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Stress granules and neurodegeneration

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Abstract

Recent advances suggest that the response that RNA metabolism plays in stress has an important role in the pathophysiology of neurodegenerative diseases, particularly amyotrophic lateral sclerosis, frontotemporal dementias and Alzheimer disease. RNA-binding proteins (RBPs) control the utilization of mRNA during stress, in part through the formation of membraneless organelles termed stress granules (SGs). These structures form through a process of liquid–liquid phase separation. Multiple biochemical pathways regulate SG biology. Major signaling pathways regulating SG formation include the mammalian target of rapamycin (mTOR)–eukaryotic translation initiation factor 4F (eIF4F) and eIF2α pathways, whereas pathways regulating SG dispersion and removal are mediated by valosin-containing protein and the autolysosomal cascade. Post-translational modifications of RBPs also strongly contribute to the regulation of SGs. Evidence indicates that SGs are supposed to be transient structures, but the chronic stresses associated with ageing lead to chronic persistent SGs that appear to act as a nidus for the aggregation of disease-related proteins. We suggest a model describing how intrinsic vulnerabilities within cellular RNA metabolism might lead to the pathological aggregation of RBPs when SGs become persistent. This process might accelerate the pathophysiology of many neurodegenerative diseases and myopathies, and suggests new targets for disease intervention.

Introduction

For years, the pathological processes contributing to the accumulation of aggregates in neurological diseases were thought to result mainly from non-physiological aggregation of proteins prone to misfolding, which then accumulated because of progressive, age-related deficits in the proteostatic systems, including the proteasomal and autophagic systems.

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Author contributions

B.W. researched data for article. B.W. and P.I. provided substantial contributions to discussion of the article's content, wrote the article, and reviewed and edited the manuscript before submission.

Competing interests

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Although there is no doubt that dysfunction of proteostasis contributes to neurodegenerative diseases, the field has been revolutionized by the emerging biology characterizing the response of RNA metabolism to stress.

Controlling the localization and utilization of macromolecules is essential to cell biology. Sequestration of most molecules into organelles is achieved by surrounding the organelles with lipid membranes. Indeed, the nucleus, mitochondrion, lysosome, peroxisome, Golgi apparatus, synaptic vesicle and many other cellular structures are surrounded by lipid membranes. In contrast to many intracellular molecules, RNA is rarely surrounded by a lipid membrane. The localization of RNA is generally controlled through the binding of RNA to RNA-binding proteins (RBPs), which themselves have the ability to coalesce through a process of liquid–liquid phase separation (LLPS) (Box 1)[1,2]. The resulting membraneless organelles are termed RNA granules. There are many different types of RNA granules, but stress granules (SGs) appear to be particularly relevant to neurodegenerative diseases and myopathies. Various genes encoding RBPs that function in the SG response have mutations that are associated with motor neuron diseases, such as amyotrophic lateral sclerosis (ALS)[3]. These proteins also accumulate as primary pathologies in a broad range of other neurodegenerative diseases[4]. Finally, therapeutic approaches that inhibit SG accumulation protect against disease progression in animal models of neurodegenerative diseases[5,6].

This Review covers the biology of SGs and related translational stress responses and presents a model for how dysfunction of these pathways contributes to many neurodegenerative diseases. We also review the emerging biology of LLPS and membraneless organelles and show how the fundamental biology of these processes renders RBPs (and other nucleotide-binding proteins) prone to aggregation and subsequent neurodegenerative responses.

The principle diseases discussed below are ALS, Alzheimer disease (AD) and frontotemporal dementia (FTD). ALS is a motor neuron disease that is characterized by a rapidly progressive loss of motor neurons in the spinal cord, an associated devastating loss of motor function, muscle wasting and the accumulation of intraneuronal protein aggregates predominantly containing TAR DNA-binding protein 43 (TDP43), an RBP[7]. AD is characterized by a progressive loss of cortical neurons, with an associated loss of cognitive function that presents as a loss of executive function and memory loss[8]. The predominant pathologies in AD are neuritic plaques and neurofibrillary tangles (NFTs). Neuritic plaques are extracellular aggregates that are composed of a 4 kD peptide, termed amyloid-β (Aβ), whereas NFTs are intra-neuronal aggregates that comprise the microtubule-associated protein tau. Aβ is generated by cleavage of the amyloid precursor protein and Aβ-mediated toxicity is thought to cause neuronal injury and degeneration, which elicits tau phosphorylation, oligomerization and, ultimately, NFT formation[9]. FTD is characterized by the progressive loss of neurons in the frontal cortex and is associated with behavioural abnormalities (for example, social disinhibition and memory loss)[10]. The most common forms of FTD are FTD-tau and FTD-TDP, which are characterized by tau pathology (including NFTs) and TDP43 pathology, respectively. While Aβ induces tau dysfunction and aggregation in AD, the proximal cause of neurodegeneration in FTD-tau is the dysfunction and aggregation of tau[10].

RNA granules

The composition of ribonucleoproteins (RNPs) is a consequence of their nuclear and/or cytoplasmic histories. Nascent RNAs emerging from transcription sites in the nucleus are immediately decorated with proteins that, in many respects, will determine their functions and future life cycle. Some protein components of selected RNPs coalesce through the process of LLPS (Box 1) and promote efficient packaging of RNAs into RNA granules or, more generally, membraneless organelles[1]. Support for the existence of LLPS comes from studies of RNA granules present in cells showing that these structures are highly dynamic and behave like droplets[11]. The classification of membraneless organelles varies based on their localization, composition and proposed functions. Examples of RNA granules connected to cytoplasmic mRNA metabolism include SGs, processing bodies (P bodies), transport granules, storage granules, activity-dependent granules and myo-granules (Fig. 1)[12,13]. Membraneless organelles also form in the nucleus and are referred to as nuclear bodies, comprising an area of considerable interest in its own right and thoroughly reviewed elsewhere[14]. The best-characterized nuclear-based membraneless organelles are nucleoli (sites of rRNA processing and ribosome biogenesis), Cajal bodies, nuclear speckles, paraspeckles, promyelocytic leukaemia (PML) bodies and gemins[14]. The gemins are of particular relevance to motor neuron diseases because they contain survival motor neuron protein (SMN1), which helps assemble spliceosomal small nuclear RNPs (snRNPs), comprising small nuclear RNAs (snRNAs) in complex with snRNP-specific proteins (sites for pre-mRNA splicing) (Fig. 1)[15,16].

RNA is a common denominator for much of the discussion above. Like RBPs, RNA can self-assemble through a process of phase transition and phase separation. For example, transcripts with extended hexanucleotide repeat domains (as are found in myotonic dystrophy, or in ALS that is caused by expansions in the gene *C9ORF72*) are highly efficient at self-assembly owing to the multivalent base-pairing[17,18]; these transcripts readily phase separate both in vitro and in cultured cells (exemplified by the formation of nuclear or cytoplasmic RNA foci)[19–21]. RNA also controls the phase separation of RBPs. In the nucleus, phase separation of the SG-associated RBP FUS occurs only at intermediate RNA concentrations (100–200 ng/μl)[22]. The RNA recognition motifs (RRMs) inherent to RBPs also regulate LLPS, in part by binding RNA to increase the local RNA concentration; however, these domains also bind proteins, such as chaperones and nucleocytoplasmic transporters (importin and transportin), which directly control the tendency of RBPs to self-associate[23–25]. These RRMs also serve as sites of control by post-translational modification; arginine methylation by protein methyl transferases promotes chaperone binding, which prevents phase separation[24,25]. These studies demonstrate how proteins and RNAs are able to self-generate dynamic and heterogeneous molecular seeds in cells that further self-organize into visible, higher-molecular RNP complexes.

Stress granules

Composition and dynamics.

As indicated above, SGs are cytoplasmic members of the RNA granule family (Fig. 1). They have critical roles in mRNA metabolism and translational control and have been implicated

in the pathogenesis of many diseases, including cancer, neurodegeneration, inflammatory disorders and viral infections[26–29]. Historically, the term ‘stress granule’ was used to describe phase-dense cytosolic particles that contain polyadenylated mRNAs, poly(A)-binding protein (PABP) and T-cell intracellular antigen 1 (TIA1)[30,31]. These proteins coalesce with mRNAs to form SGs upon heat-shock stress or under sodium arsenite treatment, which exerts multiple types of stresses, including oxidative stress. In mammalian cells, SGs are typically 200–400 nm in diameter, but they can range in size from about 100 to 1000 nm. Early studies suggested that sodium arsenite-induced or heat-shock-induced formation of SGs in mammalian cells was strictly dependent on the phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2α), providing the first indication that SG functions are connected to mRNA translation and localization (Box 2)[31]. Since SGs quickly dissolved upon stress removal, these cytoplasmic foci were also postulated to be sites of temporary mRNA storage and triage[32].

Compositional analysis of SGs has revealed that the core components of SGs are messenger ribonucleoprotein (mRNP) complexes, including translationally arrested pre-initiation complexes (PICs) consisting of small 40S (but not large 60S) ribosomal subunits, mRNAs and associated translation initiation factors (Fig. 2, Box 2)[33,34]. Besides PICs, many RBPs contribute to SG assembly and disassembly. Some of these RBPs, termed SG nucleators, are required for the condensation of PICs into SGs and act by directly binding to mRNAs and/or interacting with the SG-associated translational machinery[35,36]. Other RBPs play auxiliary roles by bringing specific mRNAs into SGs via sequence-specific interactions with their mRNA targets. Proteomic and genetic screens have identified hundreds of proteins as components of SGs or factors contributing to their assembly[34,37]. However, gene expression or deletion experiments suggest that only PICs and a limited number of specific RBPs are essential for the formation of a stable SG ‘core’[38,39]. These key mRNPs containing untranslated transcripts are hypothesized to act as ‘seeds’ that further oligomerize to form SG cores[33,40]. Once such cores are assembled, they can recruit more SG nucleators (Fig. 2) and serve as a platform for the formation of more dynamic peripheral ‘shell’-like structures around them, consisting of other proteins and mRNPs[40].

The most important feature of SGs is their dynamic nature. SGs quickly assemble in response to biotic or abiotic stresses (within minutes to hours) and quickly disassemble when the stress is removed (Box 2). Such microscopically visible dynamic behaviour is reflected by the behaviour at the molecular level, at which the majority of SG components are in dynamic equilibrium with the cytosol. Studies using fluorescent recovery after photobleaching (FRAP) analysis indicate that proteins shuttle in and out of SGs with residence times ranging from seconds (for example, TIA1) to minutes (for example, fragile X mental retardation protein (FMRP)) (Box 2)[30,41].

SGs are in dynamic equilibrium with polysomes[30], an actively translating fraction of cytoplasmic ribosomes. When polysomes are disassembled in response to stress or pharmacological interventions (for example, following treatment with the antibiotic puromycin, which causes premature protein synthesis termination), they increase the pool of untranslated mRNPs in PICs, which favours SG assembly. By contrast, an increase in mRNPs in the polysome-associated fraction or inhibition of translation elongation disfavours

SG assembly (for example, by treatment with cycloheximide, which interferes with the translocation step of protein synthesis blocking translation elongation, and freezing ribosomes on mRNAs) (Fig. 3)[30]. Thus, formation of SGs is tightly connected to the translational status in the cell, and the dynamic link between SG and translational control distinguishes SGs from many other RNA granules.

The assembly of SGs is a consequence of a multistep process that starts from the recognition of stress and leads to translational arrest, resulting in the formation of PICs[40]. The translational arrest is achieved by the inhibition of translation initiation, which is a highly regulated step in translation. Two major signaling pathways regulate translation initiation as well as SG and polysome dynamics: eIF2α phosphorylation and the assembly of the cap-binding eIF4F complex (consisting of translation initiation factors eIF4E, eIF4G and eIF4A, reviewed in REF.[42]) (Fig. 3). However, the large variety of different stresses and cell types suggests a far more pleiotropic network of signalling pathways regulating protein synthesis and SG and polysome dynamics.

Regulation of stress granules.

Under favourable conditions, the efficient translation of mRNAs is achieved by interactions between the cap-bound eIF4F complex on the 5’-end of the mRNA and the 3’-poly(A) tail-bound PABP (Fig. 3a). The assembly of the eIF4F complex on the cap structures of mRNAs is an early checkpoint in the translation initiation[42]. This step is under the stringent control of a major serine/threonine kinase, the mammalian target of rapamycin (mTOR)[43–45]. mTOR is both a sensor and a regulator of cellular metabolism, coupling needs in protein synthesis to the input from the different signalling pathways monitoring nutrient, energy and oxygen levels[46]. When conditions are optimal for growth and proliferation, mTOR constitutively phosphorylates eIF4E-binding protein (4E-BP); this phosphorylation event prevents 4E-BP from binding to eIF4E and allows the formation of the eIF4F complex (Fig. 3a)[47]. Under stress, mTOR is inactivated, leading to the accumulation of hypophosphorylated 4E-BP, which avidly binds eIF4E, causing displacement of the scaffolding protein eIF4G and the RNA helicase eIF4A from mRNA cap structures (Fig. 3b)[47]. In addition to physiological stimuli, diverse chemical compounds (for example, sodium selenite, hydrogen peroxide and chemotherapy drugs such as vinca alkaloids) also promote mTOR inactivation and eIF4E–4E-BP interactions[43–45]. As a consequence of eIF4E–4E-BP complex assembly, translation initiation and polysome formation is inhibited, leading to the formation of PICs, which recruit additional RBPs to form the ‘seeds’ that are required for SG assembly[48].

The availability of the ternary complex comprising eIF2, GTP and the initiator methionine tRNA ($tRNA_i^{Met}$) is another major checkpoint of translation initiation (Fig. 3b)[42]. The ternary complex delivers initiator $tRNA_i^{Met}$ to the 40S ribosomal subunit to recognize the start AUG codon on mRNAs. In response to stress, eIF2α kinases are activated to target serine at position 51 of eIF2α for phosphorylation[49]. These eIF2α kinases are key components of the integrated stress response, a common adaptive system that monitors and integrates different intracellular and extracellular signals regulating cellular translation. Four eIF2α kinases are activated by different stresses: general control nonderepressible 2 kinase

(GCN2) monitors levels of charged tRNAs and nutrient stress (for example, starvation)[50], PKR-like ER kinase (PERK) monitors levels of unfolded proteins and ER stress[51], protein kinase R (PKR) monitors the presence of double-stranded RNAs and viruses[52], and heme-regulated eIF2α kinase (HRI) monitors the availability of heme and redox state[53]. Once phosphorylated (p-eIF2α), eIF2α inhibits the actions of eIF2B, the guanine exchange factor that reloads the ternary complex with GTP, thus decreasing the amount of this complex that is translationally competent (Fig. 3b)[54]. In turn, reduced levels of the ternary complexes inhibit recognition of AUG start codons on mRNAs and translation initiation. Non-canonical PICs that lack specific translation initiation factors (eIF2 and eIF5) and charged $tRNA_i^{Met}$ are assembled on untranslated mRNAs as a consequence of eIF2α phosphorylation[55]. Because the elongating ribosomes already engaged in translation are not affected by p-eIF2α, they 'run off' the mRNA, leading to disassembly of polysomes[49]. Such an influx of untranslated mRNAs and translationally arrested PICs leads to SG assembly.

Prior research has identified other pathways controlling SG assembly, and new pathways continue to be identified. The eIF4 cascade stands out as potentially relevant to neurodegenerative diseases. The RNA helicase eIF4A, and initiation factors eIF4B and eIF4E appear in protein-interaction networks with tau and TDP43 (REFS[56–59]). Scattered reports also identify mutations in the gene encoding eIF4G that are associated with Parkinson disease[60], although this association has been disputed[60,61]. The putative role of the eIF4 pathway in Parkinson disease can be tested experimentally using translation initiation inhibitors that interfere with its functions and cause SG formation, including natural lipid inflammatory mediators 15-deoxy-Δ(12,14)-prostaglandin J2 (REF.[62]), steroid hippuristanol[63], and the xenobiotic agents pateamine A and silvestrol (Fig. 3b)[64]. Small non-coding RNAs termed tRNA-derived stress-induced RNAs (tiRNAs) also target the eIF4 pathway (Fig. 3b). tiRNAs are produced when stress induces angiogenin (ANG; a ribonuclease) to cleave tRNAs[65]. tiRNAs inhibit translation by targeting the eIF4F complex, causing its displacement from the cap structures of mRNAs, and such tiRNA-induced untranslated mRNAs are then packed with help of the RBP Y-box-binding protein 1 (YB1) into SGs[66].

Stress granules in neurons.

SG biology in neurons appears to differ from SG biology in dividing cells. The formation of SGs in cell lines occurs rapidly after they are exposed to stress[30]. Indeed, stress induces RBPs such as TIA1 to exit the nucleus and appear to form SGs immediately[30]. These same RBPs are also largely nuclear in the normal brain[67]. However, neurons in diseased brain tissue frequently exhibit diffuse cytoplasmic expression of nuclear RBPs with only a fraction of RBPs coalesced into cytoplasmic granules[68,69]. These observations suggest that the stable cytoplasmic translocation of nuclear RBPs is an important part of RBP biology, at least in neurons (Fig. 4). This stable cytoplasmic localization could derive from two simple factors, although other factors also probably contribute. One consideration is the biology of neurons, which appears to exhibit a robust stage of stable cytoplasmic translocation of nuclear RBPs (without consolidating to form SGs or other membraneless organelles), possibly owing to the presence of cytoplasmic factors that inhibit LLPS, such as post-translational modifications, protein chaperones, specific cytoskeletal proteins or their regulators. Profilin

1 provides one example of neuron-selective regulation; profilin 1 is an ALS-linked actin-binding protein that forms SGs that co-localize with ataxin 2 (ATXN2; also associated with ALS) in neurons but not in peripheral cells[70]. ELAV-like protein 1 (ELAV1) is an example of a cytoplasmic RBP that lacks low-complexity domains (LCDs), is abundant in neurons and delays SG formation[71,72]. A second factor is the biology of disease: laboratory experiments with cells tend to use severe, acute stresses, such as heat shock or chemical treatments, whereas neurodegenerative diseases are chronic and evolve over years. The chronic nature of neurodegenerative diseases suggests that the actual stress is milder or more readily compensated for than the severe, acute stresses used in the laboratory. The intrinsic biology of neurons might combine with the prolonged, incipient nature of neurodegenerative disease to yield an intermediate form of the translational stress response that does not yield large SGs. However, the absence of overt SGs might not prevent RBPs from forming smaller, less visible complexes that regulate the translational machinery and might contain insoluble proteins. Multiple studies in model systems suggest that RBPs can regulate RNA metabolism in the cytoplasm without forming large granules; diffuse RBPs or small RBP granules might serve as a nidus for formation of the larger pathological aggregates that ultimately accumulates in neurodegenerative diseases[73,74]. This scenario highlights the potential importance of cytoplasmic translocation of nuclear RBPs in the pathophysiology of neurons and neurodegenerative disease.

Stress granules and neurodegeneration

RNA-binding protein pathology and dysfunction in disease.

A steady progression of genetic studies has linked an increasing number of RBPs to motor neuron diseases and myopathies. For example, diseases such as spinomuscular atrophy and spinocerebellar atrophy have been associated with mutations in RBP genes for over two decades[75]. Mutations in *FMRP* are the most common cause of X-linked intellectual disability[76]; large expansions of CGG repeats in the 5’ noncoding region of this gene abolish FMRP expression, but expansions in the range of 55–200 repeats allow for expression of some FMRP and give rise to a late-onset neurodegenerative disorder termed fragile X-associated tremor/ataxia syndrome (FXTAS)[77,78].

Attention focused on RBP aggregation processes after identification of TDP43 as the major pathological protein aggregate that accumulates in ALS and some FTDs[79]. This 43 kD protein, along with its 35 and 25 kD cleavage fragments, were shown to accumulate in the spinal cords of individuals with ALS[79]. Indeed, aggregation of TDP43 is readily apparent in multiple neurodegenerative disorders, FTD-TDP, familial ALS caused by mutation of *C9ORF72*, AD and chronic traumatic encephalopathy[79]. The pathological TDP43 that accumulates in each disease is typically phosphorylated at serine residues 409 and 410, and each of these diseases also shows biochemical evidence of TDP43 truncation to form 25 kD and 35 kD cleavage fragments[79]. The aggregation of FUS is evident in cases of ALS with *FUS* mutations, and a similar situation is observed for most other RBP gene disease-linked mutations, with the exception of cases of disease linked to *TIA1* mutations, which exhibit TDP43 aggregates but not TIA1 aggregates[80–83]. Thus, in each of these disorders, the major

protein that accumulates as a pathological aggregate is generally detectable in human tissues.

Soon after the discovery of TDP43 as the major pathological protein in ALS, mutations in the gene encoding TDP43 were shown to be associated with familial ALS, which proved that dysfunction of TDP43 was sufficient to cause disease[84]. Discovery of ALS-linked mutations in other RBP genes followed, including *FUS*, *HNRNPA2B1*, *EWS*, *TAF15*, *MATR3* and *TIA1* (REFS[80,85]); in addition, repeat length polymorphisms in *ATXN2* were identified as important risk factors for ALS[86]. Mutations in *ANG* also are associated with ALS; ANG functions as an RNase that generates tiRNAs and as a RBP regulating transcription of ribosomal RNAs[65,87,88]. These observations cemented the growing consensus that something about the biology of RBPs rendered them prone to cause neurodegenerative diseases.

A major conceptual advance in our understanding of the mechanisms diseases exhibiting TDP43 and ALS pathology came with the observations that TDP43 and FUS promote the formation of SGs, that disease-linked mutations in the genes encoding these RBPs lead to an excessive accumulation of SGs in cells exposed to stress, and that the pathological accumulation of these proteins in the human brain co-localizes with SG markers[26,89,90]. Of course, a true proof-of-concept awaits a demonstration that selectively inhibiting the SG pathway prevents disease progression in human patients (Box 2). The SG pathway had been studied extensively in the fields of cell biology and rheumatology[91], and related work in virology had focused on anti-viral granules[92]. However, connecting the SG pathway to neurodegenerative diseases led to the novel prediction that SGs serve as a crucible for initiating pathological protein aggregation[26,93]. The very nature of SG biology renders this pathway fundamentally prone to diseases of aggregation because the coalescing of RBPs into SGs necessarily brings aggregation-prone proteins together into local domains that concentrate RBPs 100–400-fold[22]. The high local concentration of these proteins increases the chances that amyloidogenic interactions will lead to formation of persistent pathological oligomers and fibrils and subsequent disease outcomes.

SG biology is generally considered to be an adaptive response to a transient stress, and indeed most experimental designs use a stress that is approximately 30–60 mins. This transient time course typically used in the laboratory contrasts strikingly with the chronic stress of neurodegenerative disease. ALS generally lasts 3–6 years[94]. Moreover, overt manifestations of AD can last 15 years or more, but imaging studies show that the deposition of Aβ frequently precedes overt dementia by 15 years, which suggests that the entire disease course can extend to 30 years or more[95]. Thus, these diseases clearly are not acute stresses, as used in the laboratory; they are chronic and persistent.

Kinetic studies demonstrate that SGs evolve over time (Fig. 4a). Arginine demethylation of RRMs facilitates SG formation, in part by preventing binding of transportins, which act as disaggregases, and in part by promoting phase separation via π-cation interactions[23–25]. Nucleation is followed by accretion of the secondary SG, which enlarges in size and complexity (Fig. 4a)[32,38,40,96]. As the SG persists, other proteins such as sequestosome-1 (SQSTM1; also known as p62) bind, and RBPs become post-translationally modified by

adducts such as phosphate groups or ubiquitin (Fig. 4)[97]. The structure of several reported RBPs also evolves over time in SGs, with prolonged coalescence promoting the accumulation of some RBPs with β-sheet structure, and the concomitant formation of insoluble amyloids[1,11,98,99]. These β-sheet conformations are much more stable than the amino acid sequences that promote LLPS (referred to as low-complexity aromatic-rich kinked segments (LARKS)) and lead to the accumulation of insoluble proteins[100]. The tendency of RBPs to form insoluble amyloid fibrils following persistent and/or repetitive LLPS prompts the hypothesis that membraneless organelles serve as a genesis for the accumulation of intracellular pathological inclusions in human diseases and animal models of human diseases.

Defining pathological stress granules.

Classical SGs constitute translationally inactive complexes containing mRNAs, RBPs and ribosomal components. In this section, we introduce the term ‘pathological SG’ to describe the inclusions that accumulate in pathological tissues that carry many of the proteins that define SGs *in vitro*. SGs are dynamic structures that rapidly form and disperse with acute stress, but chronic illness produces persistent stress that allows time for SGs to mature into more stable complexes. The relationship between pathological inclusions that contain SG markers and actual functional SGs has yet to be rigorously tested; in addition, there are many other types of membraneless organelles with phase-separated RBPs (such as nuclear membraneless organelles, including nuclear speckles, nuclear gems and the nucleolus) that might also promote the formation of stable amyloids. For instance, TDP43 is known to accumulate as nuclear aggregates, referred to as subtype D TDP43 pathology (occurring in a sub-variant of FTD-TDP cases), and such nuclear aggregates could evolve from nuclear membraneless organelles containing TDP43 (REF.[4]). Environmental toxicants, such as lead or mercury, might also induce pathological SGs[101].

SGs are defined by the presence of core nucleating RBPs (for example, TIA1 or RAS GTP-activating protein-binding protein 1 (G3BP1)), translation initiation factors (for example, eIF3s), core 40S ribosomal subunits (consisting of ribosomal proteins (RPSs); with the absence of 60S ribosomal subunits) and mRNAs (Fig. 2). SGs that contain phase-separated proteins are expected to be dynamic, which can be shown with imaging studies that show granule fusion or fission, and by studies that show dynamic molecular movement in the cells; for instance, by using fluorescence photobleaching[30]. Human neuropathology, however, consists of aggregated amyloid aggregates that are unlikely to exhibit dynamic motion, suggesting that pathological SGs are also unlikely to be dynamic. The aspect of the SG definition that is dependent on co-localization is readily experimentally demonstrable, and has been shown for many SG markers in human tissues and/or animal models[26,102–104]; recent work shows this convincingly using an animal model in which a $(G_4C_2)_{149}$ hexanucleotide repeat construct is expressed, which models ALS linked to *C9ORF72* mutations[105]. The hexanucleotide repeat generates dipeptide repeats due to noncanonical translation that is itself stimulated by the integrated stress response[106,107]. These dipeptide repeats enhance SG formation in cells and form pathology that co-localizes with multiple SG markers including TIA1, G3BP1 and ATXN2 (REFS[105,108,109]). Experimentally showing co-localization by immunohistochemistry is an important approach to demonstrate

the presence of SGs. Proteomic studies also provide support for the role of SGs in pathological processes because they indicate an over-representation of RBPs linked to SG biology in insoluble material extracted from pathological tissues[58,102]. However, studies of post-mortem tissue are inherently limited by their inability to address whether the SG proteins observed in the tissues were in dynamic states prior to fixation. This type of question can only be investigated in animal models.

If the aggregation of RBPs in disease evolves from membraneless organelles, then studies must document such evolution over time. Live cell imaging with cell culture are beginning to illuminate pathways leading to formation of pathological granules containing TDP43 (Fig. 4b)[110]. The first phase of the stress response leads to translocation of TDP43 to the cytoplasm where much of it associates with SGs. With continued stress, SGs and the associated TDP43 become less dynamic, forming non-fluid gels[110,111]. The TDP43 associated with these SGs further evolves to form insoluble aggregates that tend to accumulate around the edge of the SG and that are no longer associated with RNA (Fig. 4b)[110]. These aggregates of TDP43 are phosphorylated at serine residues 409 and 410, which is a hallmark of the pathological TDP43 aggregates observed in human cases of ALS and FTD, suggesting a fundamental link between these mechanisms and disease pathology[110]. A small amount of TPD43 proceeds through a parallel pathway in which it directly aggregates and becomes phosphorylated at serines 409 and 410 upon cytoplasmic translocation[110–112]. The aggregated TDP43 is capable of cross-talk with SGs as shown by the ability to modulate the equilibrium between TDP43 associated with SGs and pathological aggregates by changing binding to RNA or DNA; increased binding to oligonucleotides shifts the equilibrium in favour of SG association (by incubation with high affinity, modified DNA oligonucleotides), whereas reduced binding increases formation of pathological aggregates (by eliminating RNA recognition motifs in TDP43)[111].

A major challenge lies with translating these concepts to studies in the brain. The field needs to determine whether SGs or other membraneless organelles exhibit dynamic movement in vivo, and quantify the proportion of pathological granules that evolve through SG-mediated pathways. Until such studies are done, an intermediate step would be to follow the evolution of RBP localization and solubility over time in disease models. Such studies are in progress in multiple laboratories, including our own. In the meantime, histopathology studies demonstrate clear evidence of redistribution of RBPs from the nucleus to the cytoplasm, which presumably suggests a change in function of these proteins[113]; similar results are observed for nuclear pore proteins in diseases exhibiting TDP43 or C9ORF72 pathologies[114,115]. The aggregation of TDP43 can be shown biochemically, which is important because aggregation of TDP43 has been associated with a loss of splicing function[116]. These studies suggest a model in which the chronic stress associated with disease induces RBPs to move from soluble, functional states, such as nuclear splicesome complexes, into the cytoplasm to form SGs, which over time evolve into pathological aggregates of nonfunctional protein amyloids. This hypothesis will be tested as chemicals that inhibit the SG pathway are translated into the clinic.

Tau phosphorylation

For over 30 years, we have known that tau becomes phosphorylated by proline-directed serine/threonine kinases, such as glycogen synthase kinase-3β, cyclin-dependent kinase 5 and microtubule-affinity regulating kinases (MARKs), in response to stress; this type of phosphorylated tau is referred to as 'hyperphosphorylated tau'[117,118,119]. Our understanding of the purpose of this phosphorylation has developed with time. Under nonstressful conditions, tau mainly localizes to axons, where it binds to microtubules, promotes microtubule assembly and facilitates the formation of long processes that characterize axons. In response to stress, tau is phosphorylated near and in its microtubule domain, which prevents tau from binding to microtubules[120]. Interestingly, experimental studies show that hyperphosphorylated tau accumulates in the neuronal soma rather than in the axon during stress[121]; this appears to occur because the stress-related phosphorylation occurs on newly synthesized tau in the somatodendritic arbor[121]. Consistent with this observation, brain tissue from individuals with AD also shows an accumulation of tau hyperphosphorylation in the somatodendritic arbor rather than in axons[121]. Thus, an important function of tau phosphorylation by stress kinases appears to be concentrating newly produced tau in the somatodendritic arbor.

Tau, stress granules and the translational stress response.

Increased interactions between tau and mRNA are an important consequence of the somatodendritic localization of tau in stress, because mRNA concentrations are much higher in the somatodendritic arbor than in the axon. Tau is not classically thought of as an RBP, but it has been known to interact with RNA since the earliest days of tau research, when the presence of RNA was shown to modulate the folding pathways of tau[122]. Native tau is highly soluble, but under some conditions, it can fold along an amyloid pathway, leading to formation of tau fibrils that contain the classic cross-β sheet structure of amyloids[123]. Since the early 1990s, acidic molecules, such as heparin, dextran sulfate and arachidonic acid, were documented to stimulate tau fibrillization[124,125]. The physiological importance of the action of these molecules on tau was never clear. However, one other key acidic molecule also stimulates tau aggregation, namely RNA. The physiological significance of this observation was not appreciated until the past several years, and is still evolving. Recent studies have demonstrated that tau undergoes LLPS, just like RBPs, and, importantly, the tendency of tau to go through LLPS increases dramatically in the presence of RNA[126,127]. The abundance of RNA in SGs might contribute to the tendency of tau to associate with RBPs.

Emerging evidence indicates that somatodendritic hyperphosphorylated tau functions to regulate the ribosome and the translational stress response[103,128]. Studies have identified ribosomal subunits and RBPs as major features of the tau interactome[56,59,129]. Ribosomal proteins that bind tau include members of the 60S ribosomal subunit (RPL6–RPL8, RPL11, RPL13, RPL26–RPL29, RPL34 and RPL35) and, less frequently, members of the 40S ribosome (RPS6, RPS10, RPS19 and RPS25) and eukaryotic translation initiation factors (eIF2, eIF3A, eIF3G, eIF3J, eIF4A2, eIF4G1 and eIF4G2)[56,59,103]. Many RBPs that are associated with SGs also appear in tau interactomes, including heterogeneous nuclear RNPs

hnRNPs (hnRNPA0, hnRNPD and hnRNPU), ATP-dependent RNA helicase DDXs (DDX3, DDX5 and DDX6), RNA-binding protein EWS (EWSR1), TATA-binding protein-associated factor 2N (encoded by *TAF15*), ATXN2 and nuclear pore complex protein Nup98 (Figs 1,4)[56,59,130]. Importantly, mutations in many of the genes encoding these proteins are associated with ALS, indicating that their dysfunction is sufficient to cause neurodegenerative disease; typically the disease-related mutation increases the tendency of the protein to aggregate, to accumulate in SGs and to fibrillize. The appearance of these proteins in the tau interactome provides the building blocks for tau to regulate ribosomal function and the translational stress response.

Studies of the physiology and pathophysiology of tau provide the biological link between this protein and SGs and the translational stress response. Expressing tau promotes SG formation in neuronal cell lines and in primary neuronal cultures[103]. By contrast, reducing TIA1 levels prevents the formation of tau-positive SGs and tau-mediated toxicity[67,103]. Disease-linked mutations in *MAPT* (which encodes tau) lead to bigger, more stable SGs, much like what is observed with mutations in the genes encoding the disease-linked RBPs that bind to tau[103]. Tau hyperphosphorylation appears to have an important role in this pathway. Tau that is pseudophosphorylated at stress kinase sites (by mutating serine and threonine residues 181, 202, 205, 262, 396 and 404 to aspartate residues) co-localized with SGs and formed larger granules than did phospho-null tau (in which the serines and threonines were converted to alanines)[103]. Studies of protein synthesis show that tau also inhibits general ribosomal function, as would be expected for proteins that stimulate the translational stress response[103,128].

Stress granules and tau aggregation.

The interaction of tau with SGs has important consequences for the pathophysiology of tauopathies because the association of tau with these structures stimulates the formation of insoluble tau aggregates[103]. Tau co-localizes strongly with TIA1 and with other SG-associated proteins, including PABP, hnRNPA0, eIF3η and EWSR1[56,103]. The mechanisms underlying the stimulation of tau aggregation in SGs remains to be explicitly determined, but a reasonable hypothesis emerges from data from LLPS, cell biology and neuropathological studies[56,103,104,126,127]. SGs contain a high concentration of mRNA, which probably promotes the coalescence of tau into droplets, much like it does in vitro. However, we have yet to observe tau-forming RNA-associated granules in neurons or in the brain independent of SGs, which suggests that tau requires the presence of RBPs to become associated with membraneless organelles such as SGs. This suggests a hybrid model in which tau associates with both RBPs and RNA in SGs, but in vivo does not undergo LLPS in absence of RBPs. The tau–SG interaction manifests in vivo in co-localization of SG markers, such as RBPs, with tau pathology[56,104,131].

The analysis above treats SG pathophysiology as a uniform pathway, but it is likely that this type of reductionist model is overly simplistic. A cursory evaluation of neuropathological samples reveals a wide range of pathological distributions for RBPs and for tau. These patterns of distribution range from the earliest stage of pathology, which is evident in the cytoplasmic translocation of nuclear RBPs (for example, TDP43, hnRNPA2B1, FUS or

TIA1) without evidence of SG formation, to large pathological inclusions of RBPs, such as TDP43 (REF.[132]). The early stage stress responses yield seemingly stable, diffuse cytoplasmic distributions of nuclear RBPs, which are either monomeric or composed of many very small complexes (Fig. 4). At this stage, other stress response proteins that are normally cytoplasmic, such as tau, eIF2α or eIF3 proteins, become hyperphosphorylated or have changes (increases or decreases) in other post-translational modifications (such as acetylation, arginine methylation or adenylation)[24,25,120,133]. Late-stage pathology also exists, which is made up of insoluble RBPs or tau molecules that appear to be consolidated into relatively homogeneous pathological aggregates, in which associated proteins become excluded[56]. Studies using seeding and propagation (described below) suggest that tau pathology evolves in a manner similar to TDP43 pathology, proceeding through a SG intermediate[134].

Other pathways for stimulating tau pathology.

Other processes might lead to the formation of RBP or tau pathology through completely independent mechanisms. Evidence suggests that pathological tau can propagate among neurons, with the tau being exocytosed from one neuron, and then taken up by an adjacent neuron[135,136]. Much of the tau propagation field has focused only on detecting tau pathology, but a recent study demonstrates that strains of tau propagates differ in toxicity in vivo, with strains present in the oligomeric fraction being the most toxic and also, interestingly, colocalizing with SG markers[134]. Thus, simply detecting tau propagation is insufficient to determine whether the propagated tau promotes neurodegeneration[134]. Some evidence suggests that TDP43 also propagates[137]. The mechanism of tau propagation might be pleiotropic, involving the exocytosis of tau directly or the exocytosis of tau-containing exosomes[138]. Whether the templating that occurs during propagation occurs through a SG pathway remains to be determined.

Inflammation has also emerged as a major contributor to the pathophysiology of neurodegenerative diseases. RBPs and SGs are clearly key to the biology of immune cells [91,139]. Full knockout of *Tia1* increases the secretion of cytokines (from immune cells), such as tumor necrosis factor[91]. Little is known about SG biology in microglia. One of the few studies in this area suggested roles for TIA1 and G3BP in microglial responses[139]; this work also implicated the kinase SYK, which is thought to contribute to a pathway downstream from triggering receptor expressed on myeloid cells 2 (TREM2), a strong genetic risk factor for AD[140].

Towards an integrative model

The discovery that tau physiology interfaces with RBPs and membraneless organelles, including SGs, suggests an integrative model for the pathophysiology of neurodegenerative diseases. We propose that the pathophysiology of many neurodegenerative diseases feeds into a unified pathway that funnels through three levels of core components, with each level being characterized by a particular set of proteins that aggregate in response to the toxic signalling cascade (Fig. 5). This model shows how risk factors, such as genetic and environmental factors, feed into a central biochemical pathway that leads to disease. The

pathophysiology of the central cascade is modified by inflammation and proteostasis because these processes respond to the pathological accumulations and resulting neuronal injury.

Extracellular signals (for example, an increase in Aβ or a reduction in progranulin) are at the top of the cascade because they activate the distal pathologies. Autosomal dominant mutations in the genes linked to AD are the most penetrant because they directly increase the production of $A\beta^{141}$, whereas polymorphisms in genes such as *APOE* and *TREM2* increase the accumulation of extracellular $A\beta^{140}$. The central trunk of the cascade acts in one direction to feed forward, which means that direct stimulation of protein aggregation in the second or third phases of the cascade does not act on the first stage to induce Aβ aggregation or to reduce progranulin levels.

Intracellular factors that mediate the actions of Aβ and progranulin, mainly tau and TDP43, comprise the middle level of the central cascade (Fig. 5). Tau and TDP43 appear to respond to different signalling cascades. Tau is necessary for stress to neurons mediated by $A\beta^{142,143}$, and was also recently shown to mediate responses to glucocorticoids[131]. By contrast, TDP43 appears to respond to reduced levels of progranulin[79,144]; sequestration of TDP43 into persistent SGs appears to elicit axonal degeneration by altering the splicing of stathmin-2 (REFS[145,146]), which might be particularly important in motor neurons because of their long axons.

Risk factors that act directly on tau (for example, *MAPT* mutations or chronic brain trauma) or TDP43 (for example, dipeptide repeats) cause FTDs but do not cause AD because they do not elicit the extracellular accumulation of Aβ. Note that tau becomes hyperphosphorylated as part of the stress response, whereas TDP43 appears to become hyperphosphorylated as it forms irreversible aggregates.

The independent behaviour of tau and TDP43, as observed neuropathologically and in laboratory studies, suggests that these two proteins act in pathways that are separate, but both pathways involve RBPs, RNA metabolism and probably translational mRNA stress cascades. Indeed, disease-linked mutations in *MAPT* or *TARDBP*, which encodes TDP43, directly enhance self-aggregation and SG accumulation without requiring toxic signals from Aβ or other cell autonomous factors. The integration of tau and TDP43 with RNA metabolism is important because it provides a mechanism that connects tau and TDP43 with the many other RBPs linked to neurodegenerative disease[147].

The biochemical pathway that activates pathological tau and TDP43 responses feeds into SGs and the translational stress response, which utilize the RBPs listed at the bottom level of the central pathway (Fig. 5). The particular RBPs that accumulate in disease appear to be those exhibiting a strong tendency to aggregate, and include TDP43, FUS, EWS, TAF15, TIA1, hnRNPs, ATXN2, MATR3 and PABPs. Aggregation of these RBPs appears to cause ALS[80,85].

The control of the accumulation of SGs or pathology is fundamentally regulated at three levels: formation, maintenance and removal. Until now, we have largely focused on the formation and maintenance of SGs, but protein catabolism also regulates the accumulation

of SGs and associated pathological aggregates. Mutations in the genes critical to autolysosomal function are linked to ALS and FTD[148–151,152,153]. These genes include ubiquilin-2 (*UBQLN2;* which encodes a ubiquitin ligase), valosin-containing protein (*VCP*; which encodes a protein disaggregase), *SQSTM1* (which encodes a ubiquitin binding protein), optineurin (*OPTN;* which encodes a multifunctional protein with a role in autophagy), charged multivesicular protein 2B (*CHMP2B*) and tank binding kinase 1 (*TBK1*; which encodes a kinase in the ubiquitin pathway that phosphorylates OPTN and that might act in microglia)[148–151,154]. Most of the encoded proteins play important roles in disaggregating SGs and facilitating catabolism of the aggregated proteins that accumulate in SGs[155,156,157]. Integrating these data suggests a clear role for protein catabolism in controlling the accumulation of the persistent pathological SGs and related protein aggregates whose accumulation drives the integrated neurodegenerative disease pathway. Deficits in proteins that control the degradation of proteins in the integrated SG pathway will necessarily lead to a deleterious accumulation of these proteins.

Mutations in genes linked to cytoskeletal proteins are an additional cause of tauopathies or ALS. Such genes include *MAPT*, *TUBA4A* (encoding tubulin) *KIF5A* (encoding kinesin), *DCTN1* (encoding dynactin 1) and profilin[158–163]. Impairment of axonal transport is the classic explanation for the role of cytoskeletal mutations in neurodegeneration; this remains a cogent explanation because of the massive and distant axonal arbor supported by each neuron[3]. However, proposals for disease mechanisms must account for the late onset of neurodegenerative diseases. Severe impairment of axonal transport causes rapid early-onset disease, which suggests that mutations linked to late-onset diseases must exert subtler dysfunctions. We suggest that dysfunction in cytoskeletal proteins linked to neurodegenerative diseases might also impair critical functions regulating the biology of SGs and other membraneless organelles. We have already discussed extensively the functions of tau and its putative role in regulating SG biology. The ALS-associated mutations in profilin impair SG dynamics, and mutations in the gene encoding kinesin all occur in the cargo domain, suggesting that these mutations cause dysfunction in the ability of kinesin to bind and carry cargo, which likely includes RNA granules[70,159,164]. Similarly, disease-associated mutations in *DCTN1* impair interactions between dynactin 1 and tau[165]. These data provide potential ‘subtle’ mechanisms of action that could account for the late onset of disease in cases related to mutations in genes encoding cytoskeletal proteins.

Finally, inflammation has become a major focus of much research in neurodegeneration and is clearly a critical pathway that contributes to many neurodegenerative diseases, including AD, ALS, FTD and diseases not directly linked to RNA metabolism, such as Parkinson disease. Coverage of the vast field of inflammation is beyond the scope of this article and we suggest that readers turn to recent reviews (for example, see REF.[166]). The major point to be focused on here is that SGs or related RNA granules participate in the inflammatory pathway[91,139], and thus are expected to be impacted by immune dysfunction in neurodegeneration. Impairment of microglia function can lead to the accumulation of Aβ, increased secretion of toxic cytokines and reduced removal of injured neurons[167,168]. Whether mutations occur at the level of receptors, such as TREM2, or intracellular regulators, such as TBK1, appears to determine the level of action in the pathway where the

microglial dysfunction manifests. Regardless of where the dysfunction occurs, the result appears to accelerate the disease process.

Therapeutic approaches

The potential role of SGs in the pathophysiology of disease suggests new targets to use as putative therapeutic approaches for diseases such as neuromuscular diseases (for example, ALS) and dementias (for example, AD and FTD). SG pathways have been approached from both biased and unbiased perspectives. Two recent genetic studies using animal models demonstrate the potential therapeutic benefit of inhibiting RBP pathways. One study examined the effects of reducing ATXN2 levels in an animal model of ALS based on overexpressing wild-type TDP43 (REF.[169]). Full knockout of *Atxn2* increased the median lifespan of these animals by 80%, and antisense oligonucleotide knockdown of *Atxn2* increased median lifespan by 35%[169]. Work from the Wolozin laboratory using the P301S tau mouse model showed that a 50% reduction in TIA1 levels extended survival of these mice by 26% and fully rescued both synaptic loss and behavioural deficits at 6 months[67]. Both of these studies demonstrate that reducing the RBP level also reduced the number of cytoplasmic pathological SGs in neurons, which demonstrates that these interventions affect SG biology.

Nuclear pore biology is another target that interfaces strongly with SG biology and offers novel approaches for therapeutic intervention. Cell stress disrupts the nuclear pore complex[170], by causing the nuclear pore components to translocate to the cytoplasm where they co-localize with SGs[170]. This pathway is therapeutically interesting because inhibitors of nucleocytoplasmic transport, such as the compound KPT-276, offers protection in a *Drosophila melanogaster* model of ALS in which the hexanucleotide repeat $(G_4C_2)_{30}$ is overexpressed[170]. An analogue of KPT-276 is approved as a cancer chemotherapy and is currently being investigated in clinical trials for ALS.

Many small-molecule inhibitors targeting known SG pathways are readily available, including those targeting the eIF4 pathway (involving mTOR), the eIF2α pathway (which includes PERK and PKR pathways) and disaggregases (such as VCP, heat shock protein 104, transportin and importin). Inhibitors of many of these SG pathways already have exhibited neuroprotection in models of neurodegenerative diseases. The PERK pathway has been studied by many groups in many different contexts[171]. Inhibiting the PERK pathway slows disease progression in transgenic mouse models in which disease is induced by transmission of the prion protein or expression of mutant TDP43 or tau[5,6,172]. The prion protein and TDP43 studies both show evidence that PERK inhibition reduces eIF2α phosphorylation, suggesting that such inhibition affects the SG pathway and modulation of translation. However, PERK inhibition produces pleiotropic effects, regulating protein folding and chaperone pathways in the endoplasmic reticulum, which raises the possibility that the benefits of PERK inhibition occur through mechanisms that extend beyond the SG pathway. Inhibition of GCN2, which is another kinase that regulates eIF2α, also provided neuroprotection in a model of AD based on overproduction of Aβ[173]. Rapamycin is another broadly neuroprotective drug that inhibits mTOR and thereby is expected to inhibit a SG pathway mediated by eIF4 proteins[42]. Rapamycin has shown success in ameliorating

neurodegeneration in multiple different animal models, including disease mediated by tau or TDP43 (REFS[174,175]). Inhibitors of cyclin-dependent kinase inhibitors that block the SG response inhibit the formation of SGs, including TDP43-positive granules, in cells[133]. Finally, SGs provide a ready mechanism for unbiased, high-throughput drug screening. With the development of techniques to efficiently observe SGs in motor neuron-differentiated induced pluripotent stem cells (iPSC), these cells can now be used for chemical screens focusing on TDP43, FUS or hnRNPA2B1[176].

Conclusions

SGs are a type of membraneless organelle that naturally increase in number in response to stress. This stress-response system is designed to be transient, but the chronic stresses associated with ageing lead to chronic persistent SGs that appear to act as a nidus for the aggregation of disease-related proteins. Thus, the pathophysiology of neurodegenerative diseases might well arise out of an intrinsic vulnerability within our cellular metabolism in which RBPs control RNA metabolism by coalescing into membraneless organelles that renders them prone to pathological aggregation.

The ‘RBP cascade hypothesis’ that we presented above demands testing. Its validation requires in vivo demonstration that each step in the cascade is necessary for degeneration caused by the proteins of the prior step. The first experiments testing this hypothesis have yielded promising results. Deleting *Mapt* inhibits Aβ-induced mitochondrial dysfunction and loss of axonal transport in neuronal cultures and *in vivo*[142,143], reducing the level of TIA1 inhibits tau-mediated degeneration[67,103] and reducing ATXN2 levels inhibits TDP43-mediated degeneration[169]. Various critical questions remain. For example, are SGs or other RNA granules a necessary part of the pathological cascade? Which elements of tau and RBP dysfunction are most relevant to degeneration? Most importantly, can this cascade hypothesis lead to novel therapeutics for human disease?

Glossary

40S ribosomal subunit
This is the small subunit of the eukaryotic 80S ribosome. It contains multiple ribosomal proteins that are designated by the term RPS and is a fundamental component of the pre-initiation complex, which binds mRNAs and interacts with translation initiation factors. It plays a key role in the recognition of the start AUG codon on mRNA

60S ribosomal subunit
This is the large subunit of the eukaryotic 80S ribosome. It contains ribosomal proteins that are designated by the term RPL, and attaches to translationally competent pre-initiation complexes. It contains a peptidyl transferase centre, catalyzing the addition of amino acids onto the nascent peptides during translation

Amyloid-β
(Aβ). A 4 kD peptide that is generated by cleavage of amyloid precursor protein and accumulates as neuritic plaques in Alzheimer disease

β-sheet
A highly stable protein structure that can stack to form large macromolecular fibrils

Chronic traumatic encephalopathy
A type of neurodegeneration that appears years after exposure to brain trauma, typically resulting from repetitive insults; it is characterized by the presence of neurofibrillary tangles or aggregated TAR DNA-binding protein 43 (TDP43). The pathology tends to begin at the base of neuronal sulci where the physical forces of the trauma are concentrated

Fibrils
A large macromolecular complex of proteins that stack in a regular array of repetitive oligomers. Multiple fibrils commonly coalesce to form the hallmark structures of aggregated proteins that are observed in individuals with neurodegenerative diseases

Frontotemporal dementia
(FTD). An age-related degenerative disease in which neurons of the frontal cortex degenerate, commonly producing behavioural dysinhibition followed ultimately by death. FTD most commonly occurs sporadically, but genetic forms are most frequently caused by mutations causing progranulin haploinsufficiency, mutations in *MAPT* (which encodes tau), or expansions of the G_4C_2 hexanucleotide repeat domain of *C9ORF72*

Intrinsically disordered protein regions
Regions in proteins that have a low propensity to form secondary structures, such as α-helices or β-sheets. These regions often contain hydrophobic or low-complexity domains, and have a high propensity to aggregate

Low-complexity aromatic-rich kinked segments
(LARKS). These segments exhibit a moderate affinity for like regions, and might be a chemical structure that enables liquid–liquid phase separation. The moderate affinity promotes a dynamic association that promotes the coalescence of like proteins in granules that still exhibit extensive protein movement

Low-complexity domains
(LCDs). Protein domains that contain a small number of different types of amino acids. The LCDs that characterize RNA-binding proteins tend to contain alanine, glycine, glutamine and proline residues

Membraneless organelles
Macromolecular complexes composed of a large group of proteins that are carry out specific functions and typically are observable by microscopy. Classic membraneless organelles are RNA granules and nuclear bodies that contain RNA binding proteins and RNA

Oligomers
Small complexes composed of 2 to ~10 subunits of the same protein that are tightly associated in a repetitive manner. Oligomers appear to be more toxic than fibrils, perhaps because oligomers are smaller and more mobile than fibrils

Polysomes

Polysomes (or polyribosomes) are complexes on mRNA molecules that are formed by two or more ribosomes that are synchronously producing a new protein. The polysome represents the most actively translating fraction of translational complex

Pre-initiation complexes
(PICs). PICs contain mRNA, the 40S ribosomal complex and associated translation initiation factors. Under optimal conditions, the PIC combines with the 60S ribosomal subunit to produce 80S ribosomes, which enables translation of nascent proteins. Under stress, the PIC is bound by other RNA-binding proteins to promote stress granule formation

Propagation
The process by which disease pathology spreads among neurons in a cell-dependent manner. With propagation, a pathological aggregate secreted by a cell (classically a neuron or microglial cell) or injected into the neuropil seeds a similar aggregate in an adjacent cells. These cells can then form new aggregated protein from endogenous stores of the same protein, secrete the newly aggregated protein, and seed aggregation in yet another cell. In this manner disease pathology can propagate via multiple stages of progressive seeding

RNA recognition motifs
Domains that are present in RNA-binding proteins (RBPs) that recognize a consensus sequence of RNA. The consensus sequence is typically single-stranded and about 6–8 bases long. RBPs typically have 1 to 3 RNA recognition motifs

Templating
A process in which a particular conformation of a protein acts to induce a similar conformation in like proteins with which it comes in contact

tRNA-derived stress-induced RNAs
(tiRNAs). These are small non-coding RNAs produced by the ribonuclease angiogenin in response to stress. They represent 5’- and 3’-halves of mature cytoplasmic tRNAs. They regulate multiple aspects of RNA metabolism, including protein synthesis and stress granule formation

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Box 1 |

Liquid–liquid phase separation

Liquid–liquid phase separation (LLPS) is a phenomenon that has been known to occur with RNA since the 1960s[177], but was only recently realized to play physiologically important roles in the cell. Water forms liquid droplets because of the high concentration (55 molar) of molecules that form weak intermolecular hydrogen bonds. Many RNA binding proteins (RBPs) possess low complexity domains (LCDs) that also interact weakly. The LCDs contain mostly alanine, glycine, glutamine and proline residues, with some extra complexity arising from interspersed arginine and asparagine residues that impact on binding affinities; these regions are a subset of, and may be referred to as, intrinsically disordered protein regions (IDPRs)[178,179]. These LCDs have a bias for amino acids that have propensity for structural disorder and flexibility, thus making them highly dynamic and conformationally diverse yet allowing promiscuous interactions including self-sustained aggregation.

The low complexity amino acids in the LCDs cluster together in short sequences that are tethered together by the intervening protein-sequence-forming strings that contain ‘stickers’ (clusters of low complexity) separated by spacers[180]. The spacers contain abundant glycine and glutamine residues. Glycine residues promote droplet liquidity (making them more dynamic), whereas glutamine residues promote droplet hardening (making them less dynamic)[181]. Interactions between aromatic amino acids (tyrosines and phenylalanines) and charged amino acids (arginines or lysines) determine droplet size and saturation concentration[181]. The close proximity of these stickers means that when they undergo intermolecular binding, the effective concentration of stickers corresponding to other regions on the string is high, which promotes multiple low-affinity interactions between the LCDs of homologous RBPs (multivalent homo-typical and hetero-typical interactions). The extensive intermolecular binding via LCDs enables these RBPs to form liquid droplets even though their concentration is relatively low (typically <1 μM). These droplets occur in an aqueous environment and the process has been referred to as LLPS, demixing or a phase transition (that is, RBPs phase separating in water) (reviewed in REF.[182]).

The RNA recognition motifs on RBPs lower the concentration at which RBP phase transition can occur by providing a scaffold upon which multiple RBPs can bind in close proximity[126,183]. Thus, the ability to bind RNA or DNA is a common feature of most of the proteins that phase separate, although some proteins such as keratins and synapsin phase separate in the absence of RNA[184,185].

Work from the Rosen lab first demonstrated that aqueous solutions of signalling proteins can coalesce to form loosely organized structures that resemble droplets[186]. Following this, multiple groups demonstrated that RBPs readily form droplets through LLPS in vitro[1,11,98,99]. Studies also demonstrate that RNA granules, including stress granules, form in cell culture via LLPS mechanisms[11,96]. The cell uses the phase separated RNA–RBP complexes to carry out the many functions of RNA metabolism and led to the term ‘membraneless organelle’. These functions include RNA splicing (the splicesome)[187],

ribosomal biogenesis (the nucleolus)[183], RNA degradation (P-bodies), RNA transport (transport granules) and RNA sequestration (stress granules (SGs))[30]. The dynamic environment enabled by the multi-valent weak interactions occurring in membraneless organelles facilitates many of these biological activities.

Cryo-electron microscopy indicated that the stacking of LCDs favoured high-order protein assemblies that upon reaching a high local concentration can promote phase transition (for example, from a liquid-like state to a gel-like state or an insoluble aggregate)[100]. However, the interactions of these LCDs are also sufficiently weak that the phase-separated species remain dynamic, yielding a fluid droplet rather than a static fibrillar species[100]. These LCDs are highly enriched within the SG proteome, and occur in almost all of the RBPs that play pivotal roles in SG condensation.

Box 2 |

Experimental approaches to stress granule characterization

Overexpression of stress granule (SG) nucleators (for example, RAS GTP-activating protein-binding protein 1 (G3BP1)) or a sudden efflux of untranslated preinitiation complexes (PICs) modifies the threshold when SGs assemble in cells, and can be used to induce SG formation. Under such conditions, hypothetical SG 'cores' can be biochemically purified, characterized by mass-spectrometry approaches and analyzed by super-resolution or electron microscopy[34,38]. These types of proteomic analysis show that hundreds of proteins are components of or associated with SGs, although such approaches have a limitation of not definitively distinguishing between protein interactions occurring within the SG and protein interactions occurring between proteins that are not associated with SGs. These studies also highlight the complexity of RNA-binding protein (RBP) interactions because up to ~50% of the RBP-network members do not belong to the class of RBPs because they lack classic RNA-binding motifs. These proteins include a number of scaffolding and adaptors proteins and various enzymes such as RNA and DNA helicases and nucleases; protein and lipid kinases and phosphatases; GTPases and ATPases; ribosyl-, acetyl-, methyl- and glycosyl-transferases; and ubiquitin-modification enzymes[34,38]. The multiple signalling and metabolic molecules present within SG proteomes highlight putative roles for SGs as important RNA-centric signalling hubs that integrate stress-responsive messages to and from the ribosome, P-bodies (for RNA degradation) and other signalling cascades.

Recent in vivo proteomic approaches based on proximity labelling have been used to catalogue the proteins that associate with SGs both stably (with the SG 'core') and transiently (with the SG 'shell')[34,188]. Such methods employ enzymatic systems that label interacting proteins over time (both short or longer periods) to identify complex interactions of a SG protein under stressed versus non-stress conditions[188]. These studies identified ~250 proteins that associate with SGs, and also demonstrated the existence of complex integrated SG-associated protein networks even in the absence of stress. In response to stress, pre-existing SG-associated messenger ribonucleoprotein (RNP) particles (mRNPs) quickly coalesce into the higher-order mRNPs that fuse with other mRNPs to form microscopically visible SGs. Such homotypic and heterotypic interactions between different mRNPs result in the characteristic irregular morphology of SGs. These in vivo proteomic studies show that SGs are compositionally diverse in a cell-specific and stress-specific manner, which confirms prior studies using immunofluorescence microscopy. Neurons possess a greater diversity in RNA granule composition than non-neuronal cell types, exemplified by the enrichment in the RNA transport-associated and protein quality control-associated factors[34]. Up to 20% of these identified RNA granule-associated proteins are recruited into SGs in a stress-specific manner. These studies demonstrate the diverse composition of SG subtypes, which presumably facilitates complex metabolic responses to stress. The complexity of SG biology is particularly important in the context of neurodegenerative diseases, in which

mutations in genes encoding specific SG proteins promote the development of specific pathological defects in cellular homeostasis, culminating in the neuron loss.

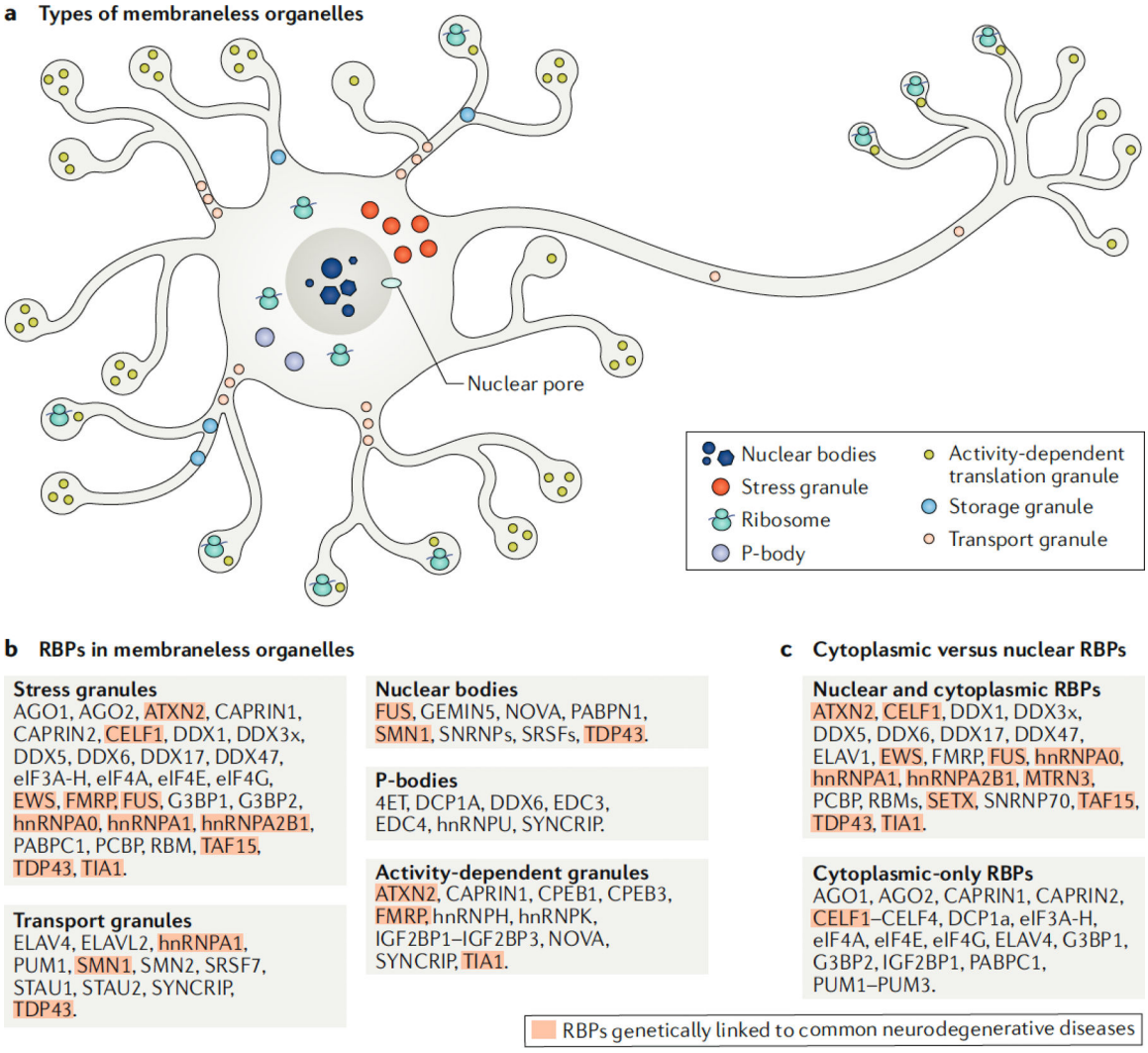


Fig. 1 |. Types of membraneless organelles present in neurons.

a | Membraneless organelles exist throughout neurons. The membraneless organelles in the nucleus are termed ‘nuclear bodies’. The nuclear pore is another membraneless organelle. In the neuronal soma, membraneless organelles comprise stress granules and P-bodies, whereas in the axon and dendrites, such organelles consist of transport granules and storage granules; note that the abundance of RNA-binding proteins (RBPs) in RNA granules is much greater in the dendrite than the axon. The synapse contains activity-dependent granules, another type of membraneless organelle, which are required for synaptic plasticity. **b** | The boxes show that RBPs are commonly associated with different types of membraneless organelles. **c** | The boxes list examples of RBPs that shuttle between the nucleus and the cytoplasm, and those that are primarily cytoplasmic. Mutations in the genes encoding the highlighted RBPs have been linked to common neurodegenerative diseases.

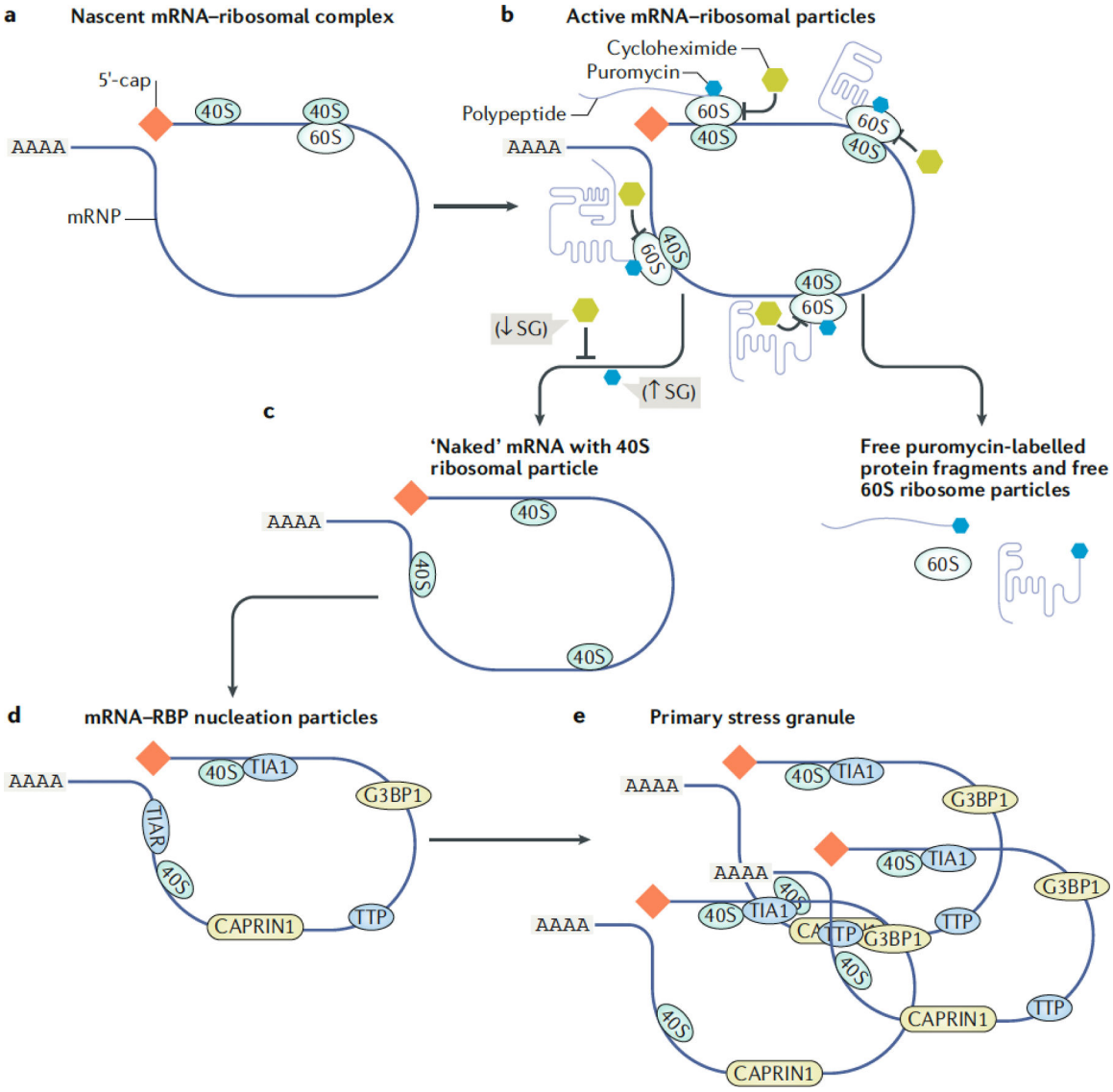


Fig. 2 |. Formation of stress granules from the mRNA–ribosomal complex.

Multiple steps are involved in the transition from a translated mRNA to a primary nucleated stress granule. **a** | Initially, the mRNA associates with the 40S ribosomal particle to form the pre-initiation complex (PIC). **b** | The PIC complex then combines with the 60S ribosome particle to form the 80S ribosome, which actively translates mRNA to make proteins. The antibiotics cycloheximide and puromycin are commonly used to study stress granule (SG) biology and exert opposite actions; cycloheximide prevents SG formation whereas puromycin promotes SG formation. **c** | Cycloheximide acts by stalling translation elongation by inhibiting ribosome translocation, which 'freezes' the mRNA covered by ribosomes and hidden from RNA-binding proteins (RBPs) in the cytoplasm, whereas puromycin breaks up the translating ribosomes by becoming incorporated into the nascent polypeptide chain, and causing separation of the 60S ribosomal subunit from the 40S–mRNA complex. **d** | The free mRNA–40S complex associates with core nucleating RNA-binding proteins (RBPs), such as T-cell intracellular antigen 1 (TIA1), RAS GTP-activating protein-binding protein 1

(G3BP1), TIA1-related protein (TIAR), tristetraprolin (TTP) and cytoplasmic activation/proliferation-associated protein 1 (CAPRIN). **e** | Stimulated by the presence of mRNAs and RBPs (and likely other factors, such as post-translational modifications), mRNA–RBP complexes begin coalescing to become primary SGs through the process of liquid–liquid phase separation. Note that polysomes and SGs exist in an equilibrium, which is regulated by the cell state (not shown).

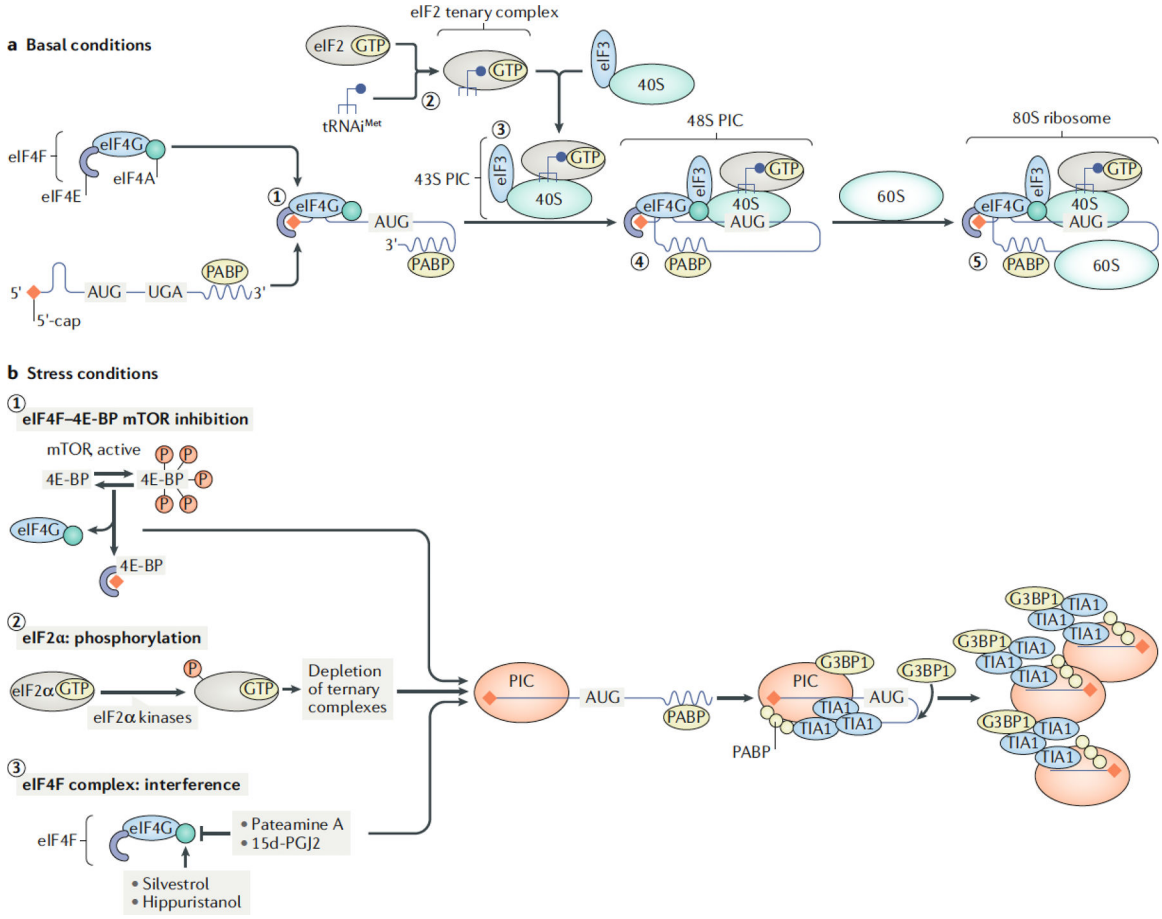
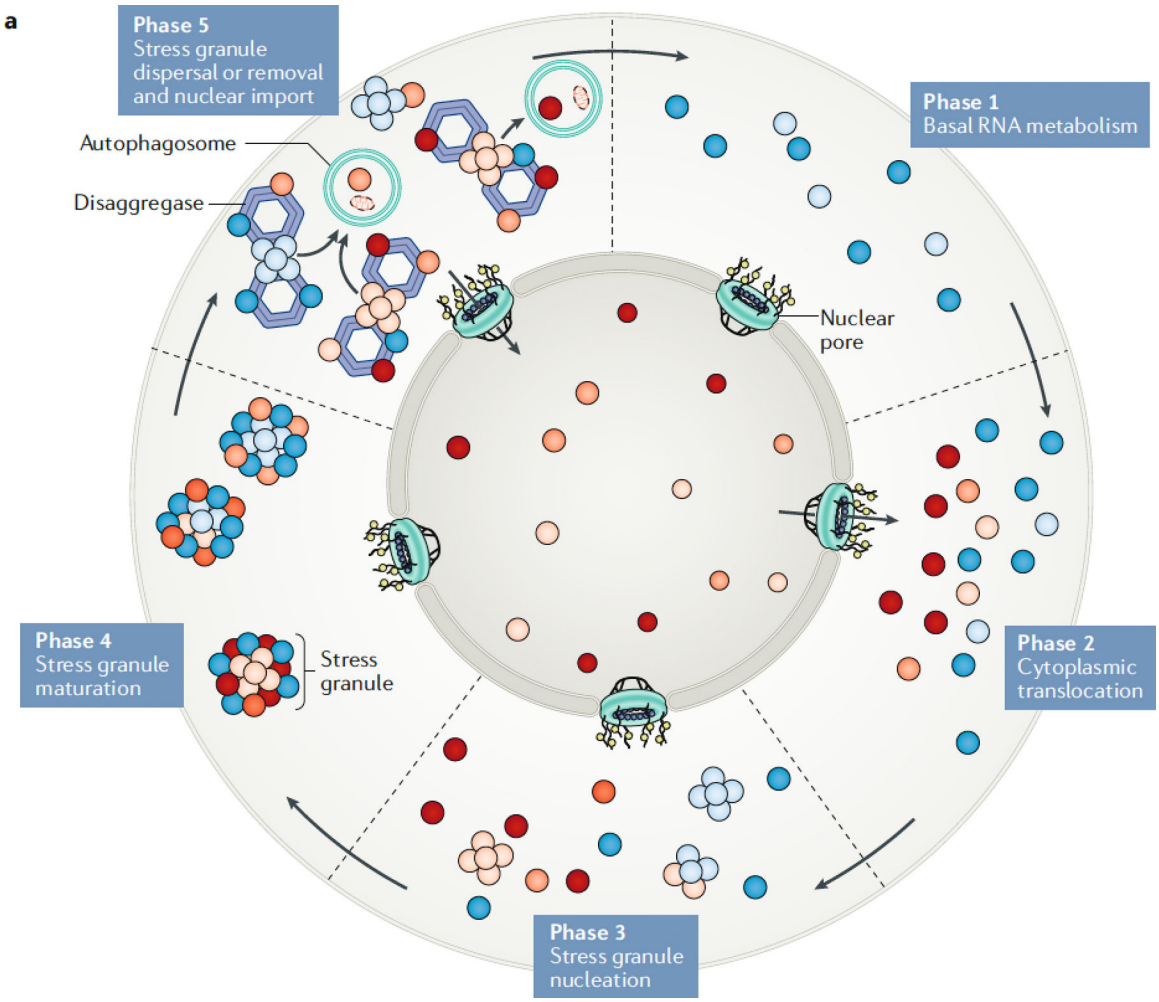


Fig. 3 |. Regulation of stress granule assembly.

a | The pre-initiation complex (PIC) has a key role in RNA translation and forms through a multi-step process. The elongation initiation factor 4F (eIF4F) complex recognizes the 5' cap structures on mRNAs (1). Meanwhile, eIF2 combines with an initiator tRNA ($tRNA_i^{Met}$) to form a ternary complex (2), which then combines with the eIF3–40S ribosome to form the 43S PIC (3). This complex associates with the eIF4F–mRNA complex to form the 48S PIC (4), which then links up with the 60S complex to initiate mRNA translation (5). **b** | Each of the three major signalling cascades regulating stress granule (SG) formation causes displacement of a key element of the PIC, allowing RNA-binding proteins (RBPs) such as T-cell intracellular antigen 1 (TIA1) to bind and nucleate SGs. Mammalian target of rapamycin (mTOR) inhibition reduces phosphorylation of eIF4E-binding protein (4E-BP), which binds eIF4E and displaces eIF4G–eIF4A from the cap structures of an mRNA (1). Phosphorylation of eIF2α prevents it from forming the ternary complex (2). Drugs (for example, pateamine A and silvestrol) interfere with eIF4F complex assembly on the mRNA cap structures by targeting the helicase eIF4A, whereas tRNA-derived stress-induced RNAs (tiRNAs) displace eIF4F complexes from mRNA (3). In each case, the incomplete pre-initiation complex (PIC) allows RBPs such as TIA1 or RAS GTP-activating protein-binding protein 1 (G3BP1) to bind to mRNA and nucleate SG formation. The nucleated SG then matures over time, as additional types of RBPs bind, each attaching to existing mRNA as

well as bringing in new mRNAs via their individual RNA recognition motifs. PABP, poly(A)-binding protein; TIAR, TIA1-related protein.



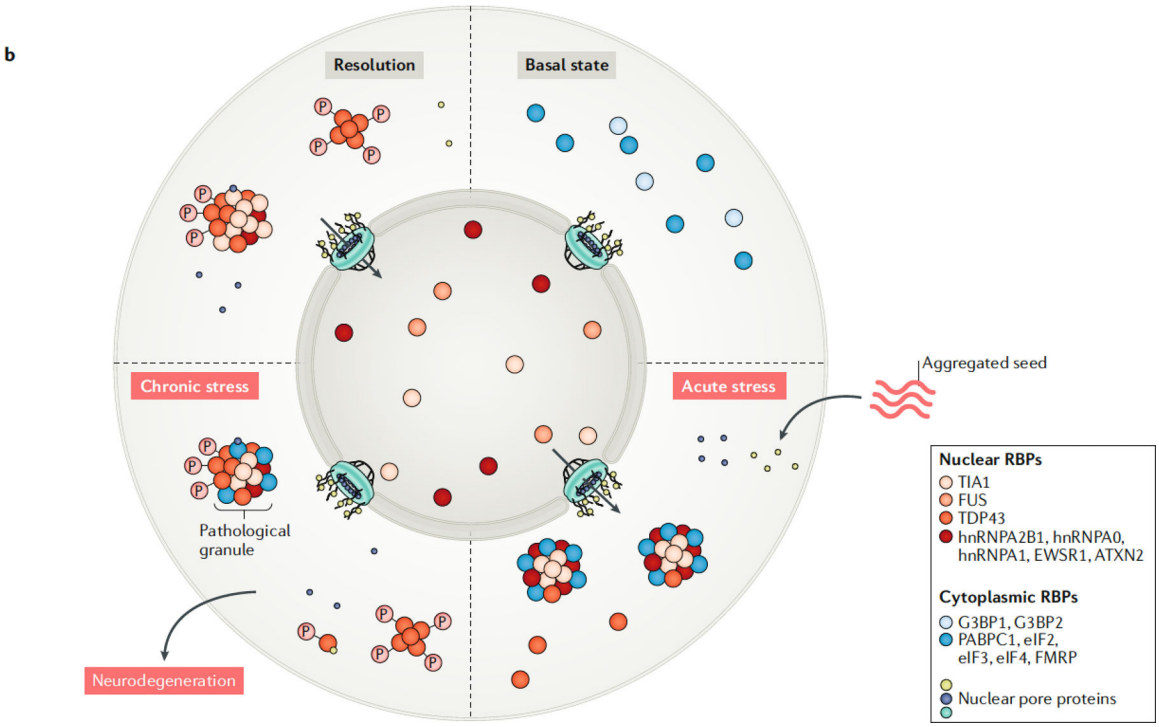


Fig. 4 |. Phases in the stress granule cycle.

a | The physiological stress granule (SG) cycle comprises several phases. Phase 1 represents basal conditions, in which nuclear RNA-binding proteins (RBPs; T-cell intracellular antigen 1 (TIA1), FUS, TAR DNA-binding protein 43 (TDP43), heterogeneous nuclear ribonucleoprotein A0 (hnRNPA0), hnRNPA1, hnRNPA2B1, EWS RNA binding protein 1 (EWSR1) and ataxin 2 (ATXN2)) perform their classical functions in the nucleus, such as participating in RNA splicing, while cytoplasmic RBPs (RAS GTP-activating protein-binding protein 1 (G3BP1) and G3BP2, polyA-bnding protein cytoplasmic 1 (PABPC1), eukaryotic initiation factor 2 (eIF2), eIF3, eIF4 and fragile X mental retardation protein (FMRP)) spread diffusely throughout the neuronal soma. In phase 2, nuclear RBPs translocate to the cytoplasm where they spread diffusely as either monomers or small complexes; neurons appear to have a strong capacity to maintain diffuse distributions of cytoplasmic RBPs without the induction of membraneless organelles. In phase 3, core nucleating RBPs (for example, TIA1, TIAR, G3BP1, and FMRP) begin to coalesce into SGs, which also include mRNA and 40S ribosomal subunits. In phase 4, the SGs mature, bringing in secondary RBPs, which include proteins such as hnRNPA0, hnRNPA1, hnRNPA2B1, EWSR1 and ATXN2. In phase 5, SG resolution begins with disaggregases (such as valosin-containing protein (VCP) and transportin), dispersing the RBPs that make up SGs. Soluble nuclear RBPs shuttle back to the nucleus, whereas RBPs that have formed insoluble amyloids are ubiquitinated and shunted to the autophagosome for disposal. **b** | The formation of pathological granules seems to differ from the cycle described above. As described above, most RBPs are in the nucleus under basal conditions. With acute stress, RBPs translocate to the cytoplasm and mostly associate with SGs. Note that in neurodegenerative diseases, seeding with extracellular fibrils can also induce cytoplasmic granules; the granules induced by seeding contain RBPs or nuclear pore proteins. With

chronic stress, the phase-separated proteins mature to become gel-like and aggregated. The aggregated TDP43 migrates to the periphery of the SGs, where it becomes phosphorylated; note that pathological tissue also exhibits aggregated TDP43 that is not associated with SGs but is also phosphorylated. Upon resolution, such as might theoretically occur with treatment, much of the pathology might disappear. Reversible components of SGs might resolve with mobile RBPs possibly returning to the nucleus. However, the most insoluble elements of the pathological granules could remain aggregated as potentially inert pathological remnants. Part b is modified with permission from Ref. 188.

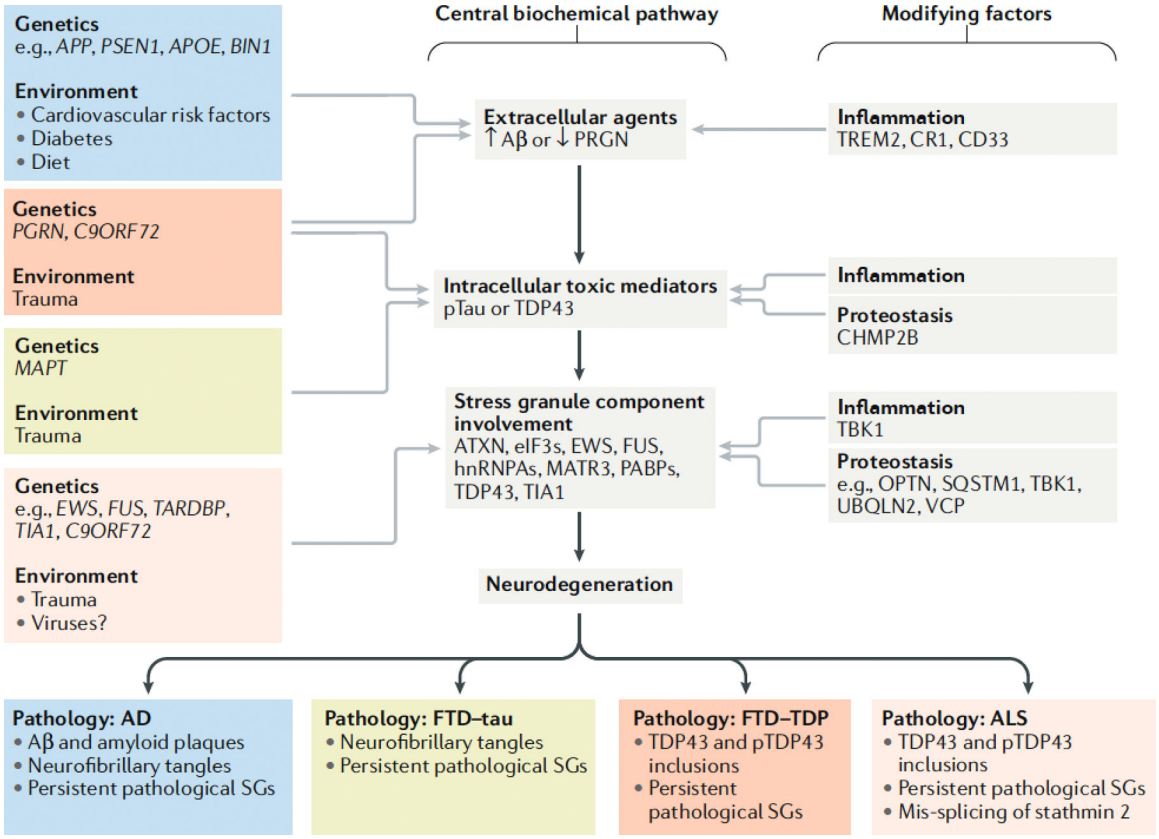


Fig. 5 |. The RBP cascade hypothesis.

The RNA binding protein (RBP) cascade hypothesis of neurodegeneration proposes that disease mechanisms feed into a central biochemical pathway that has three levels of core components. The top level of the central cascade comprises the extracellular factors that cause neuronal stress, including increased levels of oligomeric amyloid-β (Aβ) or decreased levels of progranulin (PRGN). The middle level of the central cascade contains tau (abbreviated to pTau to reflect hyperphosphorylation) and TAR DNA-binding protein 43 (TDP43), which are proteins that mediate the effects of the extracellular stresses described above. The bottom level of the central cascade comprises the RBPs that mediate the translational stress response and form stress granules (SGs). Because this entire cascade feeds forward, the pathology characterizing the top and middle levels includes the RBPs from the lower level. At the last stage, maturation of the stress response leads to involvement of many RBPs in the stress cascade. Mutations in RBP genes associated with the SG response feed directly into this level by increasing the tendency of these proteins to aggregate, producing amyotrophic lateral sclerosis (ALS; and myopathies). The chronic nature of neurodegenerative disease causes the SGs to persist. The prolonged stress response provides time for unstable proteins associated with SGs, such as tau, TDP43 and other RBPs, to evolve into highly stable amyloid conformations, which produce the classic pathological inclusions that are associated with disease (bottom row). Genetics and environmental factors feed into each stage (left). Cardiovascular factors appear to feed in at the top of the cascade, brain trauma feeds in at the middle and lower levels, and viruses might feed into the lower level because they modulate protein synthesis by co-opting the

biology of RBPs. Processes impacting after the pathological aggregates form are shown on the right column as modifying factors. These factors include mutations in genes encoding proteins that regulate proteostasis and the removal of pathological aggregates; these proteins generally function as part of the autolysosomal system. Inflammatory reactions to pathological aggregates and cellular damage play an important role at every level of the cascade. The coloured boxes at the bottom of the cascade depict the types of neuropathology resulting from the accumulation of pathological proteins and resulting neurodegeneration. The colour of each disease box (bottom row) is coordinated to reflect the respective genetic (left column), environmental (left column) and biochemical factors (central column) that ultimately leads to each particular disease. AD, Alzheimer disease; FTD-tau, frontotemporal dementia with tau pathology; FTD-TDP, frontotemporal dementia with TDP43 pathology.