions (IDRs) and proteins harboring them are named intrinsically disordered proteins (IDPs) (Li et al., 2012). Unlike globular proteins, IDPs use only a subset of the 20 amino acids, with low content of hydrophobic amino acids. To achieve phase separation, the exact amino acid sequence of IDPs is not important, while the overall composition and charge pattern are extremely relevant. Initially considered as passive segments linking structured domains, IDRs actively participate in different cellular functions, and their activity is fine-tuned by post-translational modifications (Iakoucheva et al., 2004; Collins et al., 2008).

In order to obtain the separation from nucleoplasm and cytoplasm, MLOs contain IDPs harboring low-sequence complexity domains (LCDs) (Gilks et al., 2004; Han et al., 2012; Kato et al., 2012; Toretsky and Wright, 2014). These domains are also present in yeast prion proteins (Alberti et al., 2009), from which the term "prion-like" is derived. Prions are infectious protein conformers capable of self-replication (Shorter and Lindquist, 2005). Prion-like domains (PrLDs) are a type of LCD with a tendency to self-assemble and form aggregates. Their ability to form amyloid is dependent on the PrLD rich in glycine and uncharged polar amino acids to reduce the solubility of the proteins (Alberti et al., 2009). Deletion of the prion domain precludes the formation of the prion conformer (Masison et al., 1997), while the addition of this region to a given protein is sufficient to confer prion behavior (Li and Lindquist, 2000). Remarkably, mutations in PrLD-containing proteins cause devastating protein-misfolding diseases, characterized by the formation of solid aggregates (Kim et al., 2013; Li et al., 2013; Ramaswami et al., 2013).

Proteins involved in RNA processing display high phase separation propensities (Vernon et al., 2018). Indeed, as mentioned above, MLOs contain several RBPs harboring PrLDs. Remarkably, of the 240 human proteins harboring predicted PrLDs, 72 (30%) are involved in RNA metabolism (March et al., 2016). These proteins are recently emerging in the pathology and genetics of human neurodegenerative diseases (King et al., 2012).

ATXN1 and ATXN2 were the first RBPs with a putative PrLD to be linked to the pathogenesis of neurodegenerative diseases, causing, respectively, the type 1 and type 2 spinocerebellar ataxia (Banfi et al., 1994; Lorenzetti et al., 1997), a neurodegenerative disorder characterized by an expansion of a trinucleotide CAG repeat within the coding region of the *SCA1* and *SCA2* genes (Banfi et al., 1994; Lorenzetti et al., 1997). In physiological conditions, ATXN1 is located both in the nucleus and in the cytoplasm (Servadio et al., 1995), and is able to shuttle between these two compartments. However, ATXN1 dynamics is altered by the expansion; in fact, while mutated ATXN1 is still able to enter the nucleus, its ability to be transported back into the cytoplasm is dramatically reduced (Irwin et al., 2005). On the contrary, ATXN2 is mainly localized into the cytoplasm, associated to translating polysomes or into stress granules and P-bodies, where it is involved in the regulation of translation, mRNA storage, or degradation (Orr, 2012).

Almost 10 years later, the transactive response (TAR) DNA-binding protein 43 kDa (TDP-43) was associated with

a neurodegenerative disease (Arai et al., 2006; Neumann et al., 2006). TDP-43 is a RBP containing a PrLD (amino acids 277–414, **Figure 2**), is localized in the cell nucleus, but shuttles to the cytoplasm displaying roles in transcriptional and post-transcriptional RNA processing (Buratti and Baralle, 2008, 2010). TDP-43 misfolding has been connected to the pathology of ALS and frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) (Neumann et al., 2006; Chen-Plotkin et al., 2010; Da Cruz and Cleveland, 2011). In these disorders, TDP-43 displays a cytoplasmic localization and forms aggregates; moreover, it is depleted from the nucleus of diseased neurons (Chen-Plotkin et al., 2010; Da Cruz and Cleveland, 2011).

The PrLD of FUS harbors amino acids 1–238 (**Figure 2**). Like TDP-43, FUS is mainly localized in the nucleus, but shuttles to accomplish functions in transcriptional and post-transcriptional regulation, RNA processing, and miRNA biogenesis (Bertolotti et al., 1996; Zinszner et al., 1997; Paronetto, 2013; Svetoni et al., 2016). Mutations in FUS cause familial ALS (Kwiatkowski et al., 2009; Vance et al., 2009; Da Cruz and Cleveland, 2011) and FTLD-U (Neumann et al., 2009; Mackenzie et al., 2010; Da Cruz and Cleveland, 2011; Drepper and Sendtner, 2011; Svetoni et al., 2016). In these pathologies, FUS is localized in cytoplasmic aggregates of the degenerating neurons (Mackenzie et al., 2010). FUS belongs to the FET family of RBPs, composed by FUS/TLS, EWS, and TAF15, and involved in multiple steps of RNA metabolism (Svetoni et al., 2016). FET proteins share a common domain architecture (Paronetto, 2013). Upon inhibition of transcription (Zinszner et al., 1997) or DNA damage (Paronetto et al., 2011), they translocate into the nucleoli forming dense nuclease-resistant aggregates. In Ewing sarcoma, the replacement of the RNA-binding domains of FET proteins with an ETS transcription factor due to chromosomal translocations alters their nucleic acid-binding affinities and activities, thus causing activation of a transcriptional program leading to cancer transformation (Paronetto, 2013). As mentioned, in neurodegenerative diseases, point mutations in the genes encoding FET proteins affect their localization and aggregation propensity, strongly supporting the hypothesis that phase transition contributes to the development of pathological conditions (Svetoni et al., 2016).

In 2011, mutations in the gene encoding TAF15 have been identified in ALS and FTLD-U patients (Couthouis et al., 2011; Neumann et al., 2011; Ticozzi et al., 2011). Interestingly, both TAF15 and EWS harbor a prominent N-terminal PrLD (amino acids 1–149 in TAF15; amino acids 1–280 in EWS; **Figure 2**) enriched in glutamine residues, which might enhance the formation of toxic oligomeric structures (Halfmann et al., 2011).

hnRNPA1 and hnRNPA2 are prototypical hnRNPs formed by two folded RNA recognition motifs (RRMs) in the N-terminal part of the protein and a PrLD in the C-terminal (amino acids 185–341 in hnRNPA2; amino acids 186–320 in hnRNPA1; **Figure 2**) (Kim et al., 2013), involved in the interaction with TDP-43 (Buratti et al., 2005). Missense mutations in the PrLD of hnRNPA1 and hnRNPA2 have been identified in ALS patients (Kim et al., 2013). HnRNPA2 and hnRNPA1 are prone to fibrillization, which is enhanced by disease-causing mutations (Kim et al., 2013). Notably, hnRNPA2B1 and hnRNPA1 mutations have been identified in families presenting multisystem proteinopathy (MSP) (Benatar et al., 2013; Le Ber et al., 2014), a rare complex phenotype which involves perturbation of SG dynamics and autophagic protein degradation, affecting muscle, brain, and bone (Benatar et al., 2013). MSP phenotype associates different disorders, such as frontotemporal lobar degeneration (FTLD), Paget disease of bone (PDB), inclusion body myopathy (IBM), and ALS (Benatar et al., 2013).

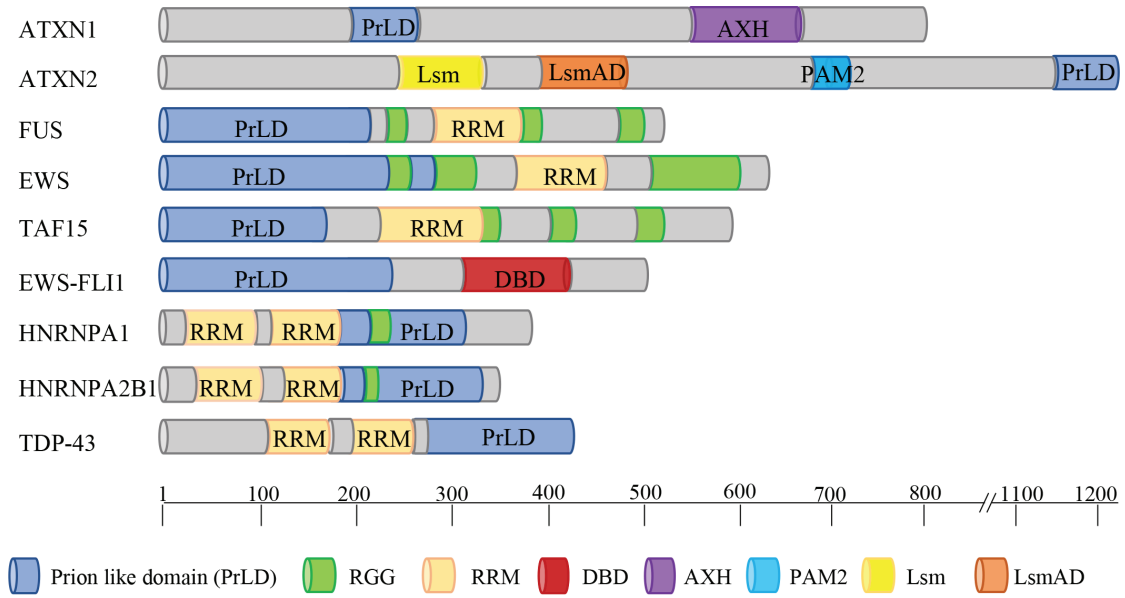
Aromatic residues play important roles in IDR interactions; they mediate short-range, aromatic interactions and promote LLPS, whereas hydrophilic residues control the solubility of IDRs and counteract LLPS (Kato et al., 2012; Xiang et al., 2015). For instance, tyrosine mutations block recruitment of hnRNPA2 and FUS IDRs into phase-separated liquids as well as into RNA granules (Kato et al., 2012; Xiang et al., 2015). The numerous tyrosine residues in FUS contribute to LLPS, and their recruitment into LLPS is controlled by phosphorylation (Lin et al., 2017). In fact, phosphorylation enables rapid transitions within the IDRs and controls the assembly/disassembly of the RNP granules (Lin et al., 2017).

Electrostatically driven phase separation can be also promoted by the interaction of arginine/glycine-rich domains with RNA. The RGG/RG repeats, often present in RBPs, usually occur in LCD. Compared to sequences from ordered proteins, these IDRs typically exhibit high levels of a subset of specific amino acids, that promote phase separation on their own. In particular, the polyvalent interactions between arginines and RNA achieve the phase separation process (Romero et al., 2001). RGG-containing regions mediate RNA binding (Kiledjian and Dreyfuss, 1992) and can be methylated by PRMTs (Bedford and Richard, 2005; Bedford and Clarke, 2009). Methylation is a post-transcriptional modification that negatively influences the capability of RBPs to bind RNA. For instance, methylation of the RGG/RG motif of FMRP reduces its affinity for RNA (Stetler et al., 2006) and its recruitment on polysomes (Blackwell et al., 2010). Moreover, methylarginines within the RG motifs of the RBP Sam68 negatively influence its affinity for the SH3 domains (Bedford et al., 2000). Arginine methylation also decreases the interaction of FUS with transportin, thus affecting its nuclear import (Dammer et al., 2012). To this regard, methylation of the RGG/RG motifs affects the localization of FUS proteins harboring ALS-linked mutations (Tradewell et al., 2012). Notably, mutations in the RGG/RG domains of FUS have been identified in familial cases of ALS (Kwiatkowski et al., 2009; Hoell et al., 2011).

The presence of RGG/RG motifs within a given protein can be regulated by alternative splicing choices (Blackwell and Ceman, 2011). For instance, isoform 12 of *FMR1* excludes exons 12 and 14, thus leading to a truncated FMRP isoform defective of the RGG domain encoded by exon 15. This FMRP isoform displays reduced localization to dendritic RNA granules (Mazroui et al., 2003; Blackwell and Ceman, 2011).

Chromosomal translocations between the *EWSR1* gene and genes encoding ETS transcription factors can cause aggressive pediatric tumors designated as Ewing sarcomas (Araya et al., 2005; Paronetto, 2013). The translocations result in chimeric proteins

A



B

Protein	Prion Domain	RRMs	RG/RGG domain	Disease Association
ATXN1	197-256			SCA1
ATXN2	1131-1223			SCA2
FUS	1-238	285-371	240-253 376-390 469-500	ALS, FTD, cancer
EWS	1-280	361-447	248-260 304-322 509-602	ALS, FTD, cancer
TAF15	1-149	234-320	334-351 405-417 495-514	ALS, FTD, cancer
HNRNPA1	186-320	14-97 105-184	218-234	ALS
HNRNPA2B1	185-341	21-104 112-184	203-215	ALS
TDP-43	277-414	106-167 191-262		ALS

FIGURE 2 | Phase Separation by RBPs. **(A)** Schematic representation of protein domains of RBPs involved in FTD, ALS, and cancer. ATXN1 and ATXN2 contain two predicted prion-like domains (ATXN1: aa197–256; ATXN2: aa1131–1,223; March et al., 2016). The FET proteins FUS, EWS, and TAF15 combine two types of low-complexity domain (LCD): prion-like domains (PrLDs) and RGG-rich domains. The two types of LCDs cooperate to drive the dynamic phase separation. FET proteins are frequently translocated in human cancers, with the resulting fusion proteins (e.g. EWS-FLI1) lacking either significant parts of the RGG-rich LCD or the RNA recognition motif (RRM) but containing the DNA-binding domain (DBD) of an ETS transcription factor (e.g. FLI1). HNRNPA1 and HNRNPA2 combine two RRM motifs with a PrLD, together with an RG domain. TDP43 contains two RRM motifs and a PrLD. Protein domains are indicated in different colors (see legend). AXH = Ataxin-1 and HMG-box protein domain; Lsm = Like RNA splicing domain Sm1 and Sm2; LsmAD = Like-Sm-associated domain; PAM2 = poly (A)-binding protein interacting motif. The location of each protein domain and the association with human pathologies are indicated in **(B)**. SCA1 = spinocerebellar ataxia type 1; SCA2 = spinocerebellar ataxia type 2; ALS = Amyotrophic Lateral Sclerosis; FTD = Frontotemporal Dementia.

harboring the N-terminal activation domain of EWS, comprehending the PrLD, fused to the DNA-binding domain of an ETS transcription factor, but lacking the C-terminal domain of EWS containing the RNA-binding regions (the RGG motifs and the RRM motif; **Figure 2**). Notably, the RGG/RG motifs of EWS display an inhibitory activity toward the DNA activation domain, thus decreasing its oncogenic potential (Li and Lee, 2000). Interestingly, the translocated PrLD deriving from EWS promotes the aggregation of EWS-FLI1 in foci (Boulay et al., 2017). The presence of aromatic residues affects the aggregation propensity of EWS-FLI1. In fact, replacement of the 37 tyrosine residues with serines removes its ability to form aggregates, and this tyrosine-replaced variant is unable to assemble active enhancers (Boulay et al., 2017). Since RNA helicases are extensively involved in phase separation dynamics (Nott et al., 2015), it is possible that the interaction between EWS-FLI1 and the DNA–RNA helicase DHX9 plays an essential role in the formation of these aggregates; thus, blocking this interaction could be a strategy to limit EWS-FLI1 oncogenic potential (Fidaleo et al., 2016). Since the ability of the EWS-FLI1 PrLD to phase separate is closely linked to its oncogenic activity, preventing or reverting phase separation properties could have therapeutic utility in Ewing sarcoma.

THERAPEUTIC APPROACHES

Phase separation displays a crucial role in neurodegenerative disorders. Several proteins involved in neurodegenerative diseases are components of MLOs and dysregulation in the formation or conservation of these components leads to pathological aggregates (Li et al., 2013; Ramaswami et al., 2013). Therefore, the development of novel therapeutic strategies to control cellular phase transition could be instrumental for the treatment of those human diseases characterized by aberrant aggregates (**Figure 3**).

In addition to RBPs, several ALS mutations have been identified in genes encoding members of the protein quality control system (PQC), including chaperones, components of the ubiquitin/proteasome, or autophagolysosomal system (Robberecht and Philips, 2013; Capponi et al., 2016; Alberti et al., 2017). These systems display a crucial role in the control of protein aggregation. Chaperones recognize SGs containing misfolded aggregated proteins (Mateju et al., 2017). When a specific chaperone mechanism is compromised, misfolded proteins and defective ribosomal products accumulate into SGs, thus altering SG dynamics and causing defects in SG disassembly (Ganassi et al., 2016). Ganassi and collaborators identified the HSPB8-BAG3-HSP70 chaperone complex as a key regulator of SG surveillance. The incidence of aberrant defective ribosomal products-containing SGs in normal conditions is very low, suggesting that the PQC is highly efficient in preventing aberrant SG formation (Ganassi et al., 2016). On the same line, Mateju and collaborators demonstrated that SGs containing ALS-associated SOD1 aggregates engage increased number of chaperones, including HSP27 and HSP70, suggesting their specific enrollment to avoid aberrant SGs (Mateju et al., 2017). Treatment with a chemical inhibitor of HSP70 increases the number of SGs containing misfolded proteins, suggesting that HSP70 hampers the accumulation of misfolded proteins and facilitates a rapid disassembly of SGs in the recovery phase (Mateju et al., 2017). Thus, surveillance of SGs by chaperones is critical for the maintenance of their normal composition and dynamics, and the stimulation of the chaperone machinery could be a useful target to disassemble MLOs. In this context, the development of potentiated chaperones could also be a suitable approach to optimize therapeutic efficacy against neurodegenerative diseases (Shorter, 2008; Jackrel et al., 2014; Yasuda et al., 2017). To this regard, Jackrel and collaborators were able to potentiate HSP104 variants from yeast (Jackrel et al., 2014; Jackrel and Shorter, 2014). In particular, the developed enhanced chaperone was able to revert TDP-43 and FUS aggregation, thus suppressing their toxicity and eliminating protein aggregates in yeast (Jackrel et al., 2014; Jackrel and Shorter, 2014). Moreover, Yasuda and collaborators demonstrated for the first time that engineered HSP104 variants are able to dissolve cytoplasmic ALS-linked FUS aggregates in mammalian cells (Yasuda et al., 2017).

Another approach to stimulate the chaperone machinery is to develop drugs able to upregulate the expression levels of heat shock proteins (HSPs). Arimoclomol (BRX-345) is a hydroxylamine derivative that facilitates the formation of chaperone molecules by enhancing the expression of heat shock genes (Vigh et al., 1997, Kieran et al., 2004). Arimoclomol treatment in *SOD1G93A* mice was shown to upregulate HSP70 and HSP90 expression, leading to a significant delay in the progression of the disease (Kieran et al., 2004). Remarkably, a phase II/III randomized, double-blind, placebo-controlled clinical trial is currently underway in familial SOD1-ALS patients (NCT00706147). Furthermore, arimoclomol treatment has been shown to induce a reduction in the pathological markers *in vitro* and amelioration of pathological and functional deficits *in vivo* of the sporadic inclusion body myositis (sIBM), a severe myopathy characterized by protein dys-homeostasis (Ahmed et al., 2016).

Since the disruption of the ubiquitin-proteasome-system and autophagy are central events in ALS, current research is now focusing on the development of drugs able to upregulate the signaling pathways involved in PQC (Barmada et al., 2014). For instance, new compounds stimulating autophagy are able to improve TDP-43 clearance and localization, thus mitigating neurodegeneration (Barmada et al., 2014). Stimulation of autophagy also enhances survival of human-induced pluripotent stem cells (iPSC)-derived neurons and astrocytes from patients with familial ALS (Barmada et al., 2014). Furthermore, great effort has recently been devoted to improving HSPB8 function and to promote the autophagy-mediated removal of misfolded mutant SOD1 and TDP-43 fragments from ALS motor neurons. To this regard, colchicine treatment enhances the expression of HSPB8 and of several autophagy players, while blocking TDP-43 accumulation in neurons (Rusmini et al., 2017). Based on these premises, a phase II randomized, double-blind, placebo-controlled, multicenter clinical trial has been activated to test the efficacy of colchicine in ALS (NCT03693781).

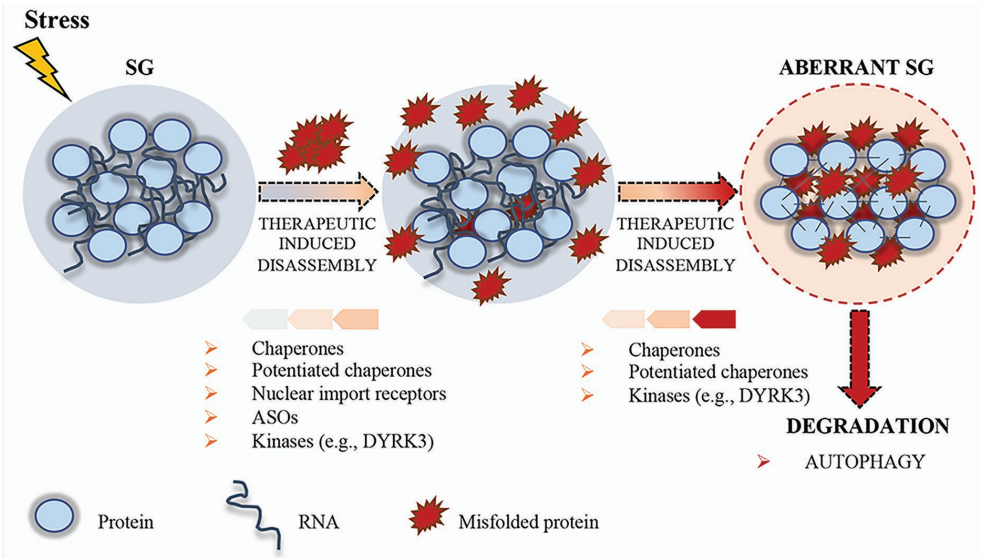


FIGURE 3 | Schematic representation of stress granule (SG) dynamics. Upon stress condition, SGs assemble; RNA and proteins accumulate in these granules until the stress persists. After removal of the stress condition, SGs disassemble, protein synthesis is reactivated after the translation inhibition induced by the stress, and RBPs can either go back to the nucleus or remain in the cytoplasm, where they carry out their normal functions. Misfolded proteins can accumulate in SGs, thus altering their physical properties. To prevent the accumulation of misfolded proteins, therapeutic strategies, such as chaperones, potentiated chaperones, protein kinases (e.g., DYRK3), nuclear import receptors, and ASOs, can be engineered to induce SG disassembly. If the disassembly does not occur, SGs become aberrant and can promote the pathogenesis of neurodegenerative disorders. Finally, aberrant SGs can undergo disassembly by chaperones, potentiated chaperones, or kinases, and, if not repaired, they undergo degradation by autophagy.

Recently, Guo and collaborators showed the relevance of nuclear-import receptors (NIRs) in the disaggregation of disease-linked RBPs with a nuclear localization signal (NLS) (Guo et al., 2018). The binding of Karyopherin-β2, also named transportin-1, to the PY-NLSs is sufficient to revert FUS, TAF15, EWS, hnRNPA1, and hnRNPA2 fibrillization, while Importin-α, in complex with Karyopherin-β1, reverts TDP-43 fibrillization (Guo et al., 2018). Karyopherin-β2 avoids the aberrant accumulation of RBPs containing PY-NLSs into the SGs and re-establishes proper RBP nuclear localization and function, thus rescuing the degeneration caused by mutated FUS and hnRNPA2 (Guo et al., 2018). Thus, NIRs might contribute to the setup of novel therapeutic strategies to restore RBP homeostasis and moderate neurodegeneration.

Recent evidences highlight the role of specific kinases in the regulation of the dissolution of SGs and other MLOs. For instance, during recovery from stressful conditions, the kinase activity of DYRK3 is required for SG dissolution and restoration of mTORC1 activity (Wippich et al., 2013). DYRK3 binds MLO proteins and phosphorylates their LCDs (Wippich et al., 2013), thus affecting the electrostatic properties of these domains and the condensation threshold of the proteins harboring them (Rai et al., 2018). Importantly, DYRK3 has been shown to act as a dissolvase of liquid-unmixed compartments (Rai et al., 2018). In fact, upon overexpression, recombinant DYRK3 was able to dissolve MLOs both in the nucleus and in the cytoplasm in a kinase-activity-dependent fashion (Rai et al., 2018). Similar to DYRK3, Casein kinase 2 (CK2) was recently found to cause SG disassembly *via* phosphorylation of the SG nucleating protein G3BP1 (Reineke et al., 2017). Thus, identification of kinases, such as DYRK3 and CK2, able to modulate MLO dissolution and/or drugs that regulate their functions may represent another interesting therapeutic approach (**Figure 3**).

Finally, a suitable approach to downregulate key regulators involved in aberrant phase transitions is the use of antisense oligonucleotides (ASOs). ASOs can be used to target pathological proteins in different mouse models (Schoch and Miller, 2017). In case of essential proteins, ASO-strategy could be engineered to target non-essential partners, involved in the regulation of phase transition (Boeynaems et al., 2018). This is the case of TDP-43 and ataxin-2. Ataxin-2 is an RBP with multiple roles in RNA metabolism, such as regulation of SG assembly (Elden et al., 2010; Kaehler et al., 2012). Reduction of ataxin-2 by ASOs affects SG dynamics and decreases recruitment of TDP-43 to SGs (Becker et al., 2017). A single administration of ASOs targeting ataxin-2 into the central nervous system is sufficient to increase the lifespan and improve motor function of TDP-43 transgenic mice (Becker et al., 2017). Since alterations in TDP-43 have been found in 97% of ALS cases and about 50% of FTD cases (Ling et al., 2013), the reduction of ataxin-2 could be used as therapeutic strategy for ALS and FTD treatment (Wils et al., 2010).

To conclude, the stimulation of chaperone machinery, the induction of pathways triggering PQC, and the development of ASOs could be exploited to set up novel therapeutic approaches for the treatment of those human diseases characterized by aberrant phase transition aggregates.

CONCLUDING REMARKS

Membrane-less subcellular organization plays a pivotal role in cellular homeostasis. LCDs, including PrLDs and RGG domains, behave as general scaffolds in assisting MLO's activity and mediating the dynamics of RNP granules. Persistence of RNP granules caused by either failure of granule removal, mutated PrLD-containing RBPs, or granule-associated misfolded proteins can lead to pathological protein aggregates, that contribute, at least in part, to the pathogenesis of neurodegenerative diseases. At the same time, chromosomal translocation can promote the formation of aberrant chimeric proteins formed by PrLD fused to transcription factors; these translocated PrLDs promote phase separation and activate transcriptional programs driving transformation (Boulay et al., 2017). On the other hand, disruption of membrane-less organization by mutations in the tumor suppressor SPOP can cause solid tumors (Bouchard et al., 2018).

Given the relevance to human health, determining how LCD proteins organize cellular compartments could be instrumental to expand our understanding of compartment formation, thus providing significant insight into neurodegenerative pathologies and cancer. Recent studies document how pernicious misfolding can be reversed by protein disaggregases (Shorter, 2008; Torrente and Shorter, 2013; Jackrel and Shorter, 2015), opening the path to novel promising therapeutic applications both in cancer treatment and in the cure of neurodegenerative disease. Yet, the exact MLO dynamics has not been completely unraveled, neither in physiological or pathological conditions.

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VV, EDP and MPP analyzed the literature and wrote the manuscript.

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