REVIEW

Role and therapeutic potential of liquid–liquid phase separation in amyotrophic lateral sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative disease selectively affecting motor neurons, leading to progressive paralysis. Although most cases are sporadic, ~10% are familial. Similar proteins are found in aggregates in sporadic and familial ALS, and over the last decade, research has been focused on the underlying nature of this common pathology. Notably, TDP-43 inclusions are found in almost all ALS patients, while FUS inclusions have been reported in some familial ALS patients. Both TDP-43 and FUS possess ‘low-complexity domains’ (LCDs) and are considered as ‘intrinsically disordered proteins’ (IDPs), which form liquid droplets in vitro due to the weak interactions caused by the LCDs. Dysfunctional ‘liquid–liquid phase separation’ (LLPS) emerged as a new mechanism linking ALS-related proteins to pathogenesis. Here, we review the current state of knowledge on ALS-related gene products associated with a proteinopathy and discuss their status as LLPS proteins. In addition, we highlight the therapeutic potential of targeting LLPS for treating ALS.

Keywords: phase separation, stress granule, motor neuron, ALS therapy

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1. Introduction

1.1 Background of Amyotrophic lateral sclerosis (ALS)

ALS is a fatal late-onset neurodegenerative disease affecting primarily upper and lower motor neurons. The average age of onset is between 51 and 66 years old (Longinetti and Fang, 2019). ALS is a progressive disease and initial muscle weakness progresses into paralysis and death of the patient, most often due to respiratory failure typically 2–5 years after displaying the first symptoms (Brown and Al-Chalabi, 2017).

1.2 Genetics of ALS

ALS cases are predominantly sporadic (sALS), with ~10% being inherited (familial ALS; fALS). Mutations in >25 genes have been linked to this disease (Nguyen et al., 2018). However, the majority of fALS can be explained by alterations in four main genes, namely C9orf72, SOD1, TARDBP, and FUS. Hexanucleotide repeat expansions in the chromosome 9 open reading frame 72 (C9orf72) gene are the most common genetic cause of fALS, accounting for ~40% of fALS (Majounie et al., 2012). Mutations in the superoxide dismutase 1 (SOD1) gene account for ~20% of fALS (Rosen et al., 1993), while mutations in the genes encoding DNA/RNA-binding proteins TAR DNA-binding protein 43 (TARDBP, encoding TDP-43) and fused in sarcoma (FUS) are causal to ~5% and ~4% of fALS, respectively (Kwiatkowski et al., 2009; Vance et al., 2009; Taylor et al., 2016).

1.3 Pathology of ALS

A characteristic pathological feature of ALS is protein aggregation in the cytoplasm of motor neurons and sometimes also in other cell types, such as glial cells (Brown and Al-Chalabi, 2017). In 97% of ALS patients, these inclusions contain TDP-43 (Neumann et al., 2006).

ALS belongs to a disease spectrum also including frontotemporal dementia (FTD), a disease affecting mainly the frontal and temporal lobes associated with dementia. Indeed, both diseases sometimes occur simultaneously (ALS/FTD) and exhibit clinical and pathological overlap, as well as common genetic causes. In post-mortem material of some FTD patients, TDP-43 or FUS aggregates can be found in the absence of pathological mutations in the genes encoding these proteins (Blokhuis et al., 2013).

During the last decades, researchers sought a unifying mechanism responsible for the complex nature of ALS pathology. However, ALS seems to be a polygenic disease in which multiple small hits (risk variants) combined with yet unclear environmental factors could lead to pathological changes in multiple pathways, concluding in motor neuron death. This multistage model is consistent with the observation that the disease is caused by a six-step process (Al-Chalabi et al., 2014). Liquid–liquid phase separation (LLPS) is one process implicated in major pathways in ALS-related pathogenicity and will be the focus of this review.
2. Potential role of LLPS in ALS

2.1 LLPS drives the formation of membraneless organelles

LLPS is the phenomenon in which a homogenous fluid de-mixes into two distinct liquid phases, one being condensed and the other one dilute, resulting in a membraneless boundary that allows for selective passage (Brangwynne, 2013; Hyman et al., 2014). This can be observed in vitro, where phase-separating proteins produce spherical droplets visible under the microscope or even by eye as a cloudy state. This state is reversible and dynamic, but the droplets can later change physical state, going from liquid to gel, and even further to solid. LLPS plays an important role in normal cell physiology and is used to regulate cellular functions such as cellular compartment control, metabolic processing, and signalling using membraneless organelles (Hyman et al., 2014; Lin et al., 2015a). Proteins that drive phase separation engage in multivalent interactions, which stem from domain–domain, domain–motif, and motif–motif interactions and direct interactions of intrinsically disordered regions (IDRs). IDRs typically do not encode a particular 3D conformation and are often composed of a limited number of amino acids and/or repetitive sequence elements. This means that they have a low sequence complexity and, therefore, these domains are termed low-complexity domains (LCDs) that are rich in charged amino acids (Kato et al., 2012; Molliex et al., 2015a). Many of these LCD-containing proteins can be found in membraneless organelles (Markmiller et al., 2018).

Stress granules (SGs) are an example of membraneless organelles (Kedersha et al., 2005). Assembly of these granules is induced in the cytoplasm in response to adverse conditions, such as immediate stresses, concentration changes, or pH fluctuations. SGs sequester free mRNA and RNA-binding proteins (RBPs) in order to halt translation and to conserve energy for acute cellular needs (Kedersha and Anderson, 2002, 2007). As conditions improve, LLPS is reversed. Advanced microscopy techniques have revealed that SGs consist of a highly concentrated core made up of mRNA and proteins, surrounded by the shell that is more dynamic in nature (Protter and Parker, 2016). Online databases for SG proteomes have been created to understand the nature of proteins collected within these organelles (http://rnagranuledb.lunenfeld.ca/). This type of analysis showed that SGs are enriched in intrinsically disordered proteins (Reijns et al., 2008; Han et al., 2012; Jain et al., 2016), but not limited to LCD proteins (Youn et al., 2019).

2.2 ALS-associated proteins aggregate into membraneless organelles

TDP-43 and FUS are associated with pathological ALS aggregates that could arise from improperly disassembled SGs (Dewey et al., 2012). TDP-43 and FUS have been associated with other membraneless organelles, such as DNA repair sites, paraspeckles, and transport granules (Andersson et al., 2008; Dormann et al., 2010). As the assembly and disassembly of LLPS are tightly regulated, it
is not surprising that the dysfunction in this process could be implicated in disease aetiology. Despite the SG being the most studied of all membraneless organelles, other noteworthy organelles assist in RNA processing and ribosomal assembly.

Here, we will summarize the information on ALS-related proteins predicted and found to phase separate and the role of this process in pathogenicity. Meanwhile, we will discuss potential therapeutic avenues targeting LLPS.

3. ALS-LLPS proteins

Many pathogenic pathways involved in ALS rely on LLPS. Indeed, evidence implicates phase separation in nucleocytoplasmic transport, RNA metabolism, DNA repair, mitochondrial function, protein aggregation, among others (Figure 1 and Table 1).

The major findings in phase separation related to ALS reveal (i) that pathogenic mutations in ALS-related genes lead to faster maturation of droplets to solid fibrillar aggregates (Johnson et al., 2009a; Conicella et al., 2016; Schmidt and Rohatgi, 2016), (ii) that several ALS-related proteins such as TIA1 (Mackenzie et al., 2017), TDP-43 (Colombrita et al., 2009), FUS, Taf15 (Andersson et al., 2008; Abramzon et al., 2012), heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and hnRNPA2 (McDonald et al., 2011), and Profilin1 (Wu et al., 2012) are found in SGs, and (iii) that the ALS pathological proteins FUS and TDP-43 have an LCD, making them prone to participate in weak and dynamic interactions (Kato et al., 2012; Molliex et al., 2015). The overexpression of these proteins produced SGs, similar to the ones formed after the addition of arsenite (Winton et al., 2008; Liu-Yesucevitz et al., 2010; Boeynaems et al., 2017). Moreover, liquid granule proteins G3BP1 and TIAR-2 are essential for granule formation implicated in axon regeneration, an important feature for motor neurons.

In Table 2, we list genes encoding proteins associated with ALS. The scores were obtained using PSPer (Orlando et al. 2019), an unsupervised tool, which provides an estimation of the probability that the target proteins undergo LLPS using a molecular mechanism similar to FUS-like proteins. The aggregation propensity for each protein was also evaluated using TANGO (Fernandez-Escamilla et al., 2004) and PASTA2 (Walsh et al., 2014). To estimate the global aggregation propensity of a protein, we used the maximum TANGO score, likely representing the aggregation propensity of the strongest aggregate-prone region (APR).

3.1 TDP-43

TDP-43 is the most studied protein in ALS, as it is the main protein found in pathological end-stage inclusions. This aggregated form of TDP-43 is ubiquitinated, mostly phosphorylated (Arai et al., 2006;
Neumann et al., 2009), and/or acetylated in ALS cases (Cohen et al., 2015). In healthy individuals, TDP-43 resides in the nucleus, binds to DNA and RNA, and is involved in mRNA stability and splicing. The most puzzling observation in ALS-related TDP-43 pathology remains the appearance of TDP-43 aggregates without mutation in the \textit{TARDBP} gene.

Structurally, TDP-43 contains a LCD in the C-terminal region, with a glycine-rich region, and a strong hydrophobic region rich in glutamine/asparagine (making it ‘prion-like’) (Gitler and Shorter, 2011). This LCD harbours most ALS-linked mutations (Kabashi et al., 2008; Sreedharan et al., 2008).

In the nucleus, TDP-43 is found in dimers, trimers, tetramers, and higher order oligomers by the interaction of their N-terminal domains (Afroz et al., 2017). In pathological conditions, TDP-43 leaves the nucleus and forms complexes that are post-translationally modified (Neumann et al., 2006, 2009a; Cohen et al., 2015; Nonaka et al., 2016). Recombinant TDP-43 readily aggregates and forms oligomeric species, which are toxic to cells (Fang et al., 2014). In addition, the C-terminal domain of TDP-43 is also known to aggregate (Johnson et al., 2009). Using solution NMR, it was shown that in the droplet state, the TDP-43 LCD forms monomeric states bearing potential for self-aggregation (Conicella et al., 2016). Increasing the concentration of the TDP-43 LCD leads to more gel-like formations. Moreover, expressing proteins with ALS-linked mutations also decreases the liquid properties of the TDP-43 LCD (Conicella et al., 2016). These observations provide direct evidence that ALS mutations disrupt the phase-separating properties of this protein.

TDP-43 was found in the SG proteome (Ayala et al., 2008; Colombrita et al., 2009), and the overexpression of TDP-43 was reported to cause spontaneous SG formation (Boeynaems et al., 2017). However, these findings are debated (Colombrita et al., 2009; Dewey et al., 2011), which could be due to the different types of constructs driving overexpression, as well as the appearance of transfection-related SGs. Recent data support TDP-43 aggregation independent of SGs, where TDP-43 can bypass SGs and produce aggregates irrespective of SG localization (McGurk et al., 2018; Gasset-Rosa et al., 2019; Mann et al., 2019).

Using biochemical assays, cleaved forms of TDP-43 of 35 kDa (C35) and 25 kDa (C25) are found, and this aberrant cleavage enhances aggregation and cellular toxicity (Zhang et al., 2009). However, these cleaved fractions have been shown to bypass SGs, C35 being partially impaired and C25 being almost fully impaired from going into SGs (McGurk et al., 2018). These data indicate that the N-terminal region of TDP-43 is important for the recruitment to SGs and could explain why fragments lacking this region are found in patient material and/or disease models. C-terminal fragments accumulate in the brains, not spinal cords, of ALS and FTD patients and are therefore described as a neuropathological signature of these diseases (Hasegawa et al., 2008; Igaz et al., 2008; Kwong et al., 2014). By generating >50000 mutations in TDP-43, variants that increase aggregation were found to strongly decrease toxicity, while toxic variants promoted the formation of liquid-like
condensates (Bolognesi et al., 2019). These results advocate for pro-aggregate species over toxic liquid condensates. However, whether or not aggregates are toxic remains an open question.

3.2 FUS

Mutations in \( FUS \) can cause fALS (Kwiatkowski et al., 2009; Vance et al., 2009). Although only contributing to 4%–5% of fALS, this gene is heavily linked to FTD-FUS and partially to ALS-FUS pathology. \( FUS \) encodes a DNA/RNA-binding protein playing a crucial role in RNA metabolism and DNA repair (Ratti and Buratti, 2016). Protein mislocalization and inclusions have been found in FUS-associated ALS, both in neurons and glia (Kwiatkowski et al., 2009; Deng et al., 2014b). However, FUS inclusions found in FTD cases do not bear disease-related mutations (Neumann et al., 2009). FUS, together with EWSR1 and TAF15, is part of the FET proteins family and has an N-terminal LCD, RGG (arginine/glycine)-rich domains, a zinc finger (ZnF) domain, and an RRM domain. The nuclear localisation signal (NLS) domain keeps FUS predominantly nuclear and harbours most ALS-related mutations (Vance et al., 2009), which were shown to interfere with the FUS nuclear/cytoplasmic balance (Dormann et al., 2010).

Like TDP-43, the FUS LCD has a prion-like motif, with a high tendency to form cross \( \beta \)-amyloid assemblies (Kwon et al., 2013). The phase-separating behaviour of the FUS LCD is impeded upon mutational change of tyrosine residues to serines (Han et al., 2012; Kato et al., 2012). In contrast to TDP-43, FUS aggregation is prevented by phosphorylation (Kwon et al., 2013; Schwartz et al., 2013; Monahan et al., 2017), which in cells occurs during DNA damage response (Deng et al., 2014a). Recombinant FUS was able to form amyloid-like cross \( \beta \) filaments \textit{in vitro} at high concentrations, but truncation of the LCD abrogates this behaviour (Kato et al., 2012). At physiological concentrations and in the presence of molecular crowding agents, the FUS LCD was also able to phase separate \textit{in vitro} (Patel et al., 2015). This suggests that LLPS precedes the cross \( \beta \) aggregation of FUS. Full-length FUS was found to form droplets \textit{in vitro}, and RNA:protein ratios were shown to modulate LLPS. Low RNA concentrations, similar to what is found in the cytoplasm, decreased the liquid-like behaviour of FUS (Burke et al., 2015; Maharana et al., 2018).

FUS methylation of arginine residues was found to disrupt LLPS and decrease its aggregation propensity (Hofweber et al., 2018; Qamar et al., 2018). Methylated FUS plays distinct pathological roles in FTD-FUS and ALS-FUS. Pathological inclusions containing FUS with asymmetrically dimethylated arginines are only found in ALS-FUS cases (Dormann et al., 2012), and inclusions containing unmethylated/monomethylated arginine at the RGG3 region of FUS are only observed in FTD-FUS cases (Suárez-Calvet et al., 2016). In addition, RGG2 arginines promote phase separation of FUS cell free systems and in cells (Bogaert et al., 2018). Inclusions in FTD-TDP and ALS-TDP, but not FTD-FUS proteinopathy, have amyloid properties, as shown by the use of aggregation dyes (Bigio et al., 2013), suggesting that LLPS could potentially account for this variability.
3.3 TAF15 and EWS

Despite TAF15, EWS, and FUS being structurally similar, they do not follow the same patterns of accumulation (Neumann et al., 2011). Some variants in *EWSR1* have been described in sALS, although follow-up studies are lacking (Couthouis et al., 2012). Furthermore, EWSR1 and TAF15 were localized to cytoplasmic puncta in sALS but were not found in aggregates (Couthouisa et al., 2011), an observation supported by the absence of these proteins in post-mortem studies. As a consequence, the contribution of these proteins to overall ALS-related LLPS seems to be limited.

3.4 SOD1

Mutations in *SOD1* were the first discovered genetic cause of ALS (Deng et al., 1993; Rosen et al., 1993) and, as such, extensive research has focused on this gene and the SOD1 protein it encodes. Localisation of SOD1 is mainly cytoplasmic. However, it was also found in the nucleus, lysosomes, and mitochondria (Tafuri et al., 2015). The main function of SOD1 is to eliminate free superoxide radicals, a major cause of oxidative stress. SOD1 pathology is based on increased aggregation, dimer destabilisation, and oligomerization of the protein. Mutant SOD1 causes ALS via a toxic gain-of-function, which could include oxidative stress, excitotoxicity, and mitochondrial dysfunction, amongst others (Wong et al., 1995). SOD1 and TDP-43 inclusions are never reported to co-localise in sALS, indicating that these are produced via different pathways (Farrawell et al., 2015).

Unlike TDP-43 or FUS, SOD1 is not directly involved in phase separation. SOD1 misfolds into amyloid aggregates, and its aggregating behaviour seems directly linked to ALS (Prudencio et al.; Bruijn et al., 1998). Accordingly, the LLPS and aggregation score of SOD1 in our study were low.

3.5 C9orf72

Individuals with C9-ALS/FTD have hexanucleotide repeats in the non-coding region of *C9orf72* ranging from 66 to >4400 units in contrast to non-disease individuals carrying 2‒30 repeats in the intronic region (Gijselinck et al., 2016; Balendra and Isaacs, 2018). These expansions are the most common genetic cause of ALS, although they occur more frequently in individuals with European descent (Ishiura and Tsuji, 2015). *C9orf72* is translated into a guanine nucleotide exchange factor, and is involved in regulating vesicular trafficking and autophagy (Iyer et al., 2018). Mechanisms proposed for C9-ALS pathology include three non-mutually exclusive hypotheses: loss-of-function through a lower expression of the *C9orf72* gene and toxic RNA gain-of-function or toxic protein gain-of-function via the generation of dipeptide repeat proteins (DPRs) due to non-ATG-mediated translation from the repeat transcripts.

The latter leads to the formation of DPRs from sense and antisense transcripts, producing five different DPRs (Ash et al., 2013; Mori et al., 2013). Arginine-rich DPRs undergo LLPS, induce phase
separation of some important ALS proteins, and are shown to disrupt mitochondrial function, RNA processing, SG dynamics (Boeynaems et al., 2017), as well as axonal transport (Fumagalli et al., 2019). These DPRs, however, cannot directly affect nucleocytoplasmic transport (Vanneste et al., 2019).

3.6 Other Proteins

3.6.1 Ataxin-2

Mutations in ATXN2 (encoding Ataxin-2) are considered as risk factors in ALS and can modify TDP-43 toxicity (Elden et al., 2010). Ataxin-2, unlike TDP-43 or FUS, is a cytoplasmic protein. This protein can contain an intermediate polyglutamine repeat expansion in ALS cases, with a repeat length between 24 and 39 (Elden et al., 2010; Lee et al., 2011). The LCD of the ATXN2 yeast orthologue PAB1-binding protein 1 (Pbp1) phase separates, forms droplets (Yang et al., 2019), and behaves similarly to the FUS LCD. However, the LCD of Pbp1 is not enriched in aromatic amino acids but contains unusually high concentrations of methionine residues, unlike FUS or TDP-43 (Kato et al., 2019). In yeast, increased expression of Pbp1 enhances and loss-of-function of Pbp1 suppresses TDP-43 toxicity (Elden et al., 2010). Furthermore, the redox state controls the phase-separating properties of Pbp1 via reversible oxidation of its methionine-rich LCD (Kato et al., 2019). Ataxin-2 is involved in RNA metabolism and was identified as a component of SGs (Kaehler et al., 2012). This could be due to its interaction with the DEAD/H-Box RNA helicase DDX6, a component of SGs and P-bodies (Nonhoff et al., 2007). IDRs in Ataxine-2 mediate LLPS and SG assembly and deletion of the IDRs is sufficient to prevent C9orf72 or FUS-induced neurodegeneration (Bakthavachalu et al., 2018)

In flies, the Ataxin-2 homologue Atx2 has a similar dose-dependent effect (Elden et al., 2010). Reduction of Ataxin-2 extends lifespan and reduces pathology in TDP-43 mice, (Becker et al., 2017) and rescues motor defects in both TDP-43 and spinocerebellar ataxia type 2 mouse models (Becker et al., 2017; Scoles et al., 2017). Ataxin-2 positive aggregates were detected in the spinal cord of ALS patients (Elden et al., 2010; Blokhuis et al., 2013). Therefore, Ataxin-2 aggregation could affect local translation by sequestering proteins and RNA, thereby changing the RNA-to-protein ratio in the cell (Nonhoff et al., 2007).

3.6.2 ADARB1

ADARB1 (also called ADAR2) is the main enzyme responsible for RNA editing in humans. It mediates adenosine-to-inosine (A-to-I) editing at the Q/R position (Higuchi et al., 2000). Inefficient RNA editing was reported in sALS cases (Aizawa et al., 2010) and ADAR2 is mislocalized and forms cytoplasmic accumulations and aggregates in the spinal cord of C9orf72 ALS/FTD patients (Moore et al., 2019). ADAR2 is typically concentrated in the nucleolus and FRAP imaging revealed that ADAR2 can shuttle between the nucleolus (a membraneless organelle) and the nucleoplasm (not a
membraneless organelle by definition) (Sansam et al., 2003). In addition, the prediction in Table 2 suggests that ADAR2 could phase separate although this has not yet been confirmed experimentally.

### 3.6.3 hnRNPA2/B1

hnRNPA2/B1, two isoforms differing from each other by 12 amino acids, are a major subclass of evolutionarily conserved RNPs belonging to the same protein family as FUS and TDP-43. hnRNPs have at least one RNA-binding motif and prion-like domain (Chaudhury et al., 2010). Mutations in the RRM domain are associated with multisystem proteinopathy and ALS characterized by TDP-43-positive cytoplasmic inclusions and increase the aggregation propensity of these proteins (Kim et al., 2013; Paul et al., 2017). In addition, hnRNPA2 LCD and the TDP-43 C-terminal domain co-phase separate and induce co-aggregation (Ryan et al., 2018). Finally, while it was suggested that hnRNPA2 undergoes amyloid aggregation (Xiang et al., 2015; Ryan et al., 2018), the resulting aggregates often appear amorphous and are not a typical amyloid in morphology.

### 3.6.4 hnRNPA1

An ALS-related variant in *hnRNPA1* was identified by linkage analysis and exome sequencing, and the mutant protein was found in aggregates in ALS patient tissue (Kim et al., 2013). Although the estimation of mutation frequencies for this gene is extremely low, hnRNPA1, similar to hnRNPA2/B1, shuttles between the nucleus and the cytoplasm and interacts with TDP-43 (Buratti et al., 2005). hnRNPA1 undergoes LLPS and the LCD is sufficient to mediate this process. However, the RRM domains influence both phase-separating properties and kinetics (Molliex et al., 2015). As shown in Table 2, this protein is predicted to phase separate and potentially aggregate, with a score just below that of hnRNPA2/B1.

### 3.6.5 Optineurin (OPTN)

OPTN is a membrane-bound protein involved in inflammation, autophagy, Golgi maintenance, and vesicular transport. Mutations in *OPTN* were found in ALS cases (Maruyama et al., 2010). These variants are mostly autosomal recessive and it is not yet clear whether they are a real cause of ALS (Kamada et al., 2014). iPSC-derived motor neurons from SOD1 and ALS patients show accumulations of insoluble OPTN, suggesting that this protein is aggregation-prone (Seminary et al., 2018). It is currently unclear whether OPTN is found in aggregates and/or whether it undergoes LLPS. However, prediction software (Table 2) generates a score close to 0 for LLPS, but a score high for aggregation, suggesting that OPTN could potentially be found in aggregate data sets.

### 3.6.6 Cell-restricted intracellular antigen-1 (TIA1)

*TIA1* has been proposed as a novel ALS-related gene, as exome sequencing in an ALS/FTD family with TDP-43 pathology identified a mutation in the TIA1 LCD (Mackenzie et al., 2017). This protein...
is commonly found in SGs (Kedersha et al., 2000). While this is compelling in terms of its link to the ALS disease mechanisms, the causality of mutations is disputed (van der Spek et al., 2018). To date, TIA1 was not detected in ALS aggregates, although TDP-43 pathology is observed in patients bearing TIA1 mutations (Mackenzie et al., 2017). The disordered domain of TIA1 undergoes LLPS in the presence of RNA (Lin et al., 2015), and ALS-related mutations in full-length TIA1 promote LLPS \textit{in vivo}, altering SG dynamics by inhibiting the disassembly (Mackenzie et al., 2017).

\subsection*{3.6.7 Kinesin heavy chain isoform 5A (KIF5A)}

Mutations in \textit{KIF5A} were recently identified in ALS patients (Brenner et al., 2018; Nicolas et al., 2018) and primarily located at the C-terminal cargo-binding domain. The mutations are rare and no studies have yet shown KIF5A in aggregates. KIF5B and KIF5C, two other homologous proteins also encoding kinesin-2 family members, were found in RNA granules (Trendel et al., 2019; Urdaneta et al., 2019). KIF5A is exclusively neuronal, which could explain why it was not found in these studies using non-neuronal human cell lines.

\subsection*{3.6.8 ANXA11}

Another relatively new risk gene for sporadic and familial ALS is \textit{ANXA11} (Topp et al., 2017). This gene encodes annexin 11, a phospholipid-binding protein involved in vesicle transport. Annexin 11 has also been shown to tether membraneless RNA granules to actively transport lysosomes via its intrinsic membrane-binding and phase-separating properties. ALS-associated \textit{ANXA11} mutations impair this tethering function and hence disrupt RNA transport (Ya-Cheng Liao et al., 2019). Annexin 11-positive protein aggregates were found in spinal cord motor neurons and hippocampal neuronal axons of an ALS patient carrying a mutation in \textit{ANXA11}, suggesting that the mutated protein is aggregation-prone (Topp et al., 2017). The predicted LLPS score of ANXA11 (Table 2) is similar to that of TIA1, a protein that was experimentally shown to phase separate.

\subsection*{3.6.9 Ubiquilin-2 (UBQLN2)}

UBQLN2 is a member of the ubiquilin family implicated in the degradation of misfolded and redundant proteins through the ubiquitin‒proteasome system and macroautophagy. Mutations in this gene were found in ALS/FTD patient cohorts, and UBQLN2 was detected in cytosolic inclusions in degenerating motor neurons of fALS and sALS patients (Renaud et al., 2019). The predicted aggregation score is very high, which is in line with these data. However, the predicted LLPS score of UBQLN2 is low (Table 2), although it was found to exhibit LLPS \textit{in vitro} at physiological conditions (Dao et al., 2018).

\subsection*{3.6.10 HSP40}
Mutations in *DNAJC7* were found in ALS cases in 2019 (Farhan et al., 2019). This gene encodes HSP40, a member of the heat-shock protein family, which, together with HSP70, facilitates protein homeostasis. More specifically, it aids in the folding of newly synthesized polypeptides and the clearance of misfolded proteins. Protein misfolding, like phase separation, is a mechanism often proposed to be involved in ALS pathology. HSP40 is depleted in fibroblasts from ALS patients carrying mutations in *DNAJC7* and localizes to SGs in HEK cells (Markmiller et al., 2018). However, its presence has not been confirmed in aggregates present in ALS patient tissue.

### 3.6.11 Matrin 3

Matrin 3 (*MATR3*) is a nuclear matrix protein, hypothesized to stabilize messenger RNA (Johnson et al., 2014). Matrin 3 has been linked to cellular transport defects in ALS due to its role in the regulation of mRNA nuclear export (Boehringer et al., 2017). This protein could potentially phase separate according to its predicted LLPS score (Table 2). While not found in SG proteomic datasets, it was shown to interact with TDP-43 in cytosolic aggregates in spinal neurons of sALS cases (Tada et al., 2018). Despite being found in aggregates, it is predicted to have a low aggregation score (Table 2).

### 3.6.12 TANK-binding kinase 1 (TBK1)

Mutations in *TBK1* (encoding TBK1) were recently identified as an important genetic cause of FTD and ALS. Loss-of-function variants in this gene are associated with cytoplasmic TDP-43 aggregates (Cirulli et al., 2015; Freischmidt et al., 2015).

Recently, it was found that a loss of TBK1 in motor neurons increases SOD1 aggregation and accelerates disease onset (Gerbino et al., 2020). However, the TBK1 protein has not yet been found in aggregates present in ALS patient tissue. No current studies support LLPS for this protein, and the predicted score was accordingly low. However, the aggregation score was similar to that of TDP-43 (Table 2).

### 3.6.13 NEK1

Gene-burden analysis identified a significant association between loss-of-function *NEK1* variants and fALS risk (Kenna et al., 2016). NEK1 has been linked to cilia formation, DNA-damage response, microtubule stability, neuronal morphology, and axonal polarity (Shalom et al., 2008; Higelin et al., 2018). The predicted score for NEK1 suggests mild LLPS behaviour but a high aggregation propensity based on its sequence (Table 2).

### 4. Potential therapeutic implications
Because the underlying mechanisms leading to ALS are still incompletely understood, the elaboration of effective treatment is arduous. Below are the current avenues involving LLPS in ALS therapeutic approaches.

4.1 Chaperones

Chaperones such as heat-shock proteins and karyopherins were proposed as a form of ‘disaggregases’ to mitigate the toxic misfolding of proteins such as TDP-43 and FUS. One example is yeast Hsp104 (Afroz et al., 2017). Using the cells’ own protein degradation and chaperone machinery, disaggregases could be generated to remove aggregated proteins. A heat-shock protein, Hsp27, inhibits FUS LLPS via weak interactions; when this heat-shock protein is phosphorylated, it causes an enhanced amyloid inhibition of FUS (Liu et al., 2020). This switchable activity of Hsp27 could serve as a potential target for therapy. Recent research identified karyopherin abnormalities associated with the mislocalization and accumulation of disease-related proteins. In addition to their classical function in nuclear import and export, karyopherins also act as chaperones preventing misfolding, accumulation, and irreversible LLPS, as shown for transportin proteins, which can protect against aberrant phase separation (Guo et al., 2018; Yoshizawa et al., 2018). Transportin has been shown to control FUS phase separation (Qamar et al., 2018) by binding to FUS proline–tyrosine NLS (PY-NLS) (Zhang and Chook, 2012) and the RGG3 domain (Dormann et al., 2012). In addition, it directly interacts with arginine, thus interfering with the weak intermolecular interactions of arginines with other residues and eventually suppressing arginine-driven phase transitions. Downregulation of Exportin 1 (XPO1), a nuclear export receptor, prevents FUS-induced neurotoxicity and reduces the LLPS propensity of FUS (Steyaert et al., 2018). Targeting karyopherins is not a novel approach but already used to treat many cancers (Çağatay and Chook, 2018).

4.2 Antisense oligonucleotides (ASOs)

ASOs are an interesting therapeutic avenue for ALS. Phase 1 clinical trial of ASO-mediated therapy showed reduced levels of mutant SOD1 (Miller et al., 2013; https://clinicaltrials.gov/ct2/show/NCT03070119, ClinicalTrials.gov Identifier: NCT03070119), while results of the C9orf72 clinical trial (Jiang et al., 2016; https://clinicaltrials.gov/ct2/show/NCT03626012, ClinicalTrials.gov Identifier: NCT03626012) are pending. The use of ASOs is consequently admissible for non-essential, monogenic gain-of-function conditions (such as SOD1), but it would not be an option for TDP-43 or FUS. A way to overcome this is targeting modifiers of these essential proteins. One example is Ataxin-2 (Becker et al., 2017), which is a modifier of TDP-43 toxicity. Indeed, Ataxin-2 ASOs abolished SGs. Because the majority of ALS patients present with TDP-43 proteinopathy, an Ataxin-2 ASO approach could be very promising, as it could be proposed to most patients. Additional targets for a therapeutic ASO approach are Stathmin-2 ASOs. RNAseq studies revealed that this gene is downregulated in TDP-43-depleted cells and
decreased in human motor neurons and spinal cord sections of ALS patients, although it is not yet clear whether it is the only and/or most important target (Klim et al., 2019; Melamed et al., 2019).

4.3 Small-molecule strategies

Small molecules to disrupt phase separation are currently explored. Using cell- and protein-based screens, it was shown that lipoamide can reduce SG protein aggregation in vitro, in Drosophila, in C. elegans, and in patient-derived motor neurons (Wheeler et al., 2019). Lipoamide and lipoic acid, two nontoxic drugs, alter FUS aggregation specifically by modulating its phase-separating behaviour. This was linked to changes in the cellular stress response of the cells, improving mitochondrial health and leading to restoration of mislocalized FUS to the nucleus. Unlike what is seen in vitro with 1,6 hexanediol, these drugs do not disrupt other membraneless organelles, a concern for developing specific SG targets, as side effects may arise from the inhibition of an essential process. Small-molecule screens in cells showed a reduction of TDP-43 in SGs, and molecules with planar moieties disrupted accumulation of ALS-associated RBPs in SGs (Fang et al., 2019). In addition, RNA can be used to suppress TDP-43 LLPS, as demonstrated by the use of bait oligonucleotide RNA (Mann et al., 2019).

4.4 Post-translational modifications

Interfering with post-translational modifications is another therapeutic approach that may be used to alter aberrant LLPS. Examples include the targeting of known phosphorylation sites of regulatory kinases (Kumar Rai et al., 2018). Similarly, alterations of methylation sites could be used to modify the phase-separating behaviour and toxicity of FUS and/or DPRs (Dormann et al., 2012; Qamar et al., 2018; Gittings et al., 2020), paving the way for new therapies. The methylation of arginine residues maintains the positive charge but diminishes its hydrogen-bonding capacity by removing a hydrogen atom for each methyl group, where the methyl group essentially adds hydrophobicity to the side chain. The use of pharmacological tools to alter arginine demethylase activity would allow the modulation of epigenetic control of transcription (Blanc and Richard, 2017).

4.5 Activation of autophagy

Autophagy activators inducing SG removal are a controversial route for potential therapeutics. There is evidence that SGs can be targeted to lysosomes for autophagy and this process can be inhibited by depletion of valosine-containing protein (VCP) encoded by VCP, a gene mutated in ALS and FTD (Buchan et al., 2013). Furthermore, this autophagic process is mediated by SG surveillance chaperone complexes, which could also represent novel therapeutic targets (Ganassi et al., 2016). The enhancement of autophagy has recently been found to reduce cytoplasmic FUS mislocalization and rescue FUS phenotypes in vivo (Marrone et al., 2019). Recently, inhibition of lysosomal mTORC1
signalling using the small molecule EN6 was found to clear TDP-43 aggregates in a lysosome-dependent manner (Chung et al., 2019).

5. Discussion

In the past five years, there has been a radical change in the view on LLPS granules. Gasset-Rosa et al. (2019) and McGurk et al. (2018) suggested that SG-independent TDP-43 foci are particularly prone to a conversion into aggregates. This suggests that SGs could be the initial checkpoints serving a protective function. This role was initially hypothesized in 2017, as chaperones were found to be present within SGs (Ganassi et al., 2016; Jain et al., 2016). Therapeutic targets have been suggested for the aggregates and SGs but lacking for oligomeric forms of these mislocalized proteins. Targeting intermediate soluble oligomers of TDP-43 should be considered at earlier time point to ensure prevention.

Novel methods for studying SGs using optodroplets confirmed that transient SGs can indeed turn into solid aggregates (Zhang et al., 2019). The tight control of SG dynamics is only recently being unravelled by looking at the relationship between G3BP1 and its binding partners (Guillé N-Boixet et al., 2020; Sanders et al., 2020; Yang et al., 2020).

In this review, we summarized known aggregating proteins and discussed their potential LLPS properties based on published evidence and predicted scores. These predictions suggest that we could investigate LLPS behaviour and LLPS-related aggregation by studying non-phase-separating ALS proteins. Indeed, SOD1 and MATR3 have low LLPS scores and are not associated to SGs, but they are found in aggregates of ALS patient tissue, despite having low aggregation score predictions. On another note, while TDP-43 and FUS share similarities in terms of domain organization, their pathologies appear to be mutually exclusive, which could potentially be explained by their differences in terms of LLPS and aggregation, as predicted by their contrasting scores (Table 2).

Neurons are post-mitotic cells and as such are particularly vulnerable to proteostasis stress, as these cells are unable to dilute their cellular content by cell division. The correct balance and function of both LLPS and clearance are therefore crucial in order to prevent the accumulation of proteins and the formation of aggregates. Because the cell body lies far from the synapse, motor neurons rely heavily on axonal transport to maintain neuronal health and to ensure proper communication between the different cellular compartments. There are three points to consider. (i) Axonal transport impairments are observed in cell lines derived from fALS and sALS patients. (ii) Pathological RNP granules in motor neurons might be due to these proteins’ unique function. Indeed, they are involved in the transport of RNA granules, relying on LLPS over long axons, and hence contain a much higher amount of RNA granules and RBPs. (iii) This LLPS occurs intentionally at low sterical hindrance to
increase transport efficiency, which unfortunately makes this system sensitive to disruptions (Wolozin, 2014).

Some ambiguities remain in ALS-LLPS research. First, many proteins found in SGs are not present in the aggregates observed in ALS patient tissue and often these proteins do not bear ALS-related pathogenic mutations. Second, SGs are currently thought to serve a protective function. Third, it is not merely the existence of SGs, which is put into question, but rather their disassembly. Perhaps the nature of these SG proteins results in partial colocalization with aggregates and, therefore, there is a bias for finding such proteins in association. In addition, while fibres have been shown to grow out of these droplets in vitro and advanced optodroplet techniques have been applied, these systems fail to show fibres growing out of the same droplets in a cellular context.

6. Future perspectives

We do not fully understand the link between TDP-43, FUS, SOD1, and DPRs, yet they are the main proteins used to classify post-mortem ALS cases. The mislocalisation of FUS and TDP-43 precedes their aggregate formation, and RBPs are unable to mislocalize without interacting with these aggregates. In sALS, FUS mislocalization is a hallmark of ALS (Tyzack et al., 2019) without the need of aggregation, suggesting that the initial mislocalization of these nuclear proteins is more significant in terms of pathogenicity than the formation of aggregates.

Moreover, it is not possible to confirm whether all these aggregates result from aberrant phase separation, as the aggresome proteome of ALS/FTD has not yet been completed. Indeed, we currently only know this for a handful of ALS aggregate-positive proteins, for which different morphologies occur in aggregates. In addition, while the impact of LLPS condensates on insoluble proteins has been studied, research on the effect of these condensates on the soluble protein state remains to be determined. Using novel techniques such as laser-capture mass spectrometry, this aggresome will likely be teased out in the near future, but the tools to study aberrant phase separation in an endogenous cell model remain limited.

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**Figure Legends**

**Figure 1** Mechanisms related to ALS associated or not with LLPS. Schematic illustration of the most important pathological processes related to ALS that rely on or are influenced by the functioning of LLPS in motor neurons. Grey boxes are for processes not directly linked to LLPS yet. Detailed evidence for the mechanisms involved in LLPS can be found in Table 1.
Table 1 Mechanisms in ALS and evidence supporting involvement of phase separation.

| Mechanism                      | LLPS | General effect                                                                 | Evidence                                                                 | Reference                                      |
|--------------------------------|------|---------------------------------------------------------------------------------|--------------------------------------------------------------------------|-----------------------------------------------|
| Autophagy                      | Yes  | Autophagy proteins regulating LLPS                                             | p62 is involved in the aggregation of polyubiquitinated misfolded proteins by LLPS and forms droplets *in vivo* with liquid-like properties | (Sun et al., 2018)                            |
|                                |      |                                                                                  | ALS-linked mutations modulate Ubiquilin-2 LLPS                          | (Dao et al., 2018)                            |
| Axonal transport               | Yes  | DPRs causing transport defects                                                  | Altered mRNA transport and local translation; LLPS influences cytoskeletal function and microtubule-based transport by steric hindrance | (De Vos and Hafezparast, 2017; Burk and Pasterkamp, 2019; Guo et al., 2019) |
|                                |      |                                                                                  | DPRs cause perturbation of kinesin-1 and dynein-1 motors along microtubules *in vitro* and *in vivo* | (Fumagalli et al., 2019)                      |
| Chaperone impairment           | Yes  | Chaperone binding impaired by LLPS proteins                                    | Kapβ2 inhibits LLPS of FUS                                              | (Yoshizawa et al., 2018)                      |
|                                |      |                                                                                  | Hsp104 mediates disaggregation of TDP-43 and FUS fibrils                 | (Shorter, 2016)                               |
|                                |      |                                                                                  | Mutant UBQLN2 lacks recognition by HSP70 causing accumulation of misfolded/aggregated proteins | (Hjerpe et al., 2016)                         |
|                                |      |                                                                                  | TNPO1/Kapβ2 are FUS chaperones mediating phase separation and SG association of FUS | (Hofweber et al., 2018)                       |
|                                |      |                                                                                  | Hsp27 chaperones FUS to undergo LLPS in response to stress-induced phosphorylation | (Liu et al., 2020b)                          |
| DNA repair                     | Yes  | DNA repair impeded                                                             | 53BP1 determines liquid-like behaviour of DNA repair compartments        | (Penndorf et al., 2018)                       |
|                                |      |                                                                                  | FUS involved in DNA repair                                              | (Wang et al., 2013)                           |
|                                |      |                                                                                  | Activation of PARP-1 directs FUS to DNA damage sites                    | (Singatalina et al., 2019)                    |
| Inflammation                   | Maybe| No effect                                                                      | No direct evidence; TIA1 and G3BP are linked with microglia function in Alzheimer’s disease | (Ghosh and Geahlen, 2015)                     |
| Mitochondrial dysfunction      | Maybe| Mitochondrial nucleoids formed by LLPS                                        | TFAM spontaneously phase separates                                      | (Smith et al., 2019)                          |
| Nucleocytoplasmic transport    | Yes  | DPRs and nuclear pore interactions influencing LLPS behaviour                  | (G4C2) repeats expression in *Drosophila* salivary glands leads to abnormal nuclear membranes | (Freibaum et al., 2015)                       |
|                                | No   |                                                                                  | PR50 and GR50 interact with nuclear pore proteins in HEK cells           | (Lee et al., 2016)                            |
|                                |      |                                                                                  | Poly-PR peptides lead to blockage of nuclear pores in U2OS cells treated with PR20 peptides | (Shi et al., 2017)                            |
|                                |      |                                                                                  | *Drosophila* salivary glands expressing (G4C2) repeats show impaired import by sequestration of RanGap in nuclear RNA foci | (Zhang et al., 2015)                          |
|                                |      |                                                                                  | HEK293 cells transfected with PR50/GR50 show sequestration to SGs of proteins involved in nuclear transport | (Zhang et al., 2018)                          |
|                                |      |                                                                                  | Poly-GR and poly-PR expressed in motor neurons and cell lines do not directly interfere with nucleocytoplasmic transport | (Vanneste et al., 2019)                      |
|                                |      |                                                                                  | Nucleocytoplasmic transport factors identified as modifiers of DPR pathology in a *Drosophila* screen | (Boeynaems et al., 2016)                      |
| Phenotype Type         | Results | Phenotypic Characteristics                                                                 | Relevant References |
|-----------------------|---------|---------------------------------------------------------------------------------------------|---------------------|
| Protein aggregation   | Yes     | Proteins in droplets evolving to protein aggregates                                          | (Patel et al., 2015; Conicella et al., 2016) |
|                       |         | ALS mutations of FUS and TDP-43 cause an acceleration of aberrant phase transition           |                     |
| RNA metabolism        | Yes     | Droplets sequestering the machinery of RNA metabolism and translation, causing significant reductions in new protein synthesis | (Mann et al., 2019) |
|                       |         | Pathological inclusions are driven by aberrant interactions between LCDs of TDP-43 that can be antagonized with RNA |                     |
|                       |         | Non-functional transcription machinery causes general reduction in protein synthesis         | (Butti and Patten, 2019) |
|                       |         | LLPS of poly(GR) and poly(PR) in vitro enhances the multivalent interactions of the liquid phase of SGs in cells | (Boeynaems et al., 2017) |
| Seeding of proteins   | Yes     | Recombinant TDP-43 seeds forming droplets of protein                                          | (Gasset-Rosa et al., 2019) |
|                       |         | Exposure to TDP-43 seeds in neuronal-like cells leads to the formation of protein droplets    |                     |

Pathogenic phenotypes are shown in blue and non-pathogenic phenotypes are shown in red. Yes/no results in the LLPS column refer to the presence/absence of direct involvement of LLPS in a particular ALS-related mechanism. DPRs, arginine-rich dipeptide repeat proteins; TNPO1, transportin 1; Kapβ2, karyopherin-β2; TFAM, mitochondrial transcription factor A.
Table 2 Genes associated with ALS/FTD with LLPS and aggregation scores.

| Gene          | LLPS score | Phase separation confirmed | Aggregation score | Found in aggregates |
|---------------|------------|----------------------------|-------------------|---------------------|
| ADAR2         | 0.242      | Not yet                    | 98.368            | Yes                 |
| ANXA11        | 0.405      | Yes                        | 76.584            | Yes                 |
| ATXN2         | 0.633      | Yes                        | 93.428            | Yes                 |
| C9orf72       | 0.137      | No (DPRs only)             | 99.952            | DPR yes             |
| DNAJC7        | 0.289      | Not yet                    | 62.24             | Unknown             |
| EWS           | 0.857      | Yes                        | 4.521             | Yes                 |
| FUS           | 0.915      | Yes                        | 4.473             | Yes                 |
| FIG4          | 0.285      | Not yet                    | 99.592            | Yes                 |
| hnRNPA1       | 0.603      | Yes                        | 92.027            | Yes                 |
| hnRNPA2B1     | 0.649      | Yes                        | 55.529            | Yes                 |
| hnRNPA3       | 0.643      | Yes                        | 92.132            | Yes                 |
| KIF5A         | 0.236      | Not yet                    | 60.092            | Unknown             |
| Matrin 3      | 0.422      | Not yet                    | 7.724             | Yes                 |
| NEK1          | 0.389      | Yes                        | 94.682            | Unknown             |
| OPTN          | 0.197      | Not yet                    | 79.727            | Yes                 |
| SOD1          | 0.223      | Not yet                    | 14.877            | Yes                 |
| SGMR1         | 0.173      | Not yet                    | 99.951            | Yes                 |
| SYNCRIP       | 0.571      | Yes                        | 74.295            | Yes                 |
| (hnRNPQ)      |            |                            |                   |                     |
| SQSTM         | 0.321      | Not yet                    | 6.455             | Yes                 |
| TARDBP        | 0.376      | Yes                        | 97.627            | Yes                 |
| TAF15         | 0.801      | Yes                        | 4.652             | Yes                 |
| TIA1          | 0.422      | Yes                        | 47.614            | Yes                 |
| TBK1          | 0.194      | Not yet                    | 96.825            | No                  |
| UBQLN2        | 0.241      | Yes                        | 98.684            | Yes                 |
| VCP           | 0.288      | Not yet                    | 61.728            | Yes                 |

Scores indicated in bold/red: highly likely to aggregate/undergo LLPS, LLPS score >0.6, aggregation score >60.
Scores underlined/inicated in organde: likely to aggregate/undergo LLPS, LLPS score >0.3, aggregation score >40.
Scores indicated in italic/beige: somewhat likely to aggregate/undergo LLPS, LLPS score >0.2, aggregation score >10.
Scores in blue: unlikely to aggregate/undergo LLPS, LLPS score <0.2, aggregation score <10.
Figure 1: Mechanisms related to ALS associated or not with LLPS
Schematic illustration of the most important pathological processes related to ALS that rely on or are influenced by the functioning of LLPS in motor neurons. Grey boxes are for processes not directly linked to LLPS yet. Detailed evidence for the mechanisms involved in LLPS can be found in table 1 below.