HHS Public Access

Author manuscript

J Mol Biol. Author manuscript; available in PMC 2019 November 02.

Published in final edited form as:

J Mol Biol. 2018 November 02; 430(23): 4685–4701. doi:10.1016/j.jmb.2018.05.003.

The role of RNA in biological phase separations

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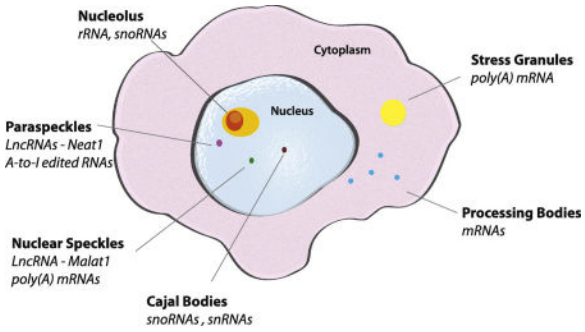
Abstract

Phase transitions that alter the physical state of ribonucleoprotein particles contribute to the spacial and temporal organization of the densely packed intracellular environment. This allows cells to organize biologically coupled processes as well as respond to environmental stimuli. RNA plays a key role in phase separation events that modulate various aspects of RNA metabolism. Here, we review the role that RNA plays in ribonucleoprotein phase separations.

Graphics Abstract



Organization of the densely packed intracellular environment requires compartmentalization. This is particularly important for gene expression as coordinated processes must occur in an ordered fashion. In eukaryotic cells, double stranded DNA (dsDNA) is sequestered in the nucleus and packaged in histones. Within the nucleus, DNA is organized into heterochromatin and euchromatin to control the relative access to the transcriptional machinery. Transcribed mRNA undergoes splicing, polyadenylation, and capping prior to export to the cytoplasm. Each of these processes is under spatiotemporal control that ensures correct processing and localization.

Just as membrane-enclosed organelles (e.g., nuclei, mitochondria, endoplasmic reticulum, golgi apparatus) serve to organize biological processes into discrete cellular domains, non-

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membrane enclosed domains play a similar role in the organization of biological activities throughout the cell. These are defined by the physical nature of their constituents and are referred to as membrane-less organelles (MLO) due to their ability to concentrate factors associated with a biological process, typically involving RNA metabolism (Figure 1). MLOs serve to concentrate their components in a way that facilitates processes such as transcription and ribosomal RNA (rRNA) processing in the nucleolus, or sequestration such as translation initiation complexes and signaling molecules in stress granules (SGs). MLOs can be visualized using phase contrast, immunofluorescence, or brightfield microscopy. The lack of a limiting membrane allows MLO constituents to rapidly exchange with the surrounding environment, allowing a dynamic response to changes in the cell state.

MLOs are assembled via a process of phase separation. During phase separation, a critical concentration of a given component (e.g., protein, RNA, ribonucleoprotein complex) allows a transition to a concentrated phase which is physically separated from a more dilute phase. This process is driven by multiple weak electrostatic, charge-charge and repetitive domain interactions that “demix” a homogenous state into two discrete phases. This process can produce a discrete liquid state within a liquid known as a liquid-liquid phase separation (LLPS), or a transition of a portion of the liquid into solid state causing a solid within a liquid. Molecules within a phase separated liquid domain which typically assumes a spherical shape, can continuously exchange with the surrounding solution while maintaining the phase separation. This is different than a transition to a solid state whose shape is internally defined by the patterning of its components which rarely exchange with the surrounding solution[1; 2].

A major advance in our understanding of biological phase transitions came with the serendipitous discovery that biotinylated isoxazole (b-isox) forms a precipitate that selectively traps proteins associated with MLOs[3; 4]. These proteins possess low complexity domains (LCD) that are enriched in amino acids Ala, Arg, Gly, Gln, Ser, Pro, Glu, and Lys. A subset of LCDs are related to prion proteins that can adopt soluble and aggregation-prone conformations to modulate biological processes. In their native state, LCDs are intrinsically disordered, but they can assume a defined tertiary structure in response to post-transcriptional modifications or interactions with partner proteins or RNAs[5]. B-isox assumes a crystalline state comprised of a lattice-like structure that selectively templates LCD-containing proteins to assume a β-strand conformation[4]. B-isox condenses mRNAs in a 3’UTR length-dependent manner[3; 4], probably reflecting the fact that longer mRNAs tend to have more binding sites for specific and non-specific RNA binding proteins. B-isox can condense mRNAs in a sequence selective manner: B-isox condenses RNAs bearing MS2 stem loops in the presence of a fusion protein composed of the MS2 stem loop-binding protein linked to a LCD that is known to phase separate in isolation[3].

Purified LCDs can form *in vitro* droplets often using low salt concentrations or in the presence of molecular crowding agents such as Ficoll or polyethylene glycol. These droplets are formed via LLPS as they: (1) deform under shear force, (2) exchange with the surrounding environment, (3) have higher component concentrations than the surrounding environment, (4) fuse with other droplets, (5) are spherical, and (6) are temperature dependent[2; 6]. Importantly, not all LCDs have the same biophysical properties. Under

conditions in which several LCDs promote LLPS, the TIA1-LCD (the prion-related domain of a ribosome recognition motif RNA-binding protein) does not undergo phase separation. In contrast, when the TIA1-LCD is expressed as a chimeric fusion protein with polypyrimidine tract-binding protein (PTB), PTB-TIA1-LCD undergoes a phase separation[7]. While RNA can coax PTB to phase separate in the absence of a LCD[8], PTB-TIA1-LCD has a lower concentration threshold for phase separation[7]. Similarly, RNA can promote the phase separation of isolated RRM and LCD domains of hnRNPA1, but the concentration required is significantly higher than that of the full length hnRNPA1 protein[9]. Finally, RNA promotes FUS (a well-characterized, sequence non-specific, RNA binding protein associated with amyotrophic lateral sclerosis (ALS)) -mediated phase separation in a non-sequence specific manner[10; 11]. RNA can undergo a phase separation event in isolation[12; 13]. Disrupting RNA:RNA interactions inhibits this process[13]. Furthermore, there is a significant overlap between RNAs that undergo phase separation *in vitro* and RNAs that interact with SG components *in vivo*[12]. These findings suggest that RNA can promote phase separation events via RNA:protein and RNA:RNA interactions.

RNA lowers the concentration threshold for the phase separation of purified PGL-3 into P-granule-like droplets. mRNA is more effective at promoting phase separaton than *in vitro* transcribed rRNA. Yet, when heated this rRNA is effective at promoting PGL-3 condensation[14], suggesting a role for complex RNA structures in granule formation. Neither the nature of these structures, nor the effect of RNA modifications (e.g., poly(A) tails, m^7GTP caps, modified nucleotides) on phase separation events is known. Liquid droplets formed by the poly Q domain-containing RNA binding protein Whi3 have different biophysical properties depending on the associated mRNA[15]. Droplets formed in the presence of a formin transcript exhibit faster fusion and reduced viscosity than droplets formed in the presence of a cyclin transcript. Both mRNAs contain the same number of Whi3 binding sites yet the formin transcript is four times longer[15]. More work is needed to understand how specific mRNAs differ in their ability to stimulate phase separation events.

Aging of phase separated MLOs can reduce their dynamic properties and produce a solid-like state. Solid-like phase separations do not exchange with the surrounding solution and often (but not always) resemble amyloid-like structures. This is the case for FUS which undergoes an LLPS *in vitro* which ages into a fibrous, b-isox-like solid. This transition occurs more rapidly when recombinant FUS contains the ALS-associated mutations G156E or R244C[16]. Similarly, the ALS-associated mutant hnRNPA1-D262V expedites the transition to a fibrillar state[9]. The kinetics of liquid to solid transition for several LLPS chimeric proteins is increased by addition of RNA[7], suggesting that RNA can drive the liquid to solid transition. In the case of TDP43, ALS-associated mutations enhance the assembly of amyloid-like β-sheet structures, a transition that is further enhanced in the presence of nucleic acids[17]. While it is clear that dynamic properties of droplets assembled *in vitro* are altered by age, mutations in component proteins, and specific RNAs, the relevance of these findings to MLOs *in vivo* remains to be determined.

Like RNA, DNA can modulate the propensity of LCDs to undergo phase separations. In the presence of dsDNA, a chimeric fusion protein between the FUS-LCD and the DNA-binding domain of the ETS transcription factor FLI assembles fibrillar structures that can be

visualized using electron microscopy[18]. In contrast, *in vitro* droplets formed by the LCD of the RNA helicase DDX4 recruit ssDNA, but not dsDNA[19], suggesting that individual unpaired bases may contribute to this phenomenon.

In vitro studies using proteins or protein domains in isolation have been used to make key observations about the biophysical properties of RNA granules. Yet, how this translates into the cellular context remains to be determined. Purified LCDs that phase separate *in vitro*, do not always drive granule assembly in cells[20]. Within the complex cytoplasmic milieu, factors such as protein and RNA interaction, subcellular localization, and post-translational modification can modulate phase separation events. Future studies are required to determine the significance of *in vitro* phase separation events and their link to specific biological functions.

Repeat expansion disorders and RNA repeat foci

While RNA repeats such as CAG, CUG, CCUG, and GGGGCC are found in the normal population, expansion of these repetitive nucleotides is associated with a subset of neurological diseases known as repeat expansion disorders. Nucleotide repeat expansions can be found in 5’ and 3’ untranslated regions, introns, and coding regions. In some cases, these repeats are translated into repetitive polypeptides (e.g., CAG repeats are translated into poly-glutamine in Huntingtin protein) (reviewed in[21; 22; 23]). Repeat expansion disorders are often associated with the appearance of RNA nuclear foci. RNA repeat expansions can recruit and sequester RNA binding proteins and directly contribute to disease pathogenesis.

In these conditions, a repeat threshold is typically associated with the assembly of RNA foci and disease pathogenesis. RNA repeats act as a template to recruit and sequester specific RNA-binding proteins. The resulting nucleoprotein complex can promote a phase separation event to produce pathological foci. CUG repeat expansions in the 3’UTR of the DM1 (also known as DMPK) mRNA[24; 25; 26] and CCUG intronic repeat expansions in ZNF9 (also known as CNBP) cause myotonic dystrophy (DM) type 1 and 2, respectively[27]. Although these are different repeat expansions that affect different genes, both diseases display discrete nuclear RNA foci that disrupt RNA metabolism. This RNA gain-of-function mechanism causes the mislocalization of proteins including the splicing factor muscle-bind 1 (MBLN1)[28]. Sequestration of MBLN1 at RNA foci disrupts alternative RNA splicing in ways that contribute to DM1 and DM2 pathogenesis (reviewed in[21; 23]). MBLN1 knockout mice display a DM-like phenotype[29] as does a mouse model that introduces 250 CUG repeats into a gene unrelated to DM1[30], implicating MBLN1 sequestration at CUG RNA foci in disease pathogenesis.

The intronic hexanucleotide GGGGCC repeat in the gene C9ORF72 is the most common cause of both inherited and sporadic ALS and frontotemporal dementia (FTD)[31; 32]. This RNA can form secondary structures including a G-quadruplex[33; 34; 35; 36]. A G-quadruplex is composed of stacked G-quartets which assemble when four guanosine residues hydrogen bond via Hoogsteen base pairing in a planar fashion. These planar structures are coordinated by specific monovalent cations and stack to form a G-quadruplex[37]. Uniquely, GGGGCC RNA can form RNA granules *in vitro* in the presence of cellular lysate in a manner similar

to b-isox[38]. The G-quadruplex structure is required for GGGGCC RNA condensation from lysates[38]. This templated pattern drives phase separation *in vitro* and promotes the assembly of nuclear RNA foci and cytoplasmic SGs in cells[38; 39]. In C9-ALS/FTD patient derived cells, GGGGCC RNA nuclear foci are recognized by a G-quadruplex specific antibody[39], suggesting that the G-quadruplex structure is preserved within RNA foci. Similarly, G-quadruplex structures encoded within 3'UTRs of selected mRNAs are enriched in neuronal granules which are targeted to, and translated in, neurites[40]. It has been estimated that a single C9-GGGGCC RNA transcript is sufficient to assemble a nuclear focus[41], suggesting that intramolecular G-quadruplexes are involved in this process. Like G-quadruplexes with GGGGCC repeats, CUG/CCUG secondary structures are also thought to play a role in phase separation[13; 23; 42].

Like LCD-containing proteins, GGGGCC, CAG and CUG repeat RNA can phase separate *in vitro*[13]. This occurs in a length dependent manner and is presumably due to base pair interactions and alternative structures including hairpins and G-quadruplex-like structures[13]. Repeat RNA-induced phase separations exhibit both liquid- and solid-like properties: they assume a spherical geometry, but rarely fuse with one another and do not exchange their constituent RNAs with the surrounding solution[13]. This is in contrast to RNA foci in cells: fluorescence recovery after photobleaching (FRAP) of MS2 tagged RNA reveals dynamic movement in and out of granules[13].

Paraspeckles

While repeat expansion disorders provide examples of disease-associated RNAs driving phase separation events, the formation of paraspeckles is facilitated by the non-pathogenetic (normal) long non-coding RNA (lncRNA) NEAT1. Paraspeckles are non-essential but are proposed to have a role increasing microRNA production by allowing a platform for miRNA processing, and sequestering paraspeckle associated proteins (such as SFPQ) to modulate gene expression in different cellular contexts such as circadian cycling[43].

Paraspeckles form in the interchromatin space that surrounds the lncRNA NEAT1 locus on chromosome 11. RNA polymerase (pol) II transcribes NEAT1.1 (3.7 kb) and NEAT1.2 (23 kb) from the same promoter. NEAT1.1 is poly-adenylated whereas NEAT1.2 encodes a tRNA-like structure at its 3' end. This structure is cleaved by RNase P[44] and the resulting 3' end of NEAT1.2 forms a triplex structure that promotes nuclear retention[45]. Whereas paraspeckles are found in most cultured cell lines, they are only found in a subset of cells in mouse tissue[46]. Mice lacking NEAT1 are viable and fertile[46] but show defects in mammary gland development and lactation[47].

NEAT1.2 acts as a structural scaffold to promote paraspeckle formation[44; 48; 49; 50]. Whereas overexpression of NEAT1.1 enhances paraspeckle formation, it is unable to scaffold their formation on its own[46]. FRAP analysis reveals that MS2 tagged NEAT1 is relatively immobile compared with paraspeckle proteins that are in dynamic equilibrium with the nuclear matrix[51]. The 5' and 3' ends of NEAT1.2 and NEAT1.1 reside at the periphery of the paraspeckle surrounding a core composed of the central region of NEAT1.2 and the paraspeckle proteins NONO, FUS, and SFPQ (Figure 2)[52; 53]. The paraspeckle proteins

NONO and SFPQ possess polymerization domains consisting of coiled-coil regions that come together to form long fibrils that are thought to coat NEAT1.2[54; 55]. Surrounding the paraspeckle core is a shell comprised of NEAT1.1, RNAs (including mRNAs and introns) enriched in GA repeats, and TDP43, an ALS-associated protein known to have roles in other phase separations[53]. The core-shell structure is further held together by patches of prion-like domains (PLDs) found in the essential paraspeckle proteins FUS and RBM14[56]. The paraspeckle phase separation is so stable that its disruption requires Trizol extraction coupled with heat or extensive needle shearing[57]. The PLD of FUS is required for this extremely stable interaction as its deletion prevents paraspeckle assembly and eases extraction of NEAT1.2[57; 58]. It is unknown whether specific sequences of NEAT1.2 or NEAT1.1 are required to promote phase separation during paraspeckle formation. Interestingly, TDP43 is recruited to paraspeckles, and binds specifically to $(GU)_x$[59]. Several $(GU)_{\geq 10}$ stretches are found in NEAT1.2, but their role in recruiting TDP43 is not known.

Paraspeckle assembly is linked to the transcription of NEAT1.2 and pol II inhibitors disrupt paraspeckle formation[51]. Interestingly, the C-terminal domain (CTD) of Pol II contains 52 heptad repeats of the low complexity sequence YSPTSPS and this domain recruits other LCD containing proteins such as FUS[18]. It possible that the CTD of Pol II attracts the LCDs of paraspeckle proteins to seed a phase separation during NEAT1.2 transcription.

In addition to NEAT1 lncRNA, adenosine to inosine (A-to-I) edited RNAs are concentrated at paraspeckles[48; 60]. The main paraspeckle protein NONO (p54/nrb) has high binding affinity for A-to-I edited RNA[61]. A-to-I editing typically occurs on double stranded nuclear RNAs, most commonly associated with inverted Alu elements[62; 63]. Addition of Alu elements to the 3'UTR of a GFP reporter transcript causes NONO binding and nuclear retention[62]. Another target of A-to-I editing is a cationic amino acid transporter 2 (mCAT2) encoding transcript. The open reading frame encoding mCAT2 is included in two distinct transcripts that use different promoters and polyadenylation signals. The CAT2-transcribed nuclear RNA (CTN) uniquely undergoes A-to-I editing in its 3'UTR which results in nuclear retention. In response to stress, the 3'UTR of CTN transcripts is cleaved, allowing escape to the cytoplasm and translation of CAT2 protein[60]. The A-to-I edited CTN-RNA localizes to paraspeckle regions[60] in a A-to-I independent manner[64]. In the absence of NEAT1 and paraspeckles, CTN-RNA interacts with paraspeckle proteins and accumulates in perinucleolar regions[64]. It is unclear whether paraspeckles contribute to nuclear retention of A-to-I edited RNAs or whether A-to-I edited RNAs modulate paraspeckle dynamics.

Interestingly, transfection of oligos with a phosphorothioate backbone can assemble paraspeckle-like structures that lack NEAT1[65]. Phosphorothioate bonds include a non-bonding sulfur instead of an oxygen in the sugar linkages and are used to promote stability from nuclease degradation in synthetic oligos[66]. It is possible that increased stability allows for promiscuous binding to paraspeckle proteins. Transfection of these oligos also promotes cytoplasmic foci of unknown composition[65].

Nuclear speckles

Interphase nuclei typically contain 20–50 dynamic phase dense foci known as nuclear speckles. Ultrastructural analysis indicates that nuclear speckles are composed of dense clusters that are connected by fibrils[67]. Nuclear speckles are proposed to have roles in promoting mRNA maturation and are localized with sites of active RNA pol II transcription. mRNA production and maturation, including transcription, splicing, polyadenylation, and mRNA export, are often coupled and the proteins involved in these processes localize to nuclear speckles[68]. The lncRNA Malat1 (also known as NEAT2) is concentrated at nuclear speckles but is not required for their assembly. The findings that splicing factors are concentrated at nuclear speckles and knockdown of the mRNA export complex TREX increases their size suggests possible roles in mRNA splicing and/or nuclear export[68]. Downregulation of Malat1 causes an increase in cytoplasmically localized poly(A) RNA[69] suggesting that Malat1 may act at nuclear speckles to modulate splicing and/or nuclear retention of mRNA.

Amyloid (A) bodies

Recently described stress-induced nuclear foci known as amyloid (A) bodies assemble in response to the transcription of stress specific lncRNAs. These lncRNAs are derived from the ribosomal intergenic spacer (rIGS) region of the ribosomal DNA locus and are transcribed under specific stress conditions[70]. The acidic and hypoxic conditions found in the tumor microenvironment induce the assembly of A bodies. In a mouse model of tumorigenesis, knockdown of one of these lncRNAs prevents tumor growth and promotes a cellular state of dormancy[70]. A-bodies are named for their amyloid-like state. Amyloids are proteins that form tight interactions through β-sheets to generate fibrils that experimentally stain with amyloid specific stains such as CongoRed. Because of the fibril state and the lack of exchange with the surrounding environment, amyloids represent a solid-like state[71]. Whereas the assembly of most amyloids is irreversible, A-bodies disassemble in cells that recover from stress, suggesting they play a dynamic role in modulating the stress response program and cell survival.

Cajal bodies

In 1900, Ramon y Cajal first identified nuclear coiled bodies that were later renamed Cajal bodies after their discoverer[72]. Cajal bodies are sites of small nuclear ribonucleoprotein (snRNP) biogenesis, including the spliceosome and the telomerase RNP complex[73]. Cajal bodies are enriched in small nuclear RNAs (snRNAs), which includes small nucleolar RNAs (snoRNAs) and small Cajal body-specific RNAs (scaRNAs). snoRNAs and snaRNAs assemble into complexes with proteins to form snoRNPs and scaRNPs, respectively. These complexes mediate 2’-O-ribose methylation and pseudouridylation of nucleotides in rRNA and spliceosomal RNAs and are required for functional complexes. snoRNAs traffic through Cajal bodies enroute to the nucleolus and in some cell types, Cajal bodies are found in association with nucleoli. Like other nuclear MLOs, Cajal bodies are tied to transcriptional activity[74]. During the cell cycle, transcriptional arrest is accompanied by the disappearance

of Cajal bodies[75]. Cajal bodies cluster around sites of snRNA transcription and intron-encoded snRNAs are then trafficked to Cajal bodies[76].

Although Cajal bodies are not observed in all cells, both snoRNPs and snRNPs are required for assembly of ribosomes and spliceosomes, respectively. An essential step in the assembly of functional spliceosomal snRNPs requires the protein coilin. Coilin is also required for assembly of Cajal bodies, and loss of coilin results in defects in splicing, and snRNP assembly[77]. Coilin is not a component of snRNP complexes, but is thought to play a role in concentrating the proteins and RNAs required for their assembly, acting as an aggregating factor using its multimodular domains[77]. iCLIP, a method of using UV crosslinking and immunoprecipitation to identify direct protein-RNA interactions, shows that coilin interacts with hundreds of snRNAs, including those targeted to the nucleolus[76], indicating coilin directly interacts with snRNA. Potentially, this direct interaction between coilin and snRNAs aids in the assembly of Cajal bodies. scaRNAs are sufficient to promote *de novo* Cajal body assembly, as an MS2-tagged scaRNA causes Cajal body formation[78]. This occurs in a manner similar to artificially tethering Cajal body proteins (coilin, SMN, etc) to DNA via a Lac operon to assess *de novo* Cajal body assembly[78].

Unlike other MLOs, the primary sequence motifs that cause retention of scaRNAs in Cajal bodies have been identified and characterized. The protein WRD79 recognizes scaRNAs promoting their localization and retention in Cajal bodies. Box H/ACA scaRNAs require the CAB box (ugAG found in the loop at the 5’ or 3’ end of scaRNA) for Cajal body localization[79]. Addition of this motif to snoRNAs results in retention in Cajal bodies[79] and mutation of this motif prevents Cajal body localization[76; 79]. Targeting of box C/D scaRNAs requires G:U wobble base pairing in a helical region of a hairpin yet is not dependent of the loop[80]. Potentially WRD79 recognizes this atypical helix caused by G:U wobble base pairs.

Nucleolus

Due to their easy detection by light microscopy, the size and number of nucleoli has been used as a cancer diagnostic for over 100 years. Only recently have we begun to appreciate the unique LLPS properties of nucleoli. Using *Xenopus laevis* oocyte germline vesicles which are similar in composition to somatic nucleoli, Brangwynne and colleagues were the first to demonstrate the dynamic and liquid-like properties of nucleoli[81].

The nucleolus is located around clusters of ribosomal DNA (rDNA) repeats known as nucleolar organization regions. These genomic regions contain clusters of repeats that code for ribosomal RNA (rRNA). The nucleolus is further organized into multiple subregions that are defined by proteins involved in rRNA processing and ribosome assembly; rDNA is transcribed in the fibrillar center, rRNA processing (cleavage and modification) to produce 28S, 5.8S, and 18S rRNAs occurs in the dense fibrillar component and assembly into a pre-ribosome occurs in the granular component (Figure 3). Further processing takes place in the cytoplasm to generate fully competent 40S and 60S ribosomal subunits.

The multiple domains of the nucleolus couple processing events within distinct LLPS compartments that maintain boundaries for spatial and temporal processing of rRNA. As

such, rRNA maturation and ribosome production take place within discrete phase separated domains. Just as concentration of the hammerhead ribozyme in an aqueous two phase system generated by the molecular crowding agents polyethylene glycol and dextran enhances activity by 70 fold[82], the super concentration of enzymes and substrates within nucleoli is likely essential for efficient rRNA processing and ribosome subunit assembly. It would be interesting to use a similar system to assess rRNA processing, yet surprisingly, the detailed events of rRNA processing remain unclear in mammalian cells[83].

The existence of nucleolar subregions raises the question of how multiple distinct phase separated domains can coexist. *In vitro* studies using purified nucleophosmin (NPM1) and fibrillarin (FIB1), proteins that localize to dense fibrillary and granular components, respectively, are instructive in this regard. In the presence of rRNA, purified NPM1 and FIB1 can independently assemble droplets *in vitro*. These droplets possess distinct biophysical properties: when mixed together, they do not fuse with one another but rather form two-layered droplets with the FIB1/rRNA phase inside an engulfing NPM1/rRNA phase, a restriction conferred by differential surface tensions associated with their respective RNA-binding domains[84].

Over 4500 proteins have been identified as components of the nucleolus[85]. Scott et al analyzed the sequence and structural features of a large number of well curated nucleolar localization signals and, based on this, developed a bioinformatics predictor of nucleolar localization signals. Their analysis showed that these signals are enriched in basic residues that are located within solvent-accessible regions of proteins[86]. Mitrea and colleagues refined this view showing that the majority of nucleolar proteins exhibit multiple segments containing two or more closely spaced arginine residues within regions predicted to be intrinsically disordered[87]. They showed that proteins with multivalent arginine-motifs interact with the granular component, NPM1, which exhibits two highly acidic regions that interact with arginine-motifs in ribosomal and other nucleolar proteins. NPM1 also contains an RNA binding domain that is required for the nucleolar localization. Nucleolar components show specificity toward rRNA binding but how this is achieved remains unclear and is difficult to test in cells due to the necessity of rDNA/rRNA to the integrity of the cell and the large number of proteins associated with the nucleolus.

Stress granules (SGs)

Stress can trigger the assembly of several MLOs. Indeed, studies defining the principles of SG assembly are now accepted as those that define the major properties of MLOs: a discrete domain[88] that is assembled by low affinity interactions between low complexity or prion related domains[89] that are visible by light microscopy[88], lack a limiting membrane[89], show molecular constituents dynamically exchanged with the surrounding environment[90]. The subsequent realization that these properties are shared by several cellular entities revealed the importance of these properties for the organization of cellular processes.

SGs are cytoplasmic assemblies containing translation initiation factors, polyadenylated (poly(A)) mRNAs, 40S ribosomal subunits, RNA binding proteins, and selective signaling molecules (reviewed in[91]). SGs assemble in response to blocked translation initiation which

results in a sudden increase in untranslated, non-polysomal mRNAs. This can result from stress-induced eIF2α phosphorylation or disruption of the eIF4F complex[88]. eIF2α phosphorylation depletes the initiator tRNA-methionine eIF2α-GTP complex, limiting translation initiation and causing downregulation of bulk translation[9293; 94; 95; 96]. The eIF4F complex (composed of eIF4E, eIF4G and eIF4A) binds the m^7GTP mRNA cap and under optimal growth conditions is the rate limiting step in translation initiation for most mRNAs (reviewed in[92]). Interestingly, not every stimulus that disrupts the eIF4F complex or causes eIF2α phosphorylation triggers SG formation: doxorubicin promotes robust eIF2α phosphorylation but not SG assembly[97] and 4EGI-1 disrupts eIF4E:eIF4G interaction but does not induce robust SG assembly[98], suggesting that additional changes are necessary to trigger the condensation step in SG assembly.

SGs are in dynamic equilibrium with active translation. SGs can be forcibly disassembled by adding emetine or cycloheximide, drugs that “freeze” ribosomes on polysomes. Conversely, SG assembly is enhanced by puromycin, a drug that disassembles polysomes[90]. It is important to note that puromycin requires a sub-SG inducing dose of stress to assemble SGs[99]. This implies that SGs are assembled when actively translating ribosomes “run off” the mRNA to release an excess of free mRNA[100]. Consistent with this, SGs are enriched in poly(A) mRNA and 40S, but not 60S, ribosomal subunits[100; 101; 102]. Thus, stalled 48S initiation complexes are core components of SGs. Interestingly, transfection of excess mRNA is sufficient to induce SGs but only in a subpopulation of cells[102]. Whether this requires the assembly of pre-initiation complexes on transfected mRNAs remains to be determined. Similarly, the G-quadruplex structure of the C9ORF72 ALS/FTD-associated GGGGCC repeat RNA or an intermolecular G-quadruplex formed from the 5’ fragments of cleaved $tRNAs^{Ala/Cys}$ promote SG formation in a structure-dependent manner[103; 104]. These findings reveal a key role for RNA in SG assembly.

SGs are thought to sequester mRNAs during stress to preserve the transcriptome, allowing resumption of translation as cells repair stress-induced damage and recover. This should minimize energy expenditure in cells intermittently exposed to stress. This process also allows cells to partition mRNAs based on the current translational needs of the cell: mRNAs encoding proteins that repair stress-induced damage are excluded from SGs and translated, whereas “housekeeping” transcripts are translationally stalled and sequestered at SGs. Consistent with this notion, mRNAs encoding the heat shock chaperones Hsp70[105] and Hsp 90[106] are excluded from SGs, while abundant housekeeping mRNAs encoding c-myc and β-actin are concentrated at SGs (Table 1)[106]. Localization of specific mRNAs to SGs has been experimentally visualized using fluorescence in situ hybridization (FISH), tracking RNA via programmable RNA-targeting Cas9, and tagging RNA with the MS2 system[105; 106; 107; 108; 109; 110; 111; 112].

While translation initiation arrest and polysome disassembly are required for SG formation, the aggregation of untranslating mRNAs at SGs requires the related proteins G3BP1 and 2. Knocking out one of these proteins decreases SG assembly, but elimination of both G3BP1/2 completely blocks SG assembly in response to most stresses[113; 114]. Caprin1 and USP10 compete for G3BP1 binding to seed SG condensation - G3BP1:USP10 prevents while G3BP1:Caprin1 promotes this phase separation event[114]. Specifically, an FGDF motif in

USP10 binds G3BP1 and this same motif is found in viral proteins that inhibit SG assembly to evade the cellular stress response[114; 115]. Furthermore, G3BP1/2 bind the 40S but not the 60S ribosomal subunit and this requires the G3BP1-RGG domain in an RNA dependent manner. This domain is also required for SG assembly[114; 116], suggesting that RNA further contributes to SG condensation yet it is unclear how. This order of events is consistent with a two-step model requiring: (1) an excess of free cytoplasmic mRNA and (2) condensation by G3BP1/2 (Figure 4). G3BP1 has been implicated in the rearrangement of ALS-associated mRNP aggregates composed of mRNA and FUS or TDP43 as judged by atomic force microscopy[102; 117], implying an ability to rearrange or disaggregate mRNPs. It remains to be determined whether this *in vitro* observation relates to SG formation in cells and furthermore whether the ability to rearrange mRNPs promotes or prevents SG formation.

G3BP1 has been reported to be post-translationally modified by methylation[118], phosphorylation[119], poly(ADP) ribosylation[120], acetylation[121], and ubiquitination[122]. PRMT1 and PRMT5 methylate multiple arginine residues in the RGG domain of G3BP1 and mutation of key Arg residues (429, 435, 443, 447, 460) to Lys prevents methylation and promotes SG formation. In contrast, Arg to Phe mutants serve as methylation mimics that prevent SG formation[123]. Consistently, inhibition of asymmetric arginine methylation, a modification most likely added by PRMT1, promotes SG formation. PRMT1, but not PRMT5, is recruited to SGs[123]. Potentially differential methylation/demethylation modulates SG assembly and shuttling in and out of SGs. As such, modulating arginine methylation may be involved in recruiting proteins to SGs[124]. Arginine methylation has been shown to alter subcellular localization as well as interactions with RNA and/or proteins[125; 126]. Gly-Arg-Gly is the consensus arginine methylation site that is enriched in RNA binding domains and LCD[125; 126], possibly linking arginine methylation to the process of phase separation.

Studies using high resolution microscopy show that SGs are structurally heterologous with central regions of higher density[101; 127]. Biochemical purification of SG cores using differential centrifugation and G3BP1 immunoaffinity purification identifies an mRNP interactome that is likely related to SGs[128]. BioID/APEX labeling, methods that allow the identification of proteins in close proximity, have uncovered an interactome that is largely pre-assembled in the absence of stress[129; 130]. In the presence of stress, the recruitment of several key proteins and RNAs may be essential for phase separation into SGs. Importantly, interactions between G3BP1 and the eIF3 complex were shown to be stress specific[129]. Knockdown of the eIF3 complex was previously shown to block SG assembly[131], further validating the importance of this interaction. Approximately 20% of G3BP1-associated proteins were found to be specific to the stress or cell type used, indicating a previously unappreciated level of heterogeneity in SGs[129].

Yeast SGs and mammalian SGs have some distinctive differences including their composition and biophysical properties: whereas yeast SGs adopt a more solid-like state, human SGs adopt a more liquid-like state[132]. Pab1, yeast PABP, acts as a stress sensing signal that alters its binding interaction with RNA in response to heat shock and low pH[133]. Interestingly, RNA prevents Pab1 phase separation *in vitro*. Mutations that decrease Pab1 phase separation cause deleterious effects on the ability of yeast to grow under stress

conditions, consistent with Pab1 acting as a stress sensor that modulates its biophysical properties[133]. It is unknown whether this mechanism of stress sensing occurs in other proteins or whether this mechanism is conserved in mammals.

Different stresses trigger unique stress responses and not all stress-induced cytoplasmic foci are SGs[99]. For example, hyperosmolar conditions (as experimentally modeled by increases in NaCl) induce cytoplasmic foci that contain classical SG proteins (G3BP1, eIF3b, mRNAs) but are not in dynamic equilibrium with active translation as judged by the effects of cycloheximide and puromycin. This complicates the prospect of therapeutically targeting SGs (or more generally phase separations in disease states such as cancer or neurodegeneration) as careful analysis of the compositional and functional effects of candidate drugs will be required.

Processing bodies (P-bodies)

While P-bodies house many mRNA decay factors including the decapping enzyme DCP1 and the exonulease Xrn1[134; 135], their assembly is not required for mRNA decay. Translation repression and decay are still carried out in yeast lacking key P-body components. Similarly, in cells lacking P-bodies, bulk mRNA decay, non-sense mediated mRNA decay, and RNA-mediated gene silencing still occur[136; 137].

Recent evidence suggests that P-bodies are primarily sites of mRNA storage. Using a method of particle sorting to purifying P-bodies, Hubstenberger et al show that approximately one third of all mRNAs are recruited to P-bodies[138]. These mRNAs are translationally repressed with a large fraction comprising mRNA regulons linked by a common biological process such as chromatin remodeling[138]. This is consistent with earlier reports that linked P-bodies to sites of translational repression[139]. P-body size and number increases with stress and decreases with removal of stress, correlating P-bodies with translational repression. mRNAs from P-bodies can then return to active translation[140]. Like SGs, P-bodies decrease in response to cycloheximide, which “freezes” polysomes resulting in a dynamic disassembly of P-bodies. This freezing of polysomes depletes the pool of non-ribosome bound mRNAs in the cytoplasm decreasing the mRNAs available to promote SGs and P-bodies. Recent evidence questions the role of P-bodies as sites of mRNA storage. Using a modified MS2 system (MBSV6) to label RNAs, Tutucci and colleagues found that during stress two mRNAs do not localized to P-bodies for storage[141]. Potentially, this indicates that the pool of mRNAs that localize to P-bodies does not change after stress, yet more experiments are needed to confirm this hypothesis.

RNA is required for the assembly and structural integrity of P-bodies. RNase treatment of purified P-bodies leads to their disruption[142]. In addition, DDX6 is required for P-body formation[143] suggesting that rearrangement of RNA is required for P-body assembly. FRAP analysis using MS2-YFP tagged mRNAs suggests that a population of mRNA is immobile or slowly exchanges with the surrounding cytoplasm and that this increases with stress. mRNAs recovered from purified P-bodies do in fact have poly(A) tails of various lengths[138]. This is contrary to observations made using FISH where poly(A) RNA is not detected in P-bodies[144]. Moreover, immunofluorescence does not detect PABP at P-bodies. These

discrepancies could be due to differential accessibility of antibodies and/or oligonucleotides used as P-body markers. Alternatively, post-translational modification of PABP, post-transcriptional RNA modification of poly(A) tails or extensive poly(A) tail base pairing with other RNAs could account for these experimental variations. Interestingly, when sequestered into phase separated domains *in vitro* miRISC more efficiently deadentylates a target RNA[145], yet whether this occurs in a cellular compartment such as P-bodies remains to be determined.

P-bodies and SGs can dock and the extent of docking is largely dependent on the stress conditions[146]. Docking can be stabilized by overexpression of TTP or BRF1[147]. TTP localization to SGs is stress specific and regulated by its interaction with 14-3-3. TTP binds to 14-3-3 when phosphorylated by MK2[146; 148]. Interestingly, in the absence of stress overexpression of cytoplasmic polyadenylation element-binding protein 1 (CPEB1) causes localization of P-body components to SGs[149] and similarly overexpression of p54/Rck drives fusion of P-bodies and SGs[150], suggesting these granules have similar properties that when modulated can be tipped to promote fusion. Furthermore some protein components are shared between SGs and P-bodies, including YB-1 and TIA1/TIAR[151; 152]. It is unclear whether mRNAs are shared or transferred between these compartments and whether RNA plays an active role in docking.

PARP

The amino acids lysine, arginine and glutamic acid can be post-translationally modified with ADP-ribose: mono(ADP)-ribosylation typically targets arginine whereas poly(ADP)-ribosylation (PAR) typically targets lysine and glutamic acid. PAR modifications can cause 2–200 (ADP)-ribose units to be added in a branching pattern. These modifications are added by poly(ADP)-ribose polymerases (PARP) and are removed by poly(ADP)-ribose glycohydrolase (PARG)[153]. Several PARP and PARG enzymes are recruited to SGs and PAR-modified proteins are enriched in SGs[120]. Similarly, a comparison of PAR modified proteins and proteins condensed by b-isox reveals a significant overlap[153; 154], indicating an enrichment in PAR modification within RNA granules.

PAR is negatively charged like RNA and can act like RNA to decrease the phase boundary and promote *in vitro* phase separation[16; 154]. PAR causes a high local density of negative charges that recruits positively charged arginine residues in RGG domains[154]. In this manner, PAR modifications can act as a *de novo* seed for phase separation. Such enrichment occurs at sites of DNA damage where PARP1 is rapidly recruited[16; 154]. This model can explain the earliest stages of the DNA damage response in which an initial recruitment of PARP1 causes rapid PAR modifications that recruit LCD proteins such as FUS, EWS, and TAF15. In cells, FUS and other LCD-containing proteins are recruited to sites of DNA damage in a PAR-dependent manner[16], suggesting PAR modifications nucleate a phase separated state at the site of DNA damage. Additional post-translational modifications, including phosphorylation and ubiquitination, play important roles in the DNA damage response. It has been suggested, but not tested, that phosphorylation by DNA damage response kinases further regulates the PAR-induced DNA damage phase separation. It has been proposed that negative charges introduced by phosphorylation reverse the phase

separation, allowing the PAR-mediated DNA damage phase separation to be transient in nature[154]. Phosphorylation has been shown to control other phase separations[8; 18]. Furthermore, many granule related proteins bind to PAR, including the SG regulating proteins G3BP1/2, and the paraspeckle proteins NONO and SFPQ. G3BP1 binds the PAR modification via its glycine-arginine rich domain[155].

RNA contribution

Recent work has greatly expanded our understanding of MLOs. With the general concepts of phase separation and the necessity of multivalent weak interactions for MLO formation in place, the field needs to address the specificity of phase separation events. If the only factors involved were weak multivalent interactions and surface tension then presumably all phase separated domains within a cell would fuse together. Yet MLOs, such as docked SGs and P bodies, appear to maintain their separate biophysical properties. Understanding what is driving these differences is a key question moving forward.

MLOs are composed of different RNA species and these RNA species contribute to the differences in cellular phase separations. A more thorough understanding of the *cis* elements or RNA secondary structures that direct MLO targeting or assembly is needed, such as the identified motifs in scaRNAs that allow for Cajal body targeting and G-quadruplexes in forming SGs. Similarly, an understanding of how LCD and RNA binding domains contribute to the assembly of distinct phase separated domains is required. While we know that LCDs are enriched in sites that can be post-translationally modified, a more thorough understanding of when post-translational modifications occur and how these post-translational modifications impact phase separation will be important moving forward. Recent connections between MLO and diseases including neurodegeneration, cancer and viral infection, have promoted further interest that will aid in expanding our knowledge.

Acknowledgments

We thank Anderson and Pavel Ivanov lab members for discussion. This work was supported by the NIH (GM111700 and CA168872 to PA, and AI007306 to MMF).

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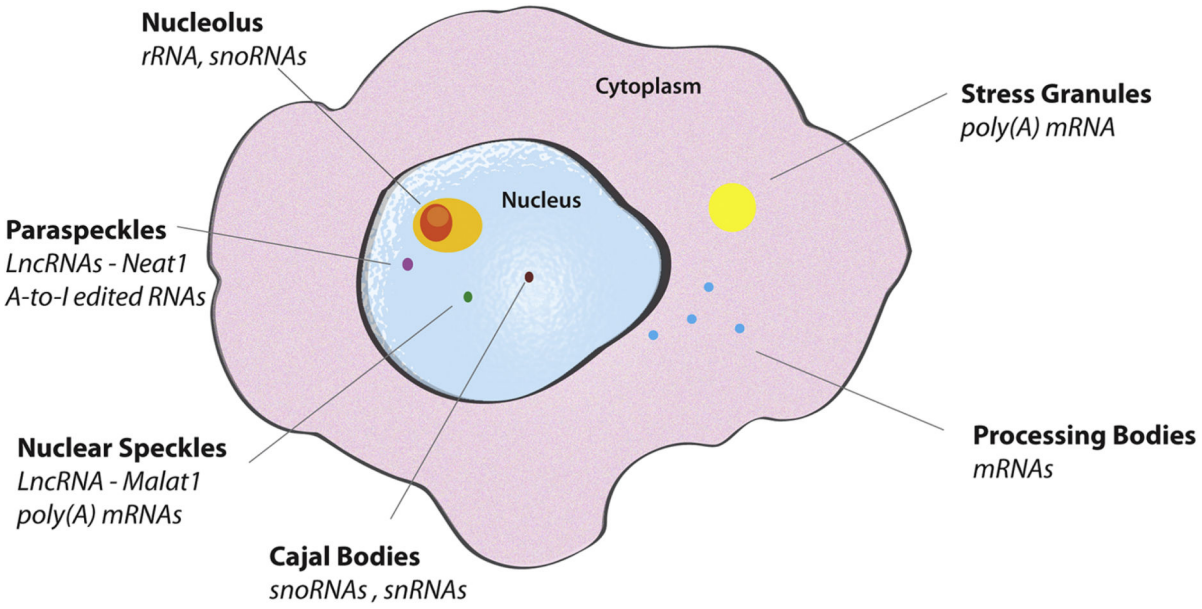

	Size	RNA	Proposed function
Cajal bodies	0.1-2 μm	snoRNA, snRNA	RNA modifications, processing and assembly of small RNA complexes
Nuclear speckles	0.5-2 μm	poly(A) mRNA, lncRNA - Malat1	mRMA maturation, including regulating splicing and mRNA export
Nucleolus	0.5-8 μm	rRNA, snoRNA	rRNA processing, RNA modifications
Paraspeckles	0.35 μm by 1-2 μm	lncRNA - NEAT1, A-to-I edited RNAs	Modulate gene expression by sequestering protein components, microRNA production
Stress granules	1-2 μm*, cores 0.2 μm	poly(A) mRNA	Stress specific mRNA storage
Processing bodies	0.1-0.3 μm	mRNA	mRNA storage

Figure 1. Membraneless organelles (MLOs)

Nuclear membraneless organelles include the nucleolus, paraspeckles, nuclear speckles and Cajal bodies. Cytoplasmic membraneless organelles including stress granules and processing bodies. * denotes that these values are reported for treatment with 0.5 mM sodium arsenite for 30 minutes, size of SGs can greatly change with time and stress used.

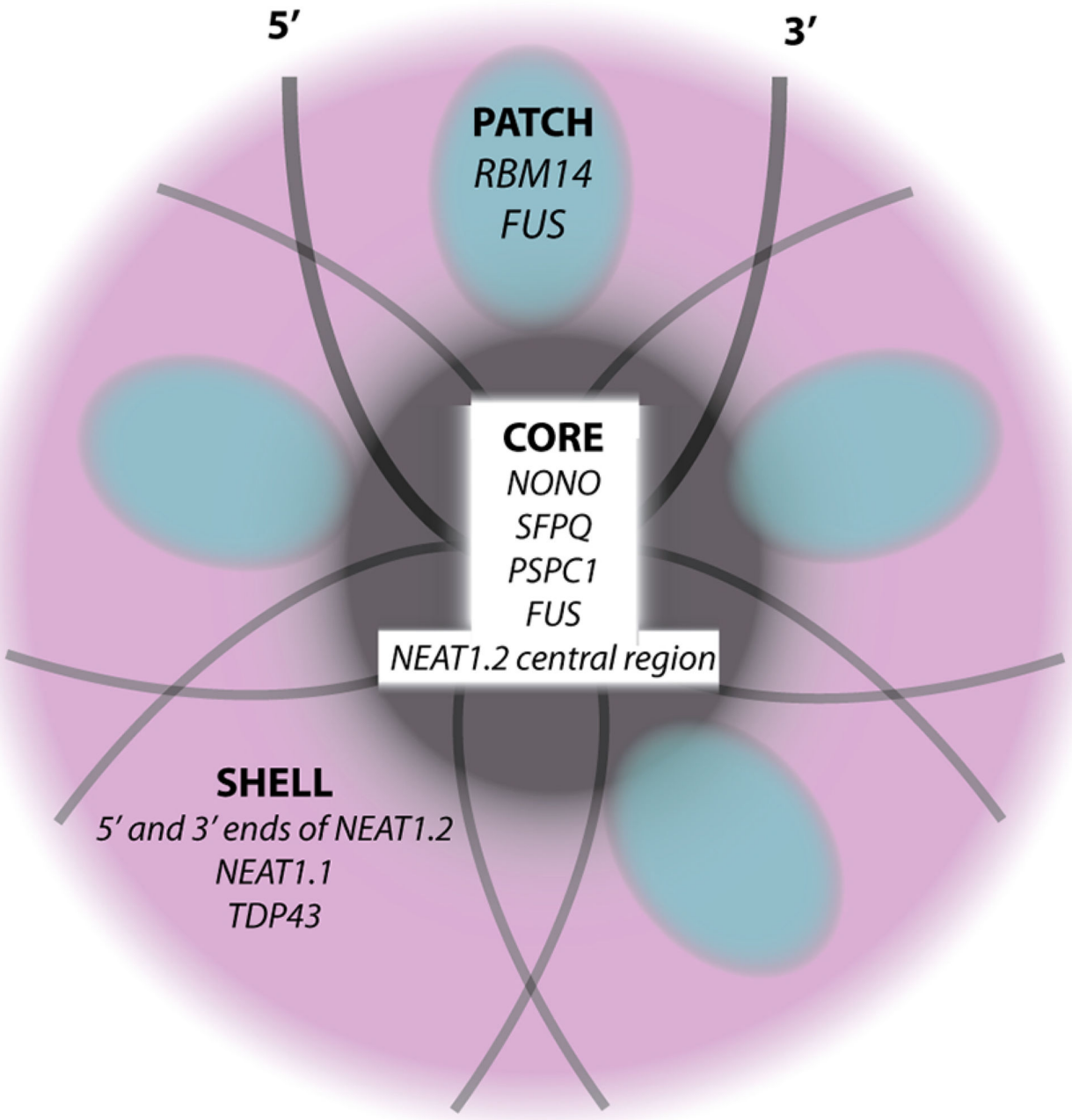

Figure 2. Paraspeckle Core-Shell Structure

The lncRNA NEAT1.2 (grey lines) acts as structural scaffold, organizing the paraspeckle core (dark grey) around its central region and the shell around its 5’ and 3’ termini. Paraspeckles are further held together by patches (blue) of LCD containing proteins including RBM14 and FUS. NONO, SFPQ and PSPC1, proteins in the DBHS (Drosophila behavior/human splicing) family, as well as FUS make up the core while TDP43 is included in the shell.

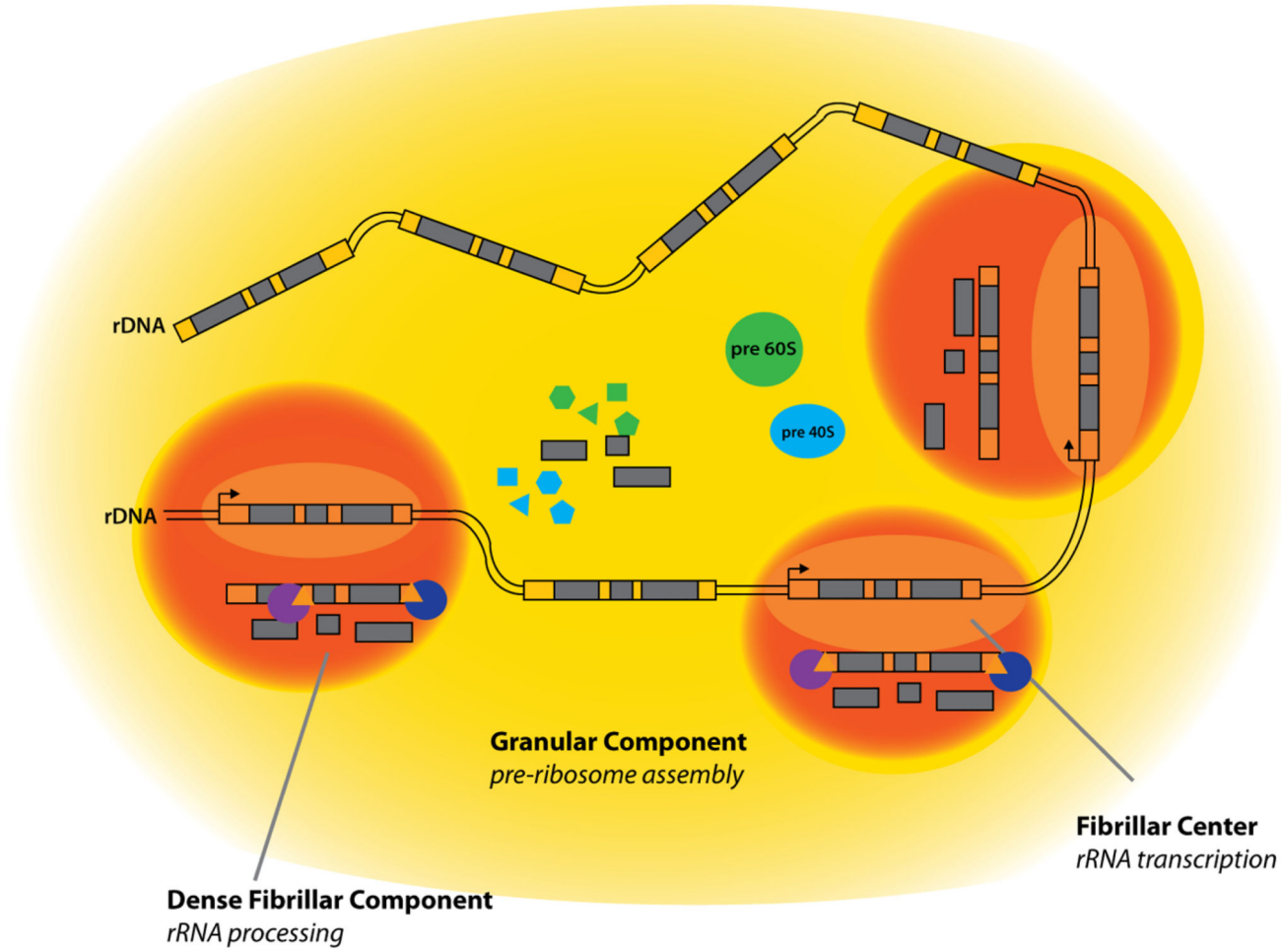

Figure 3. Nucleolar processes and structures

The nucleolus is composed of three phase separations that separate the different processes in rRNA production and maturation and are structured around rDNA. Fibrillar center (light orange) is where rDNA is transcribed into rRNA, dense fibril component (orange) where rRNA is processed, granular component (yellow) where rRNA is further processed and assembled into pre-40S (blue oval) and pre-60S (green oval) ribosomal subunits with ribosomal proteins (blue and green shapes).

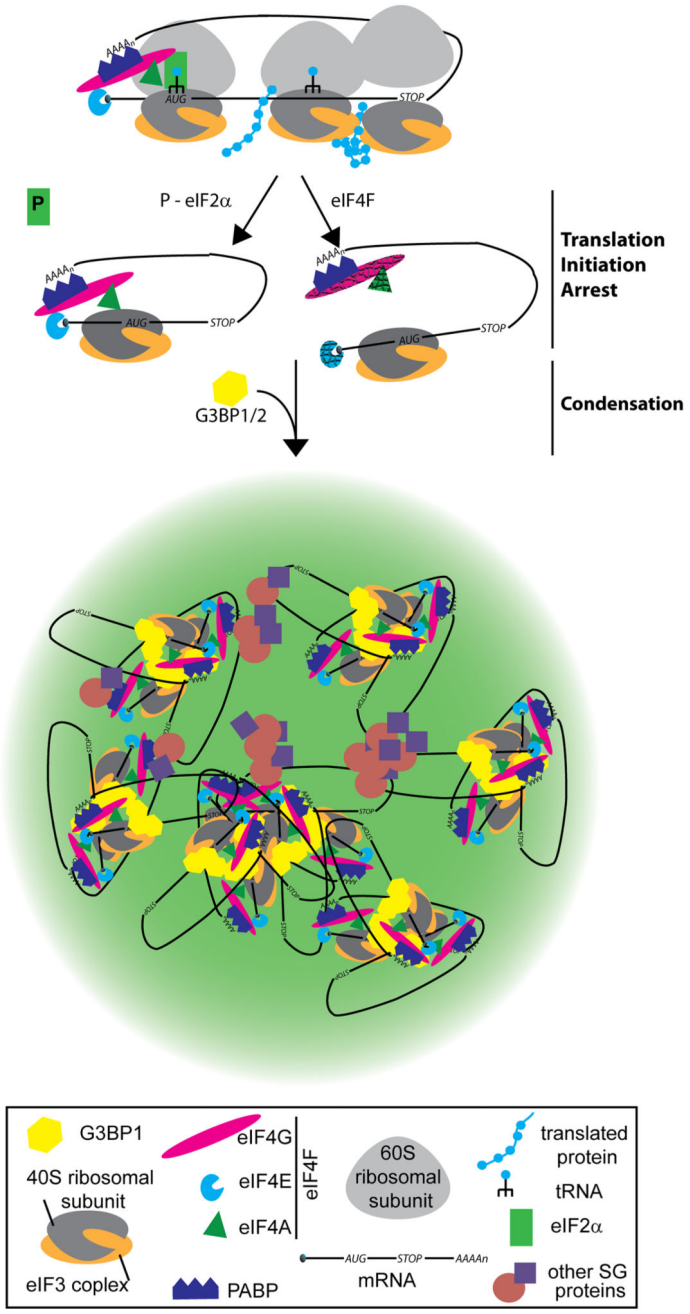

Figure 4. Stress granule assembly

Stress granules assembly requires two steps: (1) A block in translation initiation that is caused by eIF2α phosphorylation (denoted P) or modulation of the eIF4F complex (denoted with grey lines). This leads to ribosome run-off and an increase in mRNAs with translation initiation stalled 48S complexes. (2) Condensation of these translation initiation stalled mRNAs is then mediated by G3BP1. Other RNA binding proteins and LCD containing proteins are further recruited including signaling molecules.

Table 1

mRNAs localized to SGs			
Symbol	***mRNA name***	***Method of Detection***	***Ref.***
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	FISH	103
H19	Long noncoding RNA H19	FISH	103
IGF-II	Insulin-like growth factor II	FISH	103
MYC	Myc proto-oncogene protein	FISH	103
ACTB	Beta actin	MS2, FISH and RCas9	103,105,106
TFRC	Transferrin receptor protein 1	FISH and RCas9	105
CCNA2	Cyclin-A2	FISH and RCas9	105
P21	Cyclin-dependent kinase inhibitor 1	FISH	108
AHNAK	Neuroblast differentiation-associated protein AHNAK	FISH	104
DYNC1H1	Cytoplasmic dynein 1 heavy chain 1	FISH	104
NORAD	Noncoding RNA activated by DNA damage	FISH	104
COX2	Cyclooxygenase-2	FISH	109

mRNAs excluded from SGs			
Symbol	***mRNA name***	***Method of Detection***	***Ref.***
HSP90	Heat shock protein 90	FISH	103
POLR2A	DNA-directed RNA polymerase II subunit RPB1	FISH	103
HSP70	Heat shock protein 70	FISH	102
MDR	Multidrug resistance protein 1	FISH	107
CANX	Calnexin	FISH	107