Functional roles of protein phase separated assemblies in cellular stress response and proteinopathies

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is life a ride to ride

or a story to shape and confide

or chaos neatly denied

-Bill Callahan

ABSTRACT

For a long time, pertinent biological conundrums, such as the organization and compartmentalization of the cytoplasm and nucleoplasm, perplexed the scientific community. The organization of the complex biochemistry in an accurate space and time manner could not be explained without the enclosure of a membrane. In recent years, many of the processes relating to membraneless cellular organization and cytoplasmic regulation have become illuminated by the incorporation of liquid-liquid phase separation (LLPS) into biology. Phase separation entails a process by which a homogenous liquid solution of macromolecules, such as proteins or nucleic acids, separates into two distinct co-existing phases, a dense and dilute phase. LLPS thereby enables a spatiotemporal control over complex biochemical reactions, a vital process for cellular functions. Altered phase separation dynamics can lead to aberrant condensate assemblies that mature into a more solid-like state and are associated with disease. The list of cell compartments and biological processes thought to be formed and regulated through the process of phase separation has grown at a fast pace and includes a plethora of cellular functions, such as stress granule (SG) formation and disease associated protein aggregation. However, the mechanisms regulating the formation, and subsequent impact, of these phase-separated assemblies still remain elusive.

In this thesis, regulation of SG formation is explored using genome-wide phenomic screening. The results show a signaling cascade involving e.g. long-chain-base sphingolipids and ubiquitin, regulating the phase separation behavior of Lsm7 and further SG induction. Cellular consequences of cytotoxic aggregation of the ALS-associated disease protein FUS (fused in sarcoma) are also explored. A cytotoxic gain-of-function involving protein sequestration, resulting in delayed cell cycle progression, is identified. Overall, our findings elucidate the underlying mechanisms and cellular impacts of phase separated assemblies in health and disease.

Keywords: phase separation, LLPS, stress granules, Lsm7, FUS, Ccr4

SAMMANFATTNING

Hur molekyler organiseras och grupperas i den cellulära cytoplasman och nukleoplasman orsakade länge huvudbry inom det vetenskapliga samfundet. Organiseringen av den komplexa biokemiska miljön kunde inte förklaras utan att involvera omhöljande membran, som fallet är för mitokondrier eller cellkärnan. Under de senaste åren har dock en process som involverar fasseparation av vätskor kommit att klarlägga dessa frågor. Fasseparation av vätskor innebär att två separata faser uppstår ur en homogen lösning med makromolekyler bestående av till exempel proteiner och nukleinsyror. Den ena fasen innehåller därmed molekyler som inte är representerade i den andra fasen. Denna fasseparation av vätskor möjliggör således spatiotemporal reglering av komplexa biokemiska reaktioner och skapande av biomolekylära kondensat, en process som är väsentlig i åtskilliga cellulära funktioner. Rubbning av denna process kan leda till bildande av abnorma sjukdomsrelaterade kondensat som har förmågan att utvecklas till mer stabila strukturer över tid. Rapporter om biologiska processer som involverar fasseparation har ökat markant de senaste åren men många av de underliggande mekanismerna är fortfarande outforskade, såsom initieringen av stressgranuler och de cellulära implikationerna av sjukdomsrelaterad proteinaggregering.

I denna avhandling utforskar vi bland annat hur bildningen av stressgranuler regleras. Våra resultat visar att en signaleringskaskad som inkluderar exempelvis sfingolipider och ubikvitin reglerar fassepareringen av proteinet Lsm7 vilket följaktligen påverkar initieringen av stressgranuler. Vi diskuterar ytterligare de cellulära konsekvenserna av abnorm fasseparering av FUS, ett protein som har påvisats bilda sjukdomsaggregat hos patienter med amyotrofisk lateralskleros. Vi påvisar att FUS-aggregering resulterar i förändrade funktioner hos andra proteiner vilket i sin tur försenar cellcykelns fortskridning. Våra resultat belyser därmed underliggande mekanismer och konsekvenser av fasseparering i både sjukdom och hälsa.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Lindström*, M.**, L. Chen*, S. Jiang, D. Zhang, Y. Gao, J. Zheng, X. Hao, X. Yang, A. Kabbinale, J. Thoma, L. C. Metzger, D. Y. Zhang, X. Zhu, H. Liu, C. M. Gustafsson, B. M. Burmann, J. Winderickx, P. Sunnerhagen and B. Liu. Lsm7 phase-separated condensates trigger stress granule formation.

Nature Communications, **13**:3701 (2022). * Contributed equally.

II. Chen, L., **M. Lindström**, Y. Gao, X. Hao, S. Jiang, X. Yang, H. Liu, P. Sunnerhagen and B. Liu. Stress granule formation is regulated by a signaling machinery involving Sch9/Ypk1, sphingolipids, and Ubi4.

Submitted, 2022.

III. **Lindström, M.**, L. Chen, Y. Gao and B. Liu. FUS-induced abnormal protein assembly formation and cell cycle delay in *Saccharomyces cerevisiae*.

Manuscript, 2022

Not included in this thesis:

Liu, Q., X. Zhu, **M. Lindström**, Y. Shi, J. Zheng, X. Hao, C. M. Gustafsson and B. Liu. Yeast mismatch repair components are required for stable inheritance of gene silencing. *PLOS Genetics,* **16**: e1008798 (2020).

Lindström, M. & B. Liu. Yeast as a Model to Unravel Mechanisms Behind FUS Toxicity in Amyotrophic Lateral Sclerosis. *Frontiers in Molecular Neuroscience,* **11** (2018)

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AIM OF THE THESIS

Liquid-liquid phase separation (LLPS) has been implicated in the formation of a multitude of biomolecular condensates, including stress granules. LLPS is therefore considered to be a vital and ubiquitous process needed for various cellular functions. However, the mechanisms underlying the formation of these phase separated assemblies still remain elusive. Altered phase separation dynamics can further lead to aberrant condensate assemblies that have matured into a more solid-like state. These aberrant forms of condensates are associated with disease, such as the cytotoxic aggregation of human FUS (fused in sarcoma) protein in amyotrophic lateral sclerosis (ALS), further underscoring the importance of elucidating the different aspects underlying phase separated assemblies in both health and disease.

This thesis compiles a total of three research papers which demonstrate mechanisms and cellular impacts of phase separated assemblies at a genome-wide scale. The initial part of the thesis introduces the concept of phase separation and further describes its role in the formation of ribonucleoprotein granules, and more specifically stress granules and processing bodies. Next, known aspects of phase separation in a disease context are described, with further emphasis on the human disease protein FUS. The papers can therefore be divided into two main groups that explore different aspects of phase separation. In **Paper I** and **Paper II**, we explore a regulatory mechanism behind stress granule formation. In **Paper III,** focus lies on the cellular consequences of aberrant FUS protein phase separation and subsequent aggregation.

INTRODUCTION

THE CONCEPT OF PHASE SEPARATION

The development of various organelle structures is a vital feature of biological evolution. The existence of contained chemical microenvironments within the cytoplasm of eukaryotic cells enables the sequestration of molecules and proteins, thereby increasing the reaction rate of biochemical processes and critical functions. Enabled by the lipid bilayer membranes, membrane-bound organelles can harbor specific molecules in a restricted space separated from the rest of the cytoplasm[1]. This is a necessary feature underscored by the consequences of leakage from these confined spaces, such as the release of cytochrome c from mitochondria resulting in apoptosis[2].

In addition to membrane-bound organelles, such as the nucleus, endoplasmic reticulum and mitochondria, eukaryotic cells also harbor membraneless organelles, such as nucleoli, Cajal bodies, processing bodies (PBs) and stress granules (SGs). As suggested by the name, these organelles lack an encapsulating membrane[3]. Even though lacking a membrane separating them from the immediate environment, these cytoplasmic and nuclear organelles are stable and capable of controlled exchange with its surrounding[4]. But how are these structures formed and controlled without the support of a membrane?

Already in 1899, E. B. Wilson hypothesized that the cytoplasm of cells behaves like a mixture of different chemically suspended drops[5]. Subsequently, in 1999, it was suggested that a high intracellular concentration of two incompatible macromolecules should result in demixing in the cytoplasm, i.e. molecular crowding could promote liquid-liquid phase separation (LLPS) in the cytosol[6]. However, evidence in support of this was not found until 2009, when P granules in *Caenorhabditis elegans* were shown to be driven by a controlled dissolution and condensation of granule components, resulting in a structure behaving in a liquid-like manner, also referred to as condensates[7]. This process of separation of a well-mixed liquid solution into two distinct

liquid phases, where one phase contains enriched components lacking in the other phase, is the essence of LLPS[8]. Subsequently, LLPS has been shown to be involved in various processes, such as heterochromatin assembly, immune signaling, autophagy and disease development[9-12]. LLPS has been further implicated in the formation of eukaryotic membraneless organelles, such as PBs, nucleoli and SGs[7,13-15]. The process of LLPS is thereby emerging as a crucial mechanism in explaining the formation and function of membraneless organelles and phase-separated assemblies[16].

Biomolecular condensates have been found to be enriched with multivalent molecules, i.e. molecules containing multiple sites capable of potential inter- or intra-molecular interactions, referred to as multivalency[17]. Multivalency has thus been established as a driving force of phase separation[18,19]. Two types of multivalent interactions have been identified as contributing to LLPS, (1) intracellular protein-protein, protein-RNA and RNA-RNA interactions and (2) weak, transient multivalent interactions between intrinsically disordered regions (IDRs), such as cation-anion interactions, π–π interactions, π–cation interactions and dipole–dipole interactions[17,20,21]. LLPS can therefore be described as the product of the force of electrochemical gradients established through multivalent interactions within and between proteins and RNA (Figure 1). Multivalency also provides an explanation as to how the assembly of condensates can be regulated, since minor changes in the structure's stoichiometry could have forceful effects on the valency of key components[20].

It is thought that in their physiological state, certain LLPS condensates form and dissolve frequently upon specific external stimuli. However, some conditions might impair proper dissipation of LLPS droplets, such as certain mutations, chronic stress or aging. This could further result in a phase transition into a more solid-like state, of which, examples have been uncovered in a disease context[15,22].

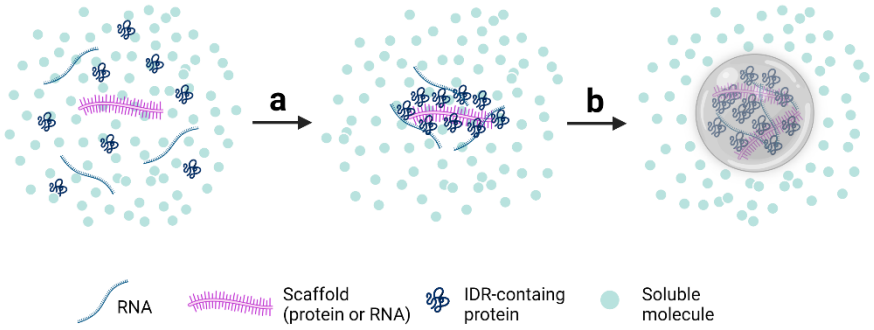

*Figure 1. Formation of biomolecular condensates. (**a**) A multivalent protein or RNA can work as a structural platform/scaffold and initiate nucleation by attracting IDR-containing proteins and RNA in a liquid solution surrounded by other small soluble molecules. (**b**) Phase separation resulting in a liquid-like condensate where the condensate is enriched in molecules not present in the surrounding. Emergence of more scaffolds, in addition to RNA-RNA, protein-RNA, protein-protein interactions. Created with Biorender.com.*

LLPS OF RIBONUCLEOPROTEIN GRANULES

Ribonucleoprotein particle (RNP) granules, termed so due to their high concentration of protein and RNA, are membraneless organelles found both in the nucleus, e.g. paraspeckles and nucleolus, and in the cytoplasm, such as SGs and PBs[23]. RNP granules are membraneless biomolecular condensates that form through multivalent interactions resulting in a demixing into two stably co-existing phases, a selective dense phase, enriched for protein and RNA, and a dilute phase[24-26]. As such, RNP granules form through a sum of various protein and RNA interactions, where many RNP granule-associated proteins contain IDRs[27]. Generally, a specific population of RNA is needed for RNPs to form. For instance, both SGs and PBs require non-translating RNAs for their formation and stability[13,28]. In accordance, RNPs also harbor specific RNA-binding proteins (RBPs), where SGs and PBs display distinct but also shared components[29,30]. The mRNP composition in these granules determines the fate

of mRNA by regulating various processes, including transport, degradation and translation[31]. It has been shown that a threshold protein and/or RNA concentration is needed for the assembly of RNP granules driven by concentration-dependent LLPS. However, these liquid-like condensates might further convert into a different state, such as hydrogel- or solid-like, if the concentration is exceeded[32,33].

It has been proposed that the dynamics of RNP components within the granule is largely controlled by two key principles. First, higher protein or RNA valency results in slower exchange rate due to increased avidity[25]. The importance of this factor is underscored by the rigid and static features of centrally located mRBPs in RNP granules, indicating these RBPs have many interactions trapping them in the center, whereas RNP surface components are more dynamic, indicative of fewer more transient interactions[34,35]. Moreover, studies indicate slow dynamics of phase separated RNAs *in vitro* as well as in RNP granules, reasonable since RNAs would be expected to be highly multivalent due to their generally increased spatial extent and mass, in comparison to proteins[34-37]. Second, tighter affinity for RNA, of any component, results in slower exchange rates. Accordingly, *in vitro* studies show that RNA affinity correlates with the presented dynamics of RBPs, further supported by *in vivo* FRAP (fluorescence recovery after photobleaching) measurements of different RNP granule components[38,39]. Furthermore, RNA self-assembly occurs readily *in vitro* and biochemical changes increasing/decreasing RNA self-assembly *in vitro* correspondingly affect RNP granule formation in vivo[40-42].

STRESS GRANULES

Eukaryotic cells are frequently exposed to fluctuating and potentially unfavorable environmental conditions, such as chemical, physical and nutritional stress, leading to e.g. starvation and cellular oxidative stress[43]. To deal with stressful conditions, cells have evolved defense and coping mechanisms enabling energy conservation and protection of macromolecules.

For short-term survival, rapid responses mainly exert their effects at the post-translational level in the form of post-translational modifications (PTMs), protein binding and protein relocalization. Medium-term survival and recovery are mainly focused on reducing the translation level of house-keeping and proliferation-linked genes, instead prioritizing the synthesis of proteins involved in stress adaptation[44,45]. Protein synthesis is a major consumer of the cell's energy production and therefore, under stress conditions, translation needs to be tightly regulated. Subsequently, cells decrease the stability and translation rate of most mRNAs under stress, whereas mRNAs needed for stress survival are stabilized and highly translated [46-49]. Long-term survival under chronic stress is characterized by transcriptional modification as the major regulatory mechanism[50,51].

The post-transcriptional stress response program also entails the formation of two evolutionarily conserved cytoplasmic mRNP granules, SGs and PBs. These stress-induced membraneless granules allow cells to adapt to diverse environmental and cellular cues. In contrast to SGs, PBs are present constitutively, however; under stress, the number of PBs increases[28,52]. SGs only form under conditions of severe stress such as temperature changes, viral infection, oxidative and osmotic stress, irradiation and starvation[53-55]. The induction of SGs is triggered by the stress-induced inhibition of translation and have been observed in isolated cells and in the context of the entire organism[56-59]. Moreover, SGs are dynamic structures that have been shown to undergo fusion and fission events, in addition to exchanging components with the cytoplasm[29]. The structure of SGs has been proposed to include two diffusely distinct compartments, a stable core substructure surrounded by a more dynamic shell[60,61]. Subsequently, SG assembly has been proposed to be nucleated by the stable core followed by the assembly of the shell[62,63]. The full function of SGs and assembly mechanism still remain unclear, however; distinct modes of mechanisms have been assigned to SGs[64].

SGs range in size from 100 to 2000 nm and contain a high local concentration of proteins and mRNA, of which most encode housekeeping proteins[65]. The composition of SGs varies based on the stress situation but usually include

translationally arrested mRNAs, translation initiation factors, RBPs and small ribosomal units[56,66]. It has been hypothesized that the close contact, within the confined space of SGs, enables an increased efficiency of certain reaction kinetics, for instance in promoting interactions between translation factors and mRNA to enhance the formation of translation initiation complexes upon stress relief[28]. Accordingly, during stress, SGs temporarily store and protect non-translating mRNA and proteins from autophagy and proteasomal degradation, thereby allowing a fast restart of translation when stress subsides[64]. In addition, SGs have been implicated as modifiers of signal transduction under stress, since specific signaling factors have been identified to be sequestered in SGs, such as members of the TORC1 signaling complex[67]. Sequestration into SGs could result in their activity in particular signaling pathways becoming altered[67,68]. Even though SGs contain many translational repressors, formation of SGs is not required for translation inhibition during stress[69,70,71].

Under normal conditions, the formation of SGs is a dynamic and reversible process needed for increased survival during stress responses[72-74]. However, prolonged stress or disease-linked mutations in proteins known to associate with SGs have been identified to alter normal SG dynamics. Accordingly, SGs are associated with a number of human diseases, including cancers, viral infections and neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS) and spinocerebellar ataxia type 2[75-77].

STRESS GRANULE ASSSEMBLY

As previously stated, SGs are formed upon stress-induced inhibition of translation[56-59]. Inhibition of translation is often induced through phosphorylation of elongation initiation factor 2 alpha (eIF2α), by one or more kinases[56,78]. Subsequently, this results in polysome disassembly and release of translation initiation factors, ribosomal subunits and RBP-coated mRNAs. Released polysome components accumulate in the cytoplasm and are further sequestered into SGs where they will be kept silent and protected from degradation until the stress subsides[72,78]. Disassembly of polysomes has

therefore been suggested to be the universal trigger of SG formation. Inhibition of translation has been shown to be mediated through various routes, such as previously mentioned phosphorylation of eIF2α, inhibition of TOR or interference with the eIF4F complex[79]. Formation of SGs can therefore be either dependent or independent of the initiation factor eIF2α[56,58]. Accordingly, observations of eIF2α-independent SG formation have been reported in both yeast and mammalian cells[80-83].

Moreover, the signaling pathways needed for SG formation can vary based on the type of stress, where specific regulators might be crucial under certain stress conditions, but not others[83]. In accordance, the composition of SGs, and exchange dynamics of individual SG components, differ depending on the type of stressor[84]. The protein composition of SGs can also vary depending on which step of the mRNA translational cycle stalling occurs[31]. Accordingly, the importance of non-translating mRNAs has been linked to the formation of SGs, where trapping mRNAs in polysomes results in failed SG formation[73]. Furthermore, the local RNA concentration has been reported to influence SG formation[85,86].

Many SG-associated proteins contain IDRs or prion-like domains (PLDs), and some have been linked to stress granule LLPS, such as the PLD of human TIA-1 protein, which has been reported to promote SG formation[87]. Specific characteristics of an IDR is the lack of a defined 3D structure, in addition to containing low diversity repeated sequences that contribute to multivalent intermolecular interactions. Many proteins with IDRs have thus been shown to phase separate on their own *in vitro*[15,88]. In accordance, RBPs with IDRs have been implicated in the formation of various RNP granules, where e.g. disruption of a key protein's IDR could lead to decreased LLPS and disrupted granule formation[89,90].

Only a fraction of SG-associated proteins is believed to be necessary in the induction and maintenance of SGs. Key proteins that are essential in driving SG formation are usually referred to as "scaffold proteins"[91]. Examples of such key RBP components of SGs have been identified in yeast and mammalian

models, e.g. PolyA-binding protein (PABP), RasGAPSH3-binding protein (G3BP), Lsm7 (Sm-like protein 7) and T-cell intracellular antigen-1 (TIA-1)[13,56,66]. Many key SG components are known to be substrates for PTMs. Subsequently, PTMs have been reported to play a role in SG regulation, where various modifications have been shown to influence SG assembly, such as e.g. demethylation of G3BP and phosphorylation of eIF2α[92,93]. Accordingly, studies have shown that a specific deubiquitinase enzyme, Ubp3 (yeast) / USP10 (human), is needed for efficient assembly of SGs[94,95]. Further identification of different components in the SG interactome is ongoing with many additional SG-related proteins being identified continuously[13,31].

STRESS GRANULE DISASSEMBLY

The process of assembly and disassembly of SGs is in equilibrium with polysomes, steered by the presence of stress[41]. The assembly of SGs has been far more extensively studied than the disassembly process[31]. However, studies have identified that distinct subsets of proteins associate with SGs during the disassembly phase, indicating that disassembly is not a mere passive dissolution of SG components, but a highly regulated process[96]. Upon stress relief, SGs can dissolve at different rates depending on the stress, e.g. within minutes after return to normal temperature after cold stress, and up to hours after heat shock[53,97]. Disassembly of SGs enables recycling of SG components, which would otherwise have had to be synthesized *de novo*, thereby allowing for a more energy efficient process[98]. Moreover, the disassembly of SGs is important for protein homeostasis and viability in cells, which is further highlighted by the reported consequences of impaired SG clearance resulting in aberrant SGs and protein aggregates, implicated in neurodegenerative disease[76,99].

It has been hypothesized that the loss of some highly multivalent components could lead to a subsequent disassembly of SGs, as a result of decreased multivalency. Accordingly, a stepwise disassembly model has been proposed, involving the titration of stalled mRNAs out of SGs, resulting in structural

instability of the protein assemblies and subsequent disassembly of visible SGs[100]. Additionally, post-translational modifications of certain essential SG node proteins have been linked to the disassembly of SGs[101,102]. For instance, during heat shock, ubiquitination is not needed for SG assembly but instead aids in SG disassembly, where loss of ubiquitination results in delayed disassembly[103]. Lastly, chaperones have been proposed to function as SG regulators, e.g. by preventing irreversible aggregation of aberrant proteins within SGs, thereby ensuring continuous SG fluidity and eventual disassembly[104,105].

As mentioned before, many RBPs contain IDRs or low complexity domains (LC) which allow for multiple interactions and a flexibility in folding. These characteristics enable the formation of dense protein-protein or protein-RNA networks[64,106]. However, this conformational flexibility also entails a proneness to aggregation, i.e. a transition into a more solid-like state is possible and could further function as a seeding mechanism to incorporate other proteins that are aggregation-prone[8,107]. Accordingly, mutations in the PLD of RBPs has been linked to increased aggregation. Furthermore, these aggregated proteins could lead to the aberrant persistence of SGs upon stress removal, leading to other cellular pathological changes[108,109]. These kind of changes in LLPS behavior have been linked to, in addition to IDR-mutations, high protein concentration and changes in temperature and pH[8,110]. In accordance, accumulating findings suggest that LLPS-driven aberrant SG assembly is associated with e.g. cancer, virus infections and neurodegenerative disease[8,76,111].

PROCESSING BODIES

mRNA turnover is a vital cellular function in regulating gene expression. The process of mRNA degradation is initiated by deadenylation of the 3′-polyadenosine (polyA) tail, predominantly performed by the deadenylases in the Ccr4-Not complex. Deadenylation is primarily followed by decapping, carried out by Dcp1 and Dcp2 decapping enzymes, a process regulated by

decapping co-activators such as the Lsm1-7/Pat1 complex. The decapped mRNA is then further 5´-to-3´degraded by the exonuclease Xrn1[112]. Non-translating mRNAs together with the mRNA decay machinery and other translation repressors accumulate into conserved cytoplasmic mRNP granules, called processing bodies (PBs), under both normal and stressed conditions[113-115]. In other words, PBs are present constitutively but drastically increase in number upon stress. PBs have been linked to various types of mRNA regulation, including mRNA decapping in regular mRNA decay, nonsense-mediated mRNA decay, AU-rich element-mediated mRNA decay, microRNA-mediated translational repression, general translation repression and mRNA storage[113,116].

Efficient mRNA degradation is correlated with the formation of PBs but macroscopically observable PBs are not necessarily required for mRNA degradation to occur[114,117]. Moreover, mRNAs stored in the large PBs visible under yeast stationary phase recycle back into translating polysomes when growth resumes, indicating mRNAs residing in PBs can re-enter the translational machinery[118]. It has therefore been proposed that PBs, in addition to hosting the mRNA decay machinery, also function as storage sites for translationally repressed mRNAs and inactive mRNA decay enzymes[113].

As mentioned before, SGs are associated with mRNA sorting and storage during stress. From SGs, mRNAs can be further routed to either reinitiation or degradation[119]. Accordingly, SGs and PBs, being sites of mRNA degradation, are spatially and functionally linked[120]. Even though SGs and PBs share and exchange several protein and RNA components, they are distinct and independent cytoplasmic structures[72,73]. Just like SGs, PBs are dynamic liquid-like structures that constantly exchange mRNAs with the surrounding cytosol and polysomes[119]. Accordingly, drugs that stabilize polysomes lead to a disassembly of both SGs and PBs, suggesting both these structures require non-translating mRNA for their persistence[78]. Multiple PB-associated proteins have been shown to undergo LLPS in vitro, such as Dcp2, Edc3 and the IDR domain of Lsm4, of which the IDR and RRM regions of Edc3 and Lsm4 have been shown to be especially important for PB maintenance[121-123]. The

importance of these LLPS-associated domains further highlights the likely role of phase separation in PB formation[122].

It has been hypothesized that the formation of SGs is dependent on a preceding formation of PBs[28]. For instance, mutations affecting PB formation often also result in SG deficiencies, whereas disrupted SGs do not necessarily affect PB formation[28]. Furthermore, upon stress, PB formation increases before SGs start to appear, often appearing close to or overlapping with PBs. However, studies in yeast have shown that SGs and PBs are regulated by different signaling pathways[13,124]. In accordance, individual SGs have been observed to form independently of pre-existing PBs[125]. Furthermore, knockdown of specific mRNAs in mammalian cells lead to suppressed PB formation without affecting SGs[71]. Lastly, SG-associated proteins have been shown to phase separate *in vitro* without the addition of PB components[13,126].

LSM1-7 COMPLEX

The conserved Lsm1-7 (Sm-like proteins) complex resides in the cytoplasm where it is involved in regulation of decapping, with the interacting partner Pat1. The Lsm components form a hetero-heptameric ring[127,128] structure that constitutes the assembled complex (Figure 2). The Lsm1-7/Pat1 complex has also been shown to protect mRNA 3'-ends from premature trimming[129]. The Lsm1-7/Pat1 complex selectively binds at the 3'-end of deadenylated mRNAs, with preferential binding of oligoadenylated RNAs over polyadenylated RNAs[130,131]. Pat1 has been shown to stabilize the binding of the Lsm1-7 complex to RNA, in addition to broadening the RNA-binding specificity[132]. Lsm1 is also known to regulate the specific RNA binding properties of the complex[133]. Loss of function of any of the complex subunits results in stabilization of mRNAs *in vivo* due to impaired 5′to 3′ mRNA decay[129]. Moreover, the Lsm1-7 complex shares components with another complex, Lsm2-8 which resides in the nucleus. The Lsm2-8 complex is involved in pre-mRNA splicing and processing of various nuclear RNAs as well as nuclear

mRNA decay[134]. Both complexes have been shown to form the ring-structure spontaneously in the absence of RNA[128].

The Lsm1-7/Pat1complex is heavily concentrated in PBs and shown to be required for efficient decapping during mRNA degradation[134]. Accordingly, the Lsm1-7/Pat1 complex components are usually considered part of the conserved core of proteins found in PBs. Accordingly, deletion of Pat1 results in reduced number of PBs[122]. Pat1 is a multifunctional protein that binds decapping-related proteins, such as Dcp1-Dcp2 and Dhh1, to activate decapping[135]. Pat1 has also been shown to interact with Xrn1 and the Ccr4-Not complex, members of the mRNA decay machinery[136]. Loss of Pat1 or Lsm1 leads to an accumulation of deadenylated and capped transcripts, indicating disturbed decapping process[137]. It has been proposed that the efficiency of recruitment of the Lsm1-7/Pat1 complex onto an mRNA could be the key determinant of which mode of decay (5' to 3'or 3' to 5') the mRNA goes through. I.e. fast association of the Lsm1-7/Pat1 complex with an oligoadenylated mRNA tail would probably lead to decapping and mRNA degradation in 5' to 3', whereas inefficient binding to the complex could result in complete deadenylation and subsequent targeting to the exosome for 3' to 5' decay[138].

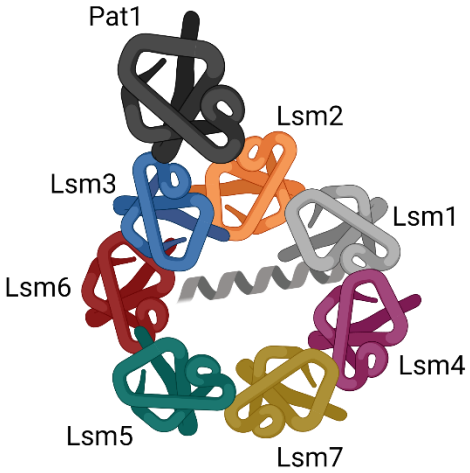

Figure 2. The heptameric ring structure of the Lsm1-7 complex. Lsm1 displays a C-terminal extension into the central channel of the complex. The interacting partner Pat1 interacts with Lsm2 and Lsm3. Adapted from Sharif and Conti, 2013. Created with Biorender.com.

CCR4-NOT COMPLEX

The Ccr4–Not (carbon catabolite repression 4 (Ccr4)-negative on TATA-less (Not)) complex is a highly conserved multifunctional assembly of proteins. The complex is heavily implicated in the rate of protein synthesis. It has been shown that the complex is involved, to different extents, in all steps controlling protein synthesis rate, including ribosomal protein and RNA synthesis, mRNA transcription as well as degradation, leading to both negative and positive regulation of global gene expression[139-141]. Additional functions linked to the complex include regulation of mRNA export, interaction with the nuclear exosome, mRNA elongation control, cell cycle regulation, as well as ubiquitin-protein transferase activity[142-144]. The role of the complex in mRNA decay has been extensively researched. Subsequently, the Ccr4-Not complex has been found to be involved in different types of mRNA decay, including generic mRNA deadenylation, nonsense mediated mRNA decay (NMD), targeted mRNA decay and deadenylation-independent decapping[140].

The Ccr4-Not complex (Figure 3) is a large L-shaped modular assembly of proteins (1.9 MDa in yeast) with nine core subunits in yeast; Ccr4, Caf proteins Caf1/Caf40/Caf130 (Ccr4 associated factor) and Not proteins Not1/Not2/Not3/Not4/Not5[145]. In humans, the complex is composed of the human orthologues CNOT1-CNOT9, in addition to three other subunits CNOT10, Tab182 (Tankyrase 1-binding protein1, TNKS1BP1) and C2ORF29 (CNOT11), which have not been identified in yeast[146,147]. Moreover, the human CNOT3 is orthologous to two yeast subunits, Not3 and Not5[148].

Not1 is the largest subunit (>200 kD) and functions as a scaffold for the others[140]. Studies have identified distinct submodules to be organized onto the Not1 backbone, Ccr4 with Caf1, Caf40, Caf130, and Not2-Not5[145]. In yeast, two deadenylase components are present in the complex, Ccr4 and Caf1. In the human complex, four deadenylase subunits are present, forming various heterodimers; CNOT7/CNOT6, CNOT7/CNOT6L, CNOT8/CNOT6, and CNOT8/CNOT6L. Therefore, either CNOT7 or CNOT8 are incorporated into the complex, perhaps through competition for the same CNOT1 binding site[149].

Lastly, CNOT4 is not as stably associated with the complex in mammalian cells, as Not4 is in yeast[150].

The Ccr4-Not complex associates with PBs in the cytoplasm and is considered to be part of the conserved core[113,151]. The localization of Ccr4-Not components to PBs is limited to stressed conditions but not unstressed cells when decay is ongoing[40,140]. It has therefore been proposed that the localization of the Ccr4-Not complex to PBs might initiate the degradation of mRNAs to avoid de-prioritized or damaged mRNAs from re-entering translating polysomes, during stress conditions[152].

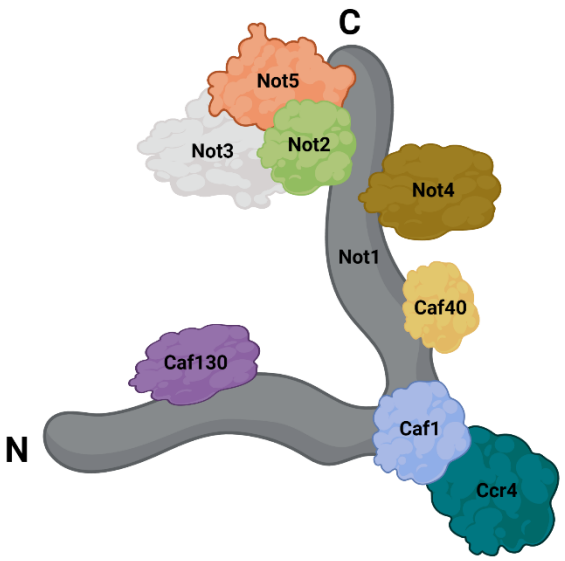

Figure 3. The Ccr4-Not complex. The expected positions of the core subunits on the Not1 scaffold. Adapted from Collart, 2016. Created with Biorender.com.

FUNCTIONS OF INDIVIDUAL COMPONENTS

In PBs, the Ccr4-Not complex partakes in mRNA decay through the deadenylase functions of the two deadenylase components, Ccr4 and Caf1[113]. The Ccr4-Not complex together with the deadenylases Pan2 and Pan3 function as the dominant deadenylase complexes, conserved from yeast to human[153,154]. Ccr4 and Caf1 are regarded as the main deadenylase complex due to the severe effects on deadenylation upon their depletion[153,155]. Studies show that Caf1 (CNOT7/8) binds to Not1 (CNOT1), thereby allowing full access of RNAs to the active site on Caf1[156]. Subsequently, Ccr4 (CNOT6/6L) binds to Caf1, not to Not1, through its leucine rich repeat (LRR) domain[156,157].

In yeast, Ccr4 is considered to be the major mRNA deadenylase in the Ccr4-Not complex, not Caf1[145,153]. Degradation of mRNA is initiated by trimming of selectively long poly(A)-tails to a length of approximately 110 nt by the Pan2/3 deadenylase dimer, after which Ccr4-Not complex takes over and completes deadenylation[158]. After the tails are shortened to an oligo(a) length of approximately 10-15 residues, the mRNA is further subjected to decapping[129]. It has been shown that Ccr4 and Caf1 play distinct deadenylation roles. Ccr4 is activated by Pab1 to shorten Pab1-protected sequences, whereas Caf1 gets blocked by Pab1 and thus only degrades naked poly(A) sequences. Accordingly, it has been established that Ccr4 can trim all mRNAs, thereby acting as a general deadenylase, whereas Caf1 is more specialized and deadenylates mRNA with reduced translation elongation and Pab1 binding[159]. Lastly, Ccr4 activity has been linked to cell cycle progression regulation[144].

Since Not1/CNOT1 functions as the complex scaffold, depletion of Not1/CNOT1 has adverse effects on the stability of the whole complex and its functions. No distinct enzymatic activity has been linked to Not1/CNOT1 but its scaffolding function is of great importance for the deadenylase activity of the complex even though CNOT6/6L and CNOT7/8 components remain intact. Accordingly, CNOT1 depletion also leads to degradation of other complex subunits, such as CNOT2, CNOT6L, CNOT7 and CNOT9[160]. Moreover, through interactions with specific RBPs, CNOT1 mediates the recruitment of

CNOT6/6L and CNOT7/8 to the selected mRNA thereby triggering degradation of target mRNAs[140].

The Not submodule (Not2-Not5) of the Ccr4-Not complex consists of a diverse set of proteins with no known enzymatic activity (except for Not4). Not much is therefore known about this submodule but it has been suggested it provides complex stability, since deletion of some of these components results in destabilization of the other complex subunits[145]. Accordingly, it has been shown that depletion of CNOT2 impacts the deadenylase activity of the complex[161]. The Ccr4-Not complex physically associates with ribosomes in yeast, and this association is enabled by Not5 recruitment to the ribosomal E-site[162]. Moreover, the interaction between the complex and ribosomes further requires specific ubiquitylation of ribosomal subunits by Not4. Loss of Not5, or this specific ubiquitylation by Not4, results in dysregulation of mRNA half-life and impairment of decapping[162]. Accordingly, deadenlyation-independent decapping of certain mRNAs is dependent on Not2, Not4, and Not5 in yeast[163].

Not4 constitutes the second major enzymatic activity of the Ccr4-Not complex, i.e. E3 ligase-mediated ubiquitylation[145]. In accordance with its E3 ligase activity, Not4 is part of the ubiquitin(ub)-proteasome pathway (UPP) involved in cellular protein turnover. Accordingly, Not4 has been shown to associate with proteasomes, and even aid in the correct assembly of the proteasome[164]. Loss of Not4 results in accumulation of polyubiquitylated, misfolded and aggregated proteins and lower levels of free ubiquitin, a phenotype consistent with a defect in proteasome function[164].

Caf130 is a yeast specific component, whereas Caf40 is conserved. The mammalian ortholog of Caf40, CNOT9, has been linked to gene expression regulation through binding to transcription factors and interaction with miRNA processing cascades[165]. However, the functions of both Caf40 and Caf130 remain poorly understood. Furthermore, the mammalian specific complex components CNOT10, CNOT11 and Tab182 (TNKS1BP1) do not appear to have any enzymatic activity[147]. However, the presence of CNOT10 and CNOT11 have been shown to promote deadenylation by stabilizing the RNA

substrate[166]. Tab182 contributes to radiation-induced DNA damage repair[140]. In addition to these integral complex components, a number of proteins have been found to associate with the complex for routine functions[140]. The results of these interactions have reported to be either activation or repression of the deadenylase activity of the complex, as well as regulatory in mRNA interaction[140].

CELL CYCLE REGULATION

In addition to the functions already mentioned, the Ccr4-Not complex has further been identified to greatly affect cell cycle progression in both mammalian and yeast cells[167]. In yeast, cell cycle progression is primarily controlled at the G1 to S phase transition[168]. Furthermore, for cell cycle progression past this transition point, yeast cells must attain a minimum cell size[169]. When cells approach the critical cell size requirement, cell cycle proteins Bck2 and Cln3 (cyclin) activate the transcription of the downstream G1-phase cyclins *CLN1* and *CLN2,* thereby initiating cell cycle progression[170,171]. To promote CLN1/2 transcription, Cln3 together with Cdc28 phosphorylates Whi5, another cell cycle regulator known to suppresses G1-phase cyclin expression, thereby enabling *CLN1/2* transcription[172]. Overexpression of *CLN3* or *BCK2* initiates premature *CLN1* and *CLN2* transcription resulting in cell cycle progression[170,171]. Moreover, deletion of *WHI5*, results in premature *CLN1/2* expression and cell cycle progression[172,173]. Furthermore, deletion of *CLN3* or *BCK2* results in delayed *CLN1/2* expression and cell cycle delay[170,171,174].

It has been shown that Ccr4 works downstream of Bck2, independently of Cln3, assisting in cell cycle coordination by negatively regulating the half-life of *WHI5* mRNAs, thereby contributing to the size-dependent timing of *CLN1/2* expression[144]. Accordingly, deletion of *CCR4* results in delayed transcription initiation of *CLN1/2* and enlarged cell size morphology[144]. Moreover, overexpression of *WHI5* is lethal in *ccr4Δ* cells and overexpression of *CLN1/2/3* or deletion of *WHI5* reduces the cell size of *ccr4Δ* cells. Double

deletion of *CLN3* and *CCR4* is lethal, suggesting that Bck2 is dependent upon Cln3 to inhibit Whi5 in the absence of Ccr4[144]. Further support for the involvement of the Ccr4-Not complex in cell cycle regulation is found in mammalian cancer cell studies where human *CNOT6/L* were identified to be dysregulated, further knockdown could significantly inhibit cell proliferation and tumorigenicity. Depletion of *CNOT6/L* resulted in a subsequent G0/G1 cell cycle phase arrest[175]. Accordingly, depletion of CNOT6 and CNOT6L in mammalian cells leads to reduced cell proliferation, with more cells being in G1 phase[176,177]. Lastly, loss of CNOT6L has been shown to induce meiotic cell cycle arrest during mouse oocyte maturation[178].

PHASE SEPARATION IN NEURODEGENERATIVE DISORDERS

Under normal conditions, LLPS is an essential and vital process in a wide range of cellular processes and systems. However, accumulating evidence[179] is linking LLPS-capable proteins to pathological aggregates found in various disease, indicating that pathological aggregates could originate from an aberrant phase separation (Figure 4). Indeed, aberrant protein aggregation is a hallmark of various neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD) and frontotemporal lobar degeneration (FTLD)[180]. Proteins implicated in these disorders, such as TAR DNA-binding protein 43 (TDP-43) and fused in sarcoma (FUS), have been shown to be able to transition from a reversible dynamic LLPS into an irreversible aggregated state[22,179]. PTMs and disease-associated mutations have been shown to regulate this shift[1]. For many of these proteins, LLPS is mainly regulated by their IDR sequences[1], where disease-linked mutations have been shown to affect condensate formation and development. The full impact of disease-linked mutations and PTMs on phase separation is not yet fully understood. However, it has been suggested that mutations that lead to a lowering of the saturation concentration, i.e. the threshold protein concentration needed for LLPS to occur, could force the

protein into a condensed state, over time favoring the transition from a reversible dynamic LLPS to an irreversible solid and aggregated state. Moreover, mutations and altered PTMs could also result in modified binding to cellular LLPS regulators[1,181].

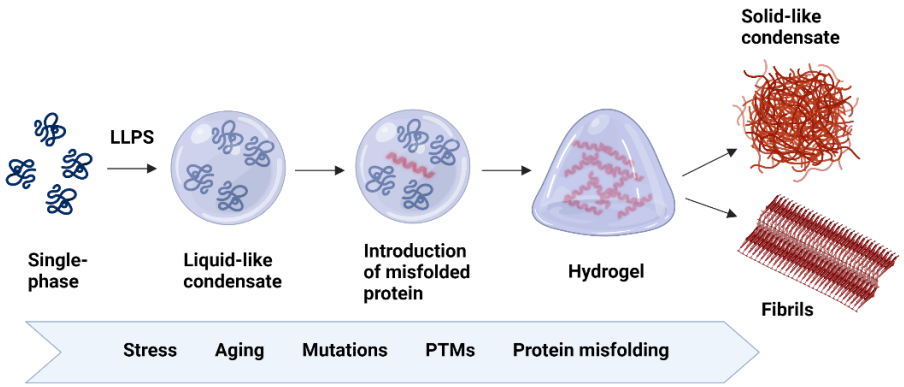

Figure 4. Aberrant phase separation. Factors such as PTMs and disease-associated mutations can trigger a transition from a reversible dynamic LLPS into an irreversible aggregated state. Created with Biorender.com.

YEAST MODELS OF NEURODEGENERATION

Saccharomyces cerevisiae has been extensively used to study the mechanisms behind neurodegenerative disease phenotypes, such as the impact of disease-linked protein misfolding and cytotoxic aggregation[182]. Yeast as a model organism has many advantages compared to more complex higher organisms, such as a fully sequenced genome, well-characterized phenotypes, easy genetic manipulation, as well as simplified culturing and handling. Moreover, pathways coupled to protein folding pathology are conserved in yeast, enabling the study of these in a more accessible model organism, underscored by the existence of a high number (approx. 20%) of orthologous gene families linked to human diseases[183,184].

Of the many experimental advantages provided by the yeast system, the main benefit is the ability to perform genome-wide high-throughput screening techniques[185,186]. These genetic modifier screens have contributed to the pioneering role yeast has played in uncovering protein deletion and over-expression phenotypes, protein localization, as well as toxicity modifiers, revealing pathways involved in human neurodegenerative disease[187-189]. Yeast high-throughput screenings are a convenient first approach, especially to uncover new mechanisms related to pathogenesis and pathophysiology, succeeded by further validation in higher organism models and potential therapeutic target discovery[183].

Yeast models of Huntington disease (HD), Parkinson's disease (PD) and Amyotrophic lateral sclerosis (ALS) have uncovered mechanisms behind toxic protein aggregation linked to pathogenesis, involving e.g. mutated huntingtin (HD), α-synuclein (PD), TDP-43 and FUS (ALS)[183]. α-synuclein was one of the first neurodegenerative-linked proteins to be characterized in a yeast model where it was identified to form cytotoxic inclusions in a dose-dependent manner[187]. Correspondingly, longer polyglutamine stretches in huntingtin has been validated to increase the protein tendency to form insoluble inclusions in yeast, whereas overexpression of wild-type TDP-43 and FUS leads to cytoplasmic mislocalization and aggregation, in accordance with their disease pathology[188,190]. Moreover, proteins involved in lipid metabolism, vacuolar degradation and vesicular transport have been identified as potential enhancers or suppressors of α-synuclein toxicity[191]. Furthermore, yeast screenings have identified many human RNA-binding proteins (RBPs) that aggregate in a toxic manner in yeast, including ALS-linked proteins FUS and TDP-43, highlighting a shared feature among most of these proteins, namely an IDR, more specifically a prion-like domain[192]. In addition, suppressors and enhancers of FUS and TDP-43 toxicity have been uncovered in yeast deletion and overexpression screens, thereby identifying possible candidate genes with human orthologs[188,190,193].

Although *Saccharomyces cerevisiae* is a simple single-celled eukaryote it has proven highly useful in a multitude of neurodegenerative studies, of which

only a fraction is listed here. Studies in yeast contribute to not only uncovering the mechanisms behind pathology, but have also suggested that yeast specific proteins, such as potentiated Hsp104 (heat shock protein 104) disaggregase variants, have the capacity to reverse the toxic phenotype linked to highly aggregation-prone human disease proteins, such as FUS, indicating that yeast proteins may eventually be used as a therapeutic agent in human neurodegenerative disease[194,195]. Indeed, the ability to actively reverse and control protein misfolding is a vital attribute when designing new therapeutic solutions for proteinopathies.

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS), also called Lou Gehrig's disease, is a neurodegenerative disease characterized by preferential degeneration of upper and lower motor neurons leading to rapidly progressing paralysis and eventual death by respiratory failure, typically 3-5 years after disease onset[196,197]. ALS is the most common motor neuron disease with a global incidence of 2.5 cases per 100,000 individuals each year, and an average age of onset of 60 years[198]. As of today, there are no cures and available treatments do not effectively slow disease progression. Most cases of ALS are sporadic with no apparent genetic link (sALS), whereas inherited ALS (familial, fALS) accounts for approximately 10% of all reported ALS cases. However, given the similarities in disease display and pathology of sporadic and familial ALS, the study of genes implicated in fALS could uncover mechanisms underlying both fALS and the more common sporadic form[199].

The degeneration of cells in ALS patients coincides with the accumulation of misfolded protein inclusions in motor neurons and oligodendrocytes, as well as neuroinflammation[200]. In ALS pathology, more than 20 genes have been linked to disease progression. Among these genes some have been identified as the most common causative genes, such as SOD1 (Cu-Zn superoxide dismutase 1), C9ORF72 (chromosome 9 open reading frame 72), TDP-43 (TAR-DNA-binding protein-43 kDa) and FUS (fused in sarcoma; also known

as TLS, translocated in liposarcoma[201,202]. SOD1 was first to be linked to fALS and much research has been focused on identifying the cause and results of mutated SOD1 in ALS pathology[203]. Eventually, other genes involved in fALS, such as the RNA-binding proteins TDP-43 and FUS, were identified[204]. Subsequently, mutations in FUS have also been identified in sALS and a subset of FTLD (frontotemporal lobar degeneration) cases[205,206].

FUSED IN SARCOMA (FUS)

FUS/TLS was first discovered as part of a chromosomal translocation associated with human myxoid liposarcomas, resulting in gene fusions and production of chimeric oncoproteins, hence the name fused/translocated in liposarcoma[207]. The *FUS* gene is located on chromosome 16 and encodes for a 526 amino acid protein ubiquitously expressed in all tissues. Moreover, FUS is a DNA/RNA-binding protein (RBP) belonging to the FET (FUS/EWS/TAF15) protein family[208]. FUS has different domains (Figure 5) linked to various functions of the protein; the N-terminal prion-like domain (PLD) that is enriched in polar amino acids (glutamine, glycine, serine and tyrosine, QGSY) and contains a glycine-rich region (sometimes referred to as an RGG domain), the conserved RNA-recognition motif (RRM, RNA-binding domain), two arginine-glycine-glycine (RGG)-repeat regions, a C2/C2 zinc finger motif as well as the nonclassical C-terminal nuclear localization signal (NLS)[209,210]. Together, the zinc finger motif, the RRM region, and the RGG regions contribute to the DNA/RNA-binding ability of FUS[199,211].

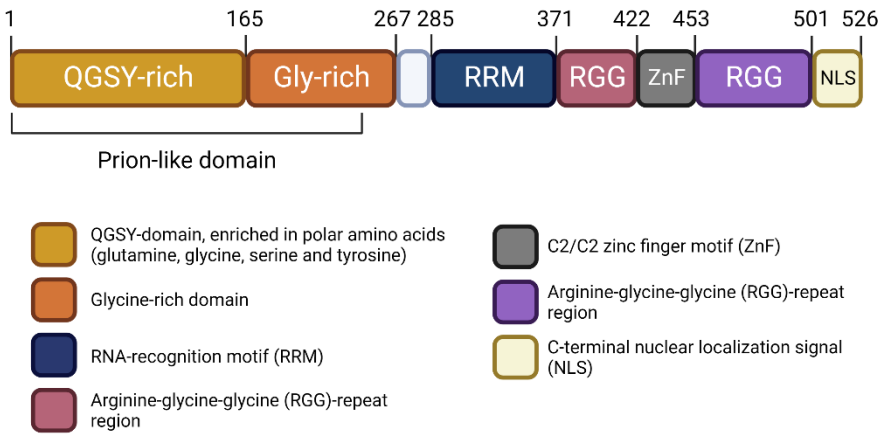

Figure 5. The different domains of FUS. The N-terminal prion-like domain (PLD) has been underlined. Created with Biorender.com.

FUS is involved in many regulatory functions, such as DNA repair, transcriptional control, RNA splicing and mRNA transport to the cytoplasm[199,212]. FUS binds to both single stranded DNA and RNA *in vitro*[213]. Accordingly, several FUS target genes have been identified through chromatin immunoprecipitation combined with transcriptomics[214]. Moreover, it has been shown that FUS binds to nascent pre-mRNAs, as well as components of the spliceosome, thereby regulating pre-mRNA fate, including pre-mRNA splicing[199]. In addition, FUS has been identified as a regulator of microRNA (miRNAs) and long noncoding RNA (lncRNAs) biogenesis through its interaction with Drosha[215]. FUS has also been identified in neuronal mRNA transport granules indicating that it plays an active role in mRNA trafficking along axons and dendrites[216]. Many FUS functions involve the binding of RNA, a direct interaction enabled by the RNA-binding domain through hydrogen bonds and ring stacking[210]. RNA binding is further supported by the zinc finger domain and the RGG motifs[211].

In neurons and glial cells, FUS is predominantly localized to the nucleus but have been shown to shuttle between the nucleus and the cytoplasm, e.g. when

being transported to dendritic spines at excitatory post-synapses[212,217,218]. Moreover, during stress, such as heat shock or oxidative stress, FUS has been shown to exit the nucleus and temporarily assemble into perinuclear SGs and PBs, eventually re-localizing to the nucleus. Accumulation of FUS in SGs is therefore a reversible process in healthy neurons[219,220]. The C-terminal NLS of FUS is known to be recognized by a nuclear transport receptor called Transportin 1/Karyopherin-β2, an interaction that results in FUS re-entering the nucleus[219,221].

As previously mentioned, FUS is normally localized to the nucleus, but has been shown to form neuronal cytoplasmic inclusions in ALS patients when mutated[222]. Aggregation of mutated FUS has been implicated in 7.5% of fALS and <1% of sALS cases, in addition to rare forms of frontotemporal lobar degeneration (FTLD)[222-224]. FTLD is the second most common form of dementia in people under 65, characterized by neuronal cell death in the frontal and temporal cortex[225]. ALS patients with mutated FUS display a particularly severe disease progression, where 60% of cases display pathogenesis by the age of 40[226].

Even wild-type FUS has been linked to cytotoxic accumulation in some cases of juvenile ALS, basophilic inclusion body disease, Huntington's disease, spinocerebellar ataxia (SCA, type 1, 2, and 3), dentatorubral-pallidoluysian atrophy, in addition to the majority of tau- and TDP-43-negative FTLD cases, as well as a subtype of FUS-FTLD[227-230]. Moreover, mutations leading to increased expression of FUS have been identified in ALS patients[231]. Further support of wild-type FUS being implicated in disease comes from studies in transgenic mice, where overexpression of wild-type human FUS results in ALS-like behavior and degeneration, including spinal cord motor neuron degeneration and progressive hind limb paralysis, followed by death, suggesting that high levels of wild-type human FUS trigger a gain-of-function toxicity[131]. Indeed, it has been proposed that the cytoplasmic mislocalization and aggregation of FUS may lead to a loss of normal nuclear protein functions and/or gain of new toxic functions in the cytoplasm[199,232].

The increasing number of findings of both wild-type and mutated FUS inclusion formation in disease broaden the spectrum of disorders linked to FUS beyond ALS and FTLD, emphasizing the importance of identifying the mechanisms behind both normal and aberrant FUS functions, thereby uncovering the pathology of FUS proteinopathies.

FUS-ALS

Known *FUS* mutations linked to ALS reside in certain distinct regions of the gene, including the N-terminal PLD, a portion of the glycine-rich region and the C-terminal NLS[209]. However, most FUS-ALS mutations are clustered in the NLS. Some of these mutations, such as P525L and R521C have been shown to contribute to cytoplasmic FUS mislocalization due to disruption of the transportin-mediated nuclear import of FUS as a result of reduced interaction with the nuclear import receptor Transportin 1[221,231]. Accordingly, disruption of the FUS/Transportin interaction has been shown to result in nuclear depletion and cytoplasmic accumulation of FUS in mammalian cells[221,233]. Moreover, ALS disease severity correlates with the extent of mutated FUS redistribution to the cytoplasm, where patients with the FUS-R495X mutation, which results in high cytoplasmic retention of FUS due to a truncated NLS, display early disease onset and severe disease development[219,233].

Furthermore, when expressed at high level (hereafter referred to as overexpression) in yeast, both wild-type and mutated FUS has been shown to mislocalize to the cytoplasm forming toxic aggregates largely due to a non-functional NLS[188,193]. Upon replacement of the non-functional NLS with a, in yeast, recognizable sequence, predominantly nuclear localization of FUS can be seen in addition to a reduction in cytotoxicity, further supporting the role of the NLS in cytoplasmic mislocalization of FUS and toxicity[188,193]. However, studies in mammalian cells have shown that FUS can enter the nucleus even though missing the C-terminal NLS, indicating that other sequences also enable import of FUS[188,234]. The importance of a functional nuclear import has thus lead to the hypothesis that impaired nuclear import and subsequent

cellular accumulation of FUS may be necessary, and potentially sufficient, for FUS pathogenesis[219,233].

As mentioned before, overexpression of wild-type FUS in yeast results in the formation of cytotoxic FUS inclusions in accordance with ALS pathology[188,193,235]. This phenotype has been utilized to identify the exact sequence domains needed for FUS aggregation and cytotoxicity in yeast. By employing a set of truncated FUS plasmids it was shown that the RRM domain, PLD and parts of the first RGG domain are required for full cytoplasmic FUS aggregation and toxicity in yeast[188]. Further studies in insect cells support the notion that the PLD is needed for the forming of FUS inclusions[22]. Moreover, *in vitro* studies using purified FUS have shown that FUS is intrinsically aggregation-prone, displaying rapid aggregation in a concentration dependent manner, regulated mainly by the PLD and first RGG domain[188]. *In vitro* FUS aggregates form large, tangled and stacked linear polymers, resembling those found in ALS and FTLD patients, with a seemingly non-amyloid nature[188,236].

The cytoplasmic aggregation of FUS has been hypothesized to further lead to a toxic protein gain-of-function in the cytoplasm. This hypothesis is supported by a multitude of studies highlighting changes in FUS function upon aggregation in the cytoplasm. For instance, aggregation of mutated FUS has been shown to affect the expression of its target genes through sequestration of these mRNAs within the cytoplasmic FUS aggregates[237]. Moreover, mutated FUS displays changed mRNA binding patterns and an increase in unique targeting of an overrepresented group of transcripts[238]. Accordingly, by disrupting the RNA binding activity of FUS, FUS-related cytotoxicity was reduced in yeast, indicating that aggregated FUS exerts some of its toxicity through binding to RNA and potential RNA sequestration[188]. Moreover, mutant FUS has been shown to suppress protein translation and alter the nonsense-mediated decay (NMD) pathway, leading to hyperactivation of NMD and thereby disruption of NMD autoregulation[239].

Furthermore, studies indicate that aggregation of mutated FUS causes an imbalance in the cytoplasmic RBP homeostasis due to interaction impairment

between FUS and other RBPs. Accordingly, RBPs of the FET family have been shown to buffer the aggregation tendency of FUS under normal circumstances but is suggested to be impaired in disease due to the disrupted RBP homeostasis, resulting in disease exacerbation. Accordingly, studies in iPSC (induced pluripotent stem cells)-derived neurons, with increased cytoplasmic FUS levels, have shown reduced protein levels of the same FET family of RBPs, findings further confirmed in ALS-patient tissue samples, as well as an impaired protein degradation machinery[226].

Another gain-of-function of FUS has been identified in regards to neuronal cell cycle progression and differentiation. Exogenous expression of human wild-type FUS in neural stem progenitor cells results in reduced growth and arrest in cell cycle progression in G1/S phase. Moreover, expression of ALS-linked FUS-P525L displays an abnormal cellular distribution in the cytoplasm and drives cell differentiation towards a glial lineage, thereby reducing the development of neurons, in addition to decreased overall proliferation[240]. Lastly, FUS is known to interact with SGs in a reversible manner in healthy neurons, however; ALS-linked FUS mutants display increased association with SGs and have been shown to bind and sequester wild-type FUS into SGs[202,219]. This finding is of special interest since SGs have been proposed to play a role in neurodegeneration as possible precursors of pathological inclusions[241].

INTERACTION WITH SGS AND PBS

It has been suggested that disease-related association of FUS with stress granules is another step in FUS pathology, where the aberrant interaction of FUS with SGs may impair cellular stress response, ultimately causing disease[241]. In accordance, a co-localization of pathological FUS aggregates with SGs has been seen in yeast, mammalian cell lines, as well as in ALS-patient tissue samples[219,242]. SGs have a protective function in healthy neurons but mutations in their components or perturbations to their overall function might convert them into overly stable structures[241]. Accordingly, the different

ALS-linked FUS mutations result in varying amount of FUS recruitment into and impact on SGs, where some result in increased number of SGs that persist longer[243,244]. Upon overexpression in yeast, wild-type and mutated FUS induce both SG and PB formation without the addition of any external stressors[188]. Moreover, studies show that overexpressed or endogenous mutant FUS can compete with SGs for their core proteins, such as G3BP1, and RNA species thereby disrupting physiological SGs[245]. External stresses have been proposed as further drivers of FUS-ALS development, supported by studies showing that FUS aggregation and partitioning into SGs is increased by different stressors[233,243,246]. Accordingly, mutant-FUS delays SG formation in mammalian cell lines under oxidative stress conditions but upon formation, SGs containing mutant-FUS are bigger, increased in number and more dynamic than those without FUS. Furthermore, deletion of either FUS-RGG domain results in impaired assembly of FUS into SGs, indicating that these domains are needed for the interaction with SGs[247].

Interaction between FUS and SGs has been established in disease, but does it connect to FUS toxicity, or is it merely a downstream result of cellular stress associated with degeneration pathology? On that matter, several yeast genome-wide screens have identified components of SGs as potential regulators of FUS toxicity, when overexpressed or deleted. For instance, overexpression of Tif2, Tif3 or Pab1, proteins involved in SG assembly in yeast, was able to reduce FUS toxicity. Another SG component, Pub1, was shown to suppress FUS toxicity when deleted[188].

In addition to being linked to SGs, FUS has been shown to be associated with PBs as well, in both healthy neurons and in a disease context. Wild-type expression of FUS in yeast induces PB formation[188]. In mammalian cell studies, wild-type FUS has been shown to clearly localize with SGs under stress, but only moderately with PBs[248]. However, pull-downs of mutated FUS in mammalian studies reveal interactions with PB-associated proteins[249]. Moreover, overexpression of SBP1, a component of PBs, has been shown to reduce FUS toxicity in yeast[193]. Therefore, SGs and PBs, or individual components of these RNP granules, might play an important role in mediating

FUS toxicity, in addition to being markers of FUS-positive inclusions in disease.

PHASE SEPARATION OF FUS

FUS is able to undergo rapid and reversible phase separation between dispersed, liquid droplet and hydrogel states[22,250,251]. *In vitro* studies using purified FUS have shown that FUS is intrinsically aggregation-prone, displaying rapid aggregation in a concentration dependent manner, regulated mainly by the PLD and first RGG domain[188]. Accordingly, further studies in insect cells demonstrate the importance of the PLD in the formation of FUS liquid droplets, as well as in the formation of aggregates in yeast[22,188]. Over time, *in vitro* FUS droplets become more viscoelastic eventually adopting a more solid-like state and ceasing to exchange molecules with the surroundings, a process referred to as maturation[15,22,37]. It is still unclear what the exact material properties of these *in vitro* hardened states are, but they are often referred to as hydrogels[250,252]. FUS hydrogels can further transition into solid-like fibrillar aggregates composed of large, tangled and stacked linear polymers resembling those found in ALS and FTLD patients, similarities further supported by their seemingly non-amyloid nature[15,22,188,228,236]. Furthermore, *in vitro* liquid droplets of ALS-FUS with mutations in the PLD convert faster into an aggregated fibrous state[15,22]. The importance of PLDs in LLPS is further underscored by it being a shared feature among multiple proteins that form aggregates in disease[235,253].

PLDs have distinct residue compositions, being enriched in polar amino acids and aromatic residues[254]. It has been proposed that PLDs drive FUS droplet and hydrogel phase separation mainly through the formation of beta-sheeted structural motifs stabilized by hydrogen bonding[250,255]. FUS demixing is further mediated by cation-π interactions between positively charged arginine residues in the C-terminal RGG domain and tyrosines in the PLD[181]. Lastly, both phosphorylation and methylation of FUS has been shown to influence FUS LLPS[181,256].

Arginine hypomethylation has been identified to lead to increased FUS phase separation, of which relevance is underscored by the significant hypomethylation of FUS in FUS-FTLD patients[181]. Moreover, the nuclear import receptor Transportin-1 acts as a chaperone of FUS, reducing phase separation and gelation of both methylated and hypomethylated FUS through its interaction with the NLS and PLD of FUS[181,257]. Transportin-1 has also been shown to reduce the association of FUS with SGs[258]. Disease-linked mutations in FUS have been identified to prevent the interaction between FUS and Transportin-1, thereby indirectly suppressing the physiological LLPS behavior of FUS and promoting aggregation[181,258]. Moreover, FUS interaction with other RBPs has been shown to prevent FUS from undergoing an aberrant liquid-to-solid transition, a regulation that is probably lost upon disease-associated mislocalization of FUS to the cytoplasm, further leading to cytotoxic FUS aggregation[226]. Lastly, Pub1 (TIAL-1 in human) has been identified as a deletion suppressor of FUS in yeast[188]. Pub1 also contains a PLD which has been shown to template the aggregation of the polyQ protein huntingtin, suggesting that FUS aggregation and cytoplasmic sequestration might be templated in a similar manner by Pub1[227,259]. It has therefore been proposed that aberrant FUS LLPS is a result of hampered Transportin-1 binding followed by impaired nuclear transport and altered protein/RNA interactions in the cytoplasm[193,233,258].

The formation of *in vitro* FUS hydrogels has been shown to be regulated by protein concentration. At physiological concentrations, FUS demixes into dynamic liquid droplets, but converts into hydrogels when the concentration is increased[15,22]. However, FUS protein concentrations, similar to the physiological nuclear FUS concentrations, give rise to FUS LLPS *in vitro*, but *in vivo* there are barely any detectable condensates. Even though the protein saturation concentration is obtained, something else prevents LLPS from happening[260].

Accordingly, RNA has been proposed to act as a regulator of FUS phase separation, highlighted by the prevalence of an RNA-binding domain in many disease-linked proteins. Indeed, small amounts of RNA has been shown to

increase FUS LLPS *in vitro*, whereas high RNA to protein ratios prevent FUS phase separation[260]. It has been further hypothesized that in environments with high RNA concentration, such as the nucleus or SGs, specific highly structured RNAs could act as scaffolds to enable FUS phase separation despite high RNA/protein ratio. Lastly, addition of RNA to *in vitro* FUS droplets has been shown to keep the droplets in a soluble state, preventing fibril formation[260]. RNA certainly appears to be an important regulator of FUS LLPS, with the ability to prevent the formation of solid assemblies that can cause disease. However, studies in yeast have shown that when impairing the RNA-binding ability of FUS, through residue substitutions in the RRM domain, FUS toxicity is drastically mitigated without affecting the cytoplasmic aggregation of FUS. This indicates that, in yeast, binding of RNA by FUS is not needed for LLPS and aggregation[188].

The correlation between FUS inclusion formation and toxicity is further complicated by mouse models of juvenile FUS-ALS displaying motor neuron loss without the occurrence of FUS aggregates, and studies in *Drosophila* where neither wild-type FUS nor ALS-linked FUS expression resulted in any cytoplasmic inclusions even though toxicity was observed[261,262]. Moreover, identified suppressors of FUS toxicity in yeast do not significantly alter the aggregation of FUS, suggesting that these genes act downstream or in parallel to FUS aggregation, in addition to potentially modifying the dynamics or composition of FUS inclusions in a subtle way[188]. Nevertheless, a decrease in toxicity often coincides with a decline in aggregation capacity of truncated FUS constructs in yeast[188,263].

MAIN FINDINGS

PAPER I

Stress granules (SGs) are membraneless organelles that form in response to stress and are needed for cellular stress management. Liquid-liquid phase separation (LLPS) has been implicated in the formation of these ribonucleoprotein granules. However, the exact regulatory mechanisms behind SG initiation, and the role of phase separation, still remains elusive. In this study, a genome-wide imaging-based screen was performed to identify SG co-localizing proteins under 2-deoxy-D-glucose (2-DG) stress. Further studies into one of the SG-associated proteins, Lsm7, uncovered a mechanism needed for SG assembly.

- The genome-wide imaging-based screen uncovered previously unreported proteins that form foci, co-localizing with SGs (Pab1), under 2-DG stress.

- The highly conserved RNA-binding protein Lsm7 was among the screen candidates and was identified as needed for promoting SG formation.

- The ability to undergo LLPS and form dynamic condensates, a process driven by the intrinsically disordered region and hydrophobic clusters within the Lsm7 sequence, is needed for Lsm7's role in inducing SG formation.

- Lsm7 condensates appear to work as seeding scaffolds, promoting Pab1 demixing and subsequent SG initiation, seemingly mediated by RNA interactions.

In conclusion, Lsm7 was identified as an early phase separation component involved in promoting SG formation, most likely through modulating RNA interactions and/or acting as a seeding scaffold.

PAPER II

In Paper I, we uncovered that the RNA-binding protein Lsm7, displays SG regulatory functions. However, the signaling cascade leading to Lsm7 activation remained unexplored. Reports indicate that certain signaling components, RNA-binding proteins and regulatory proteins function in SG initiation; however, the crosstalk between these remains elusive. Therefore, in this study, we wanted to identify the upstream pathway leading to Lsm7 activation and subsequent SG formation under 2-DG stress. Consequently, two genome-wide imaging-based screens were performed to uncover genes regulating SG formation. Subsequently, the identified candidates led to the uncovering of upstream signaling components that form a possible signal transduction pathway in regulating SG formation.

- TORC1/2 signaling, through Sch9 and Ypk1 was identified as an early step in the pathway.
- Upon 2-DG stress, Sch9 and Ypk1 activation leads to reduced levels of LCB (long-chain base) sphingolipids.
- Reduction in LCBs further results in derepression of the deubiquitinase Ubp3, and subsequent downregulation of the ubiquitin gene UBI4.
- Further, Ubp3 was shown to positively regulate Lsm7 phase separation by suppressing Ubi4, resulting in SG formation.

In conclusion, key components in a SG regulatory pathway, under 2-DG stress, were identified. Characterized by an interplay between Sch9/Ypk1, LCBs, Ubp3, Ubi4 and Lsm7 regulating SG formation.

PAPER III

Mislocalization and cytoplasmic aggregation of the human DNA/RNA-binding protein FUS (fused in sarcoma) is a hallmark of aberrant FUS phase separation behavior, implicated in amyotrophic lateral sclerosis (ALS) and other neurodegenerative disorders. When overexpressed in yeast, wild-type FUS forms cytotoxic aggregates, reminiscent of the ALS phenotype. We utilized the FUS phenotype presented in yeast to elucidate the cellular effects of toxic FUS aggregation. We performed a genome-wide imaging-based screen to identify proteins, which upon FUS induction, form protein assemblies co-localizing to FUS aggregates. Further studies into one of the screen candidates, Ccr4, uncovered a toxic gain-of-function for FUS.

- The genome-wide imaging-based screen uncovered proteins that form assemblies, co-localizing with FUS aggregates. PB and SG-associated proteins, involved in regulation of mRNA metabolism, were enriched among the screen candidates.

- The Ccr4-Not complex subunit, Ccr4, physically interacts with FUS, forming co-localizing assemblies upon cytoplasmic FUS aggregation. A process which is driven by the N-terminal domain of FUS and can be enhanced by ALS-associated mutations in FUS.

- Prolonged FUS exposure leads to Ccr4 protein downregulation and assembly transition into a less dynamic state, further hampering Ccr4-mediated cell cycle regulation.

- Subsequently resulting in delay of cell cycle progression, contributing to the FUS toxicity phenotype.

In conclusion, induction of FUS results in cell cycle delay from G1 to S phase, a process which can be due to Ccr4 sequestration and Ccr4 protein downregulation, and subsequent loss of Ccr4 cell cycle regulatory functions, resulting in contribution to the FUS toxicity phenotype.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Already at the end of the 19th century it was hypothesized that the cytoplasm of cells behaves like a mixture of various chemically suspended drops. However, evidence of the vastness and full impact of cellular liquid-liquid phase separation (LLPS) did not emerge until a decade later. In recent years, accumulating reports implicate phase separation in the formation of a wide range of biomolecular condensates involved in a multitude of cellular functions, as well as a potential driver of disease. Being such a young research field, many regulatory pathways and functional implications still remain undescribed.

The work in this thesis presents insights into the molecular mechanisms driving condensate formation needed for physiological cellular functions, such as stress granule formation upon stress, but also highlights implications of aberrant phase separation, including dysregulation of stress granules and cytotoxic aggregation of the human protein FUS (fused in sarcoma). The initial approach in all three papers was a high-content genome-wide screening that could elucidate the underlying pathways involved. Further studies into the identified candidates subsequently led to uncovering novel underlying mechanisms.

In **Paper I-II**, we uncover a regulatory pathway behind stress granule formation (SG) under glucose limiting conditions (2-deoxy-D-glucose). The genome-wide imaging-based screening performed in **Paper I** identified previously unreported proteins that form co-localizing foci with SGs (Pab1). The highly conserved RNA-binding protein Lsm7 was among the screen candidates and its role in SG formation was established to be through the forming of liquid-liquid phase-separated condensates that further triggered Pab1 demixing and subsequent SG initiation. We further hypothesized that the promotion of Pab1 demixing could be due to Lsm7 working as a seeding scaffold or through mediating RNA interactions. An informative future study

would be to describe the probable link between Lsm7, Pab1 and RNA in phase separation and SG formation. We have shown that Lsm7 can rescue Pab1 demixing *in vitro* upon addition of RNA. However, whether inhibition of the RNA-binding domain of Lsm7 would result in a loss of this rescue effect, due to impaired Lsm7-RNA interactions, would give further useful insight into this process. Another pertinent future study would be to investigate the roles of the key components identified in **Paper I-II** in SG formation under a variety of different stress conditions.

In **Paper II**, we looked to uncover the upstream pathway leading to Lsm7 activation and phase separation, resulting in SG formation. In this study two separate genome-wide imaging-based screenings were performed to identify genes that regulate SG formation. An early step in the pathway was found to be governed by Sch9 and Ypk1, downstream effectors of TORC1/2. Upon stress activation of Sch9 and Ypk1, the level of long-chain base (LCB) sphingolipids is reduced which further leads to derepression of the deubiquitinase Ubp3 and downregulation of the ubiquitin gene *UBI4*. This subsequently results in activation of Lsm7 phase separation and SG formation. The potential existence of other regulatory components in this pathway could be studied by revisiting the screening results and characterizing other candidates and whether they can regulate the key components identified in this paper. It also remains unknown how reduced ubiquitin levels lead to Lsm7 phase separation. Since ubiquitin and polyubiquitin has been reported to influence the LLPS behavior of some SG components it would be pertinent to investigate the role of ubiquitin on Lsm7 phase separation further. A possibility would be to employ the *in vitro* setup described in **Paper I** to look at the effects of added free ubiquitin on Lsm7 phase-separated droplets, and/or define potential ubiquitination/deubiquitination of Lsm7 itself.

In **Paper III**, we focused on establishing the cellular impact of aberrant FUS protein phase separation and subsequent aggregation. A genome-wide imaging-based screening assay was performed and uncovered proteins forming protein assemblies co-localizing with FUS aggregates. The highly conserved deadenylase Ccr4 was identified as a hit and shown to physically interact with

FUS, forming assemblies that transition into a more solid-like state over time. It was also established that prolonged induction of FUS results in Ccr4 protein downregulation. We identified that such sequestration and downregulation most likely contribute to the FUS-induced delay in cell cycle progression from G1 to S phase, and contributing to the FUS toxicity phenotype. Further studies describing the phase separation link between Ccr4 and FUS assembly formation, and the impact of RNA on this**,** would elucidate the relationship between Ccr4 and FUS even further, and discern whether RNA is involved in the phase separation process. An *in vitro* assay, similar to the one described in **Paper I,** could help to answer this further. Another valuable addition would be to determine the extent of FUS-induced toxicity due to cell cycle delay. Growth assays of overexpressed downstream cell cycle activators could possibly discern this. Lastly, determining any potential loss of other functions of Ccr4, due to the association with FUS, could yield valuable information to decipher the FUS-related toxicity in neurodegenerative disease.

Pertinent future studies related to **Paper I-III** would be to investigate the functions of identified conserved key components in mammalian model systems. This would further deepen the knowledge of phase separation-related mechanism in both health and disease.

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