RNP Granule Formation: Lessons from P-Bodies and Stress Granules

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It is now clear that cells form a wide collection of large RNA–protein assemblies, referred to as RNP granules. RNP granules exist in bacterial cells and can be found in both the cytosol and nucleus of eukaryotic cells. Recent approaches have begun to define the RNA and protein composition of a number of RNP granules. Herein, we review the composition and assembly of RNP granules, as well as how RNPs are targeted to RNP granules using stress granules and P-bodies as model systems. Taken together, these reveal that RNP granules form through the summative effects of a combination of protein–protein, protein–RNA, and RNA–RNA interactions. Similarly, the partitioning of individual RNPs into stress granules is determined by the combinatorial effects of multiple elements. Thus, RNP granules are assemblies generally dominated by combinatorial effects, thereby providing rich opportunities for biological regulation.

Ribonucleoprotein (RNP) granules are a diverse set of biomolecular assemblies of RNA and protein that form within cells. RNP granules in the nucleus, such as Cajal bodies, the nucleolus, and paraspeckles, regulate various steps of RNA and RNP processing (Table 1; Trinkle-Mulcahy and Sleeman 2017; Fox et al. 2018). Cytoplasmic granules, such as P-bodies and stress granules, may regulate RNA translation, RNA degradation, signaling pathways, and stress responses (Sheth and Parker 2003; Kedersha et al. 2013; Protter and Parker 2016; Samir et al. 2019). RNP transport granules in neurons and myo-granules in skeletal muscle are thought to facilitate RNA transport and localized translation (Kiebler and Bassell 2006; Vogler et al. 2018). Furthermore, germ granules, such as *Caenorhabditis elegans* P-granules and *Drosophila* polar bodies, form in the germline during development, contain maternally encoded mRNAs, and help specify germ cell fate (Voronina et al. 2011; Wang and Seydoux 2014; Trcek and Lehmann 2019).

RNP granules are also found in bacteria. In *Caulobacter*, bacterial RNP bodies (BR-bodies) form under stress and can be considered similar to eukaryotic stress granules (Al-Husini et al. 2018). It is likely only a matter of time before more RNP granules are discovered in bacteria.

Aberrant RNP granules are also pathological hallmarks of numerous neurodegenerative and neuromuscular diseases, including amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), and inclusion body myopathy (Arai et al. 2006; Neumann et al. 2006; Weihl et al. 2008; Dickson et al. 2011). These aggregates often contain many of the same components that are found in canonical RNP granules, such as stress granules and

splicing speckles, suggesting that aberrant granules may arise from canonical granules and thereby contribute to neurodegenerative diseases (Taylor et al. 2016; Hsieh et al. 2019; Wolozin and Ivanov 2019).

An unanswered question is how RNP granules assemble, and what interactions allow for multiple distinct RNP granules to exist in cells at any given time. Herein, we review the composition, assembly, and how RNPs are targeted to RNP granules using stress granules (SGs) and P-bodies (PBs) as model systems to understand these issues.

RNP GRANULE COMPOSITION

Proteins in RNP Granules

Three types of approaches have been used to identify components of RNP granules (Fig. 1). First, and sometimes the initial identification of the granule, has been the identification of locally concentrated proteins by immunofluorescence or by the tagging of proteins with green fluorescent protein (GFP). More complete descriptions of the proteins in RNP granules has come from mass spectroscopy of RNP granules purified by differential centrifugation, with or without immunopurification, or by using particle sorting to fractionate granules (Jain et al. 2016; Hubstenberger et al. 2017). A third approach has been to use proximity labeling approaches such as Bio-ID or APEX labeling (Markmiller et al. 2018; Youn et al. 2018), although such proximity labeling approaches are unable to distinguish between interacting proteins within the granule or outside the granule, which can be an issue because even proteins that are highly concentrated in gran-

Table 1. Overview of select ribonucleoprotein (RNP) granules function and composition

RNP granule	Cell type/modulators	Function	Protein composition	RNA composition	References
Nucleolus	Found in all cell types during interphase	Site of rRNA transcription, pre-rRNA processing, ribosome assembly	snRNPs, ribosomal proteins, and ribosome biogenesis factors: RNA polymerase I, DNA topoisomerase, fibrillarin, nucleolin, p52; RNA-modifying enzymes	snoRNAs, rRNAs	Yasuzumi et al. 1958; Chamberland and Lafontaine 1993; Scheer et al. 1993; Mélése and Xue 1995; Maden and Hughes 1997; Dundr and Olson 1998; Olson et al. 2000; Fatica and Tollervy 2002; Desterro et al. 2003
Cajal body	All cell types	snRNP assembly, processing	coilin, Sm proteins, SMN, NIOPP140, snRNPs	snRNAs, scaRNAs, snoRNAs	Gall et al. 1999; Narayanan et al. 1999; Sleeman and Lamond 1999; Hebert and Matera 2000; Carmo-Fonseca 2002; Darzacq et al. 2002; Verheggen et al. 2002; Schaffert et al. 2004; Cioce and Lamond 2005; Morris 2008; Staněk et al. 2008; Shaw et al. 2014; Hebert and Poole 2016; Love et al. 2016; Trinkle-Mulcahy and Sleeman 2017
Paraspeckle	All cell types, increase in stress	Unknown	RNA-binding proteins: PSPC1, p54nrb, SFPQ, RBM14, CPSF6	<i>NEAT1</i> lncRNA, hyperedited RNAs	Prasanth et al. 2005; Bond and Fox 2009; Clemson et al. 2009; Fox and Lamond 2010; Nakagawa et al. 2011; Naganuma et al. 2012; Naganuma and Hirose 2013; Imamura et al. 2014; Fox et al. 2018
P-bodies	Constitutive but increase in stress	Translation regulation, RNA storage, degradation	mRNA decapping enzymes, exonucleases, RNA-binding proteins, NMD factors, translation repressors, RNA helicases	Enriched for nontranslating mRNAs, biased for AU-rich RNAs, RNA composition similar to stress granules under stress, enriched for RNAs with short poly(A) tails	Schwartz and Parker 1999; Sheth and Parker 2003, 2006; Decker et al. 2007; Eulalio et al. 2007; Teixeira and Parker 2007; Buchan et al. 2008; Pilkington and Parker 2008; Hubstenberger et al. 2017; Courel et al. 2019; Matheny et al. 2019
Stress granules	Induced by stress such as viral infection, heat/cold shock, oxidative stress, starvation	Unknown, hypothesized to regulate translation, RNA stability, cell survival, cell signaling	RNA-binding proteins: G3BP1, UBAP2L, PABPC1, TIA-1, IDR-containing proteins, RNA helicases, translation factors	Enriched for longer and poorly translated mRNAs	Kedersha et al. 1999, 2000, 2005, 2013, 2016; Gilks et al. 2004; Buchan and Parker 2009; Hofmann et al. 2012; Buchan et al. 2013; Jain et al. 2016; Wheeler et al. 2016; Khong et al. 2017; Namkoong et al. 2018; Protter et al. 2018; Matheny et al. 2019; Moon et al. 2019, 2020; Cirillo et al. 2020

Continued

RNP GRANULE FORMATION

3

Table 1. Continued

RNP granule	Cell type/ modulators	Function	Protein composition	RNA composition	References
P-granules	<i>Caenorhabditis elegans</i> germline	Germ cell fate specification, RNA silencing	RNA-binding proteins: PGL-1, PGL-3, MEG-3, LAF-1, IDR-containing proteins, RNA helicases	Maternal mRNAs, poorly translated mRNAs	Voronina et al. 2012; Seydoux 2018; Ouyang et al. 2019; Putnam et al. 2019; Lee et al. 2020
Polar granules	<i>Drosophila</i>	Germ cell fate, RNA segregation	RNA-binding proteins: Vasa, Tudor, eIF4A, Aubergine, ME31B, TER94, Oskar	<i>Nanos</i> , <i>cyclin B</i> , <i>polar granule component</i> , <i>oskar</i> mRNAs	Mahowald 1971; Hay et al. 1988; Ephrussi and Lehmann 1992; Nakamura et al. 1996; Ferrandon et al. 1997; Thomson and Lasko 2004; Arkov et al. 2006; Jones and Macdonald 2007; Vanzo et al. 2007; Thomson et al. 2008; Little et al. 2015
Myogranules	Regenerating skeletal muscle	Unknown, hypothesized to facilitate localized translation	RNA-binding proteins: TDP-43, FMRP; IDR-containing proteins	Unknown, hypothesized to be long, sarcomeric mRNAs	Vogler et al. 2018
<i>C9ORF72</i> RNA foci	Neurons, glia, fibroblasts	Aberrant	Hypothesized to contain RNA-binding proteins	<i>C9ORF72</i> G ₄ C ₂ repeat, potentially entire transcript and/or other RNA species	DeJesus-Hernandez et al. 2011; Fratta et al. 2012; Almeida et al. 2013; Gendron et al. 2013; Mori et al. 2013; Kumar et al. 2017; Česník et al. 2019
Myotonic dystrophy type 1 (DM1) granules	Skeletal muscle, neurons	Aberrant	MBNL1, MBNL2	<i>DMPK</i> transcript	Taneja et al. 1995; Wang et al. 1995; Timchenko et al. 1996; Davis et al. 1997; Jiang et al. 2004
Bacterial RNP bodies (BR-bodies)	<i>Caulobacter</i> , other α -proteobacteria form in stress	RNA degradation, rRNA processing	RNA degradeosome proteins: RNase E, Aconitase, RhlB	Nontranslating RNAs	Al-Husini et al. 2018

(rRNA) Ribosomal RNA, (snRNP) small nuclear ribonucleoprotein, (snoRNA) small nucleolar RNA, (snRNA) small nuclear RNA, (scaRNA) small Cajal body–specific RNA, (lncRNA) long noncoding RNA, (mRNA) messenger RNA, (NMD) nonsense-mediated decay.

ules can have a majority of molecules outside the granule in the cell (Wheeler et al. 2017). Taken together, these approaches have revealed several features of RNP granule proteomes.

RNP granules contain three types of RNA-binding proteins (RBPs). First, there are RBPs that contribute to the assembly of the granule by providing protein-based cross-links between different RNAs or by forming a protein-based assembly. Second, there are RBPs that bind to RNAs resident to a granule and as such are simply carried into the granule by the RNAs. Third, there can be RBPs, such as YB-1 (Bounedjah et al. 2014) and eIF4A for stress granules (Tauber et al. 2020), that bind to RNA and compete for RNA–RNA interactions that would otherwise promote RNP granule formation.

Some RBPs are found in more than one RNP granule. This can be understood for RBPs that bind RNAs and are carried into granules. However, RBPs that play a role in the formation of a specific granule are generally only found in that granule. For example, G3BP1 or Edc3, which are important for SG or PB formation (Tourrière et al. 2003; Decker et al. 2007), respectively, are only

found in those granules (Kedersha et al. 2005; Decker et al. 2007). Furthermore, coilin, which is essential for Cajal body (CB) formation, is found specifically within CBs (Raška et al. 1991; Sleeman et al. 2003).

RNP granules can also contain other types of proteins. For example, stress granules are known to contain a number of proteins involved in cellular signaling pathways and SG formation can influence cell signaling (Kedersha et al. 2013). Similarly, SGs can contain proteins involved in metabolism (Jain et al. 2016; Bergovich et al. 2020). Moreover, the nucleolus contains a wide variety of proteins involved in a number of physiological processes, such as dealing with misfolded proteins (Boisvert et al. 2007; Frotin et al. 2019). Taken together, the compositional studies suggest that cells have taken advantage of the formation of RNP granules to create localized concentrations of proteins involved in various processes, thereby increasing their effectiveness.

Although multiple RNP granules have been described as having very complex proteomes, the composition of RNP granules may often be much simpler. For example, yeast and mammalian PBs have been described to have more

Figure 1. Overview of the three major approaches to determining the protein or RNA composition of ribonucleoprotein (RNP) granules. One approach involves probing for individual proteins or RNAs by fluorescence imaging methods (*left*). A second approach involves purifying RNP granules, followed by RNA sequencing or mass spectrometry to determine the RNA or protein compositions, respectively (*middle*). The third approach relies on tagging RNAs or proteins that interact with known granule proteins, followed by purification of tagged molecules and RNA sequencing or mass spectrometry (*right*).

than 50 or 100 different proteins resident to them, respectively (Hubstenberger et al. 2017). However, a quantitative analysis revealed that only 14 proteins are highly concentrated within yeast PBs, and the other approximately 36 proteins are present at low amounts (Xing et al. 2020). This is significant because 300–500 different proteins have been identified in SGs (Jain et al. 2016) and 200 proteins have been identified in the nucleolus (Thul et al. 2017). An important area of future research will be quantitative analyses of the abundance of these components to determine the primary constituents of any given RNP granule.

RNAs in RNP Granules

The RNAs present in RNP granules have been identified by three general approaches (Fig. 1). In one case, individual RNAs are examined by single-molecule fluorescence in situ hybridization (smFISH) or by tagging the RNAs with a fluorescent system such as MS2. A second

approach has been to purify RNP granules and then sequence the copurifying RNAs (Hubstenberger et al. 2017; Khong et al. 2017; Namkoong et al. 2018). A third approach is to identify RNAs that interact with a key component of an RNP granule, inferring that this will identify RNA present in that assembly. This latter approach, followed by smFISH to validate the inferred partitioning, has worked well for the identification of mRNAs in *C. elegans* P-granules (Lee et al. 2020). These experiments have revealed the following features of RNP granules.

First, as inferred from their definition, every RNP granule contains a set of RNAs, although they can vary between different RNP granules. For example, both SG and PBs in the cytosol are made up of a wide distribution of nontranslating messenger RNAs (mRNAs) and long noncoding RNAs (lncRNAs) (Hubstenberger et al. 2017; Khong et al. 2017). Cajal bodies contain a population of small Cajal body-specific RNAs (scaRNAs), small nucleolar RNAs (snoRNAs), and small nuclear RNAs (snRNAs) (Darzacq et al. 2002; Schaffert et al. 2004; Staněk and

Neugebauer 2004; Trinkle-Mulcahy and Sleeman 2017). Some RNP granules appear more specific to particular RNAs. For example, paraspeckles are thought to essentially contain multiple copies of the *NEAT1* RNA (Clemson et al. 2009). Similarly, RNP foci observed from repeat expansion RNAs are inferred to be made up solely of those RNAs with repeat expansions (Česnik et al. 2019). However, given the tendency of RNA to interact with other RNA molecules (Van Treeck et al. 2018; Tauber et al. 2020), one anticipates that additional RNAs, perhaps in relatively nonspecific manners, will be localized to these structures.

Second, some RNP granules form locally when a new population of RNA is produced. This is observed for the nucleolus, wherein the production of nascent ribosomal RNA (rRNA) transcripts is required for the formation of the nucleolus (Mélèse and Xue 1995; Falahati et al. 2016). Similarly, the formation of the histone locus body (HLB) occurs when histone mRNAs are produced at high rates (Shevtsov and Dundr 2011). Paraspeckles also require *NEAT1* transcription for formation and maintenance (Mao et al. 2011; Naganuma and Hirose 2013). One anticipates that anytime a transcript is produced at high rates, and from repeated genes, it would generate a transient RNP granule as a result of the interactions between RNAs and RBPs.

Third, because the assembly of RNPs into granules may be a relatively promiscuous process, one should expect that even highly specialized RNP granules will contain other RNAs. For example, the formation of P-granules in *C. elegans* is critical for the delivery of a few specific mRNAs to the germline precursor cells, yet P-granules contain a large number of mRNA molecules that are simply carried along for the ride and may contribute to the formation of P-granules by acting as scaffolds for RBPs or through RNA–RNA interactions (Lee et al. 2020). In other words, because RNP granule formation requires a critical concentration to form above the activity of RNA decondensers (Tauber et al. 2020), one way of achieving the critical concentration is to include additional nontranslating RNA and lncRNAs that are not necessary to be transported per se but allow the formation of the granule.

HOW DO RNP GRANULES FORM?

It is now clear that RNP granules form from a summation of multivalent interactions between individual RNPs (Fig. 2) (Mittag and Parker 2018; Van Treeck and Parker 2018). The multivalent nature of these interactions allows for the formation of large assemblies containing up to hundreds of RNPs, as in the case of stress granules (Khong

Types of protein-protein interactions

Types of RNA-RNA interactions

Figure 2. Summation model for RNP granule formation. Protein–protein interactions such as those between intrinsically disordered regions (IDRs), LARKs, short linear motifs (SLiMs), or well-folded domains contribute to granule formation, as do RNA–RNA interactions such as Watson–Crick base pairs and non-Watson–Crick base pairs, and possibly helical stacking between RNAs. Increasing or decreasing the availability of any one type of interaction can induce or prevent RNP granule formation.

et al. 2017). RNPs can interact either through protein–protein, protein–RNA, and/or RNA–RNA interactions.

Protein–protein interactions promoting RNP granule formation occur between RBPs bound to different RNAs and can involve well-folded protein domains (for review, see Mittag and Parker 2018). For example, the dimerization of G3BP1 or EDC3 promotes SG formation in mammals (Tourrière et al. 2003) or PBs in yeast (Ling et al. 2008), respectively. Similarly, depletion of coilin prevents Cajal body formation (Kaiser et al. 2008; Walker et al. 2009; Nizami et al. 2010). However, many RNP granule proteins contain intrinsically disordered regions (IDRs) that can also contribute to RNP granule assembly (Gilks et al. 2004; Decker et al. 2007; Lee et al. 2020). The IDRs of RBPs can promote RNP granule formation in multiple ways, including forming short linear motif (SLiM) interactions with well-folded domains (Jonas and Izaurralde 2013) or by forming local regions of structure that stabilize interactions (Hughes et al. 2018).

Purified RNP granule proteins, or their IDR domains, can also self-assemble in vitro through liquid–liquid phase separation (LLPS), wherein weak multivalent interactions of the IDRs partition those proteins into a concentrated phase in solution (Elbaum-Garfinkle et al. 2015; Lin et al. 2015; Molliex et al. 2015; Nott et al. 2015; Patel et al. 2015; Lee et al. 2020; Martin et al. 2020). This suggested that IDRs also promote the formation of RNP granules, and other macroscale assemblies, through weak, dynamic interactions. Some IDRs weakly interact with many proteins, referred to as “promiscuous” interactions (Protter et al. 2018). Promiscuous IDRs promote LLPS in vitro and RNP granule formation in cells when coupled to proteins undergoing specific interactions by an increase in avidity of the overall complex (Protter et al. 2018). Such promiscuous interactions are not stereospecifically defined, and therefore an individual IDR can interact with different components of the complex to enhance assembly (Protter et al. 2018).

Proteins affecting RNP granule assembly are controlled by multiple posttranslational modifications (reviewed in Protter and Parker 2016). For example, upon arsenite stress, G3BP and UBAP2L, two RBPs that contribute to mammalian SG formation, are arginine-demethylated within their RGG RNA-binding domains, which is likely to increase their RNA binding and thereby promote SG assembly (Tsai et al. 2016; Hofweber and Dormann 2019; Huang et al. 2020). RNP granule formation can also be affected by phosphorylation (Berchtold et al. 2018), acetylation (Gal et al. 2019; Saito et al. 2019), SUMOylation (Jongjitwimol et al. 2016), and glycosylation (Ohn et al. 2008). Thus, multiple posttranslational modifications of proteins affect the assembly of RNP granules.

In addition to RNA serving as a scaffold for the recruitment of RBPs, intermolecular RNA–RNA interactions also contribute to RNP granule assembly. A role for intermolecular RNA–RNA interactions in RNP granules was suggested by the observations that RNA molecules can self-assemble in vitro (Aumiller et al. 2016). For example, base-pairing allows oligonucleotides of repeat expansion RNAs to self-assemble in vitro, suggesting that intermolecular RNA–RNA interactions contribute to the formation

of nuclear RNP foci of repeat expansions RNAs (Jain and Vale 2017). Moreover, when purified cellular RNA is incubated under approximately physiological concentrations and salts, RNA assemblies form whose composition is biased toward longer RNAs and largely matches the stress granule transcriptome (Van Treeck et al. 2018). Because longer RNAs are more prone to self-assembly through RNA–RNA interactions, this observation provides a possible biophysical explanation for why SGs and PBs are generally enriched in longer RNAs (Hubstenberger et al. 2017; Khong et al. 2017; Namkoong et al. 2018; Matheny et al. 2019). However, based on the ability of proteins to target RNAs to RNP granules (see below), one anticipates that, on average, longer RNAs will also have more binding sites for proteins that contribute to RNP recruitment. In the *Drosophila* embryo, homotypic intermolecular base-pairing between *oskar* or *bicoid* mRNAs determines whether those mRNAs localize to RNP granules in the anterior or the posterior of the embryo (Ferrandon et al. 1997; Jambor et al. 2011). This latter observation demonstrates that cells can use specific intermolecular RNA–RNA interactions for RNP granule assembly.

Cells have developed multiple mechanisms to modulate intermolecular RNA–RNA interactions and thereby control the process of RNP granule formation. For mRNAs, this includes elongating ribosomes, which limit the association of mRNAs with SGs and PBs (see below). Similarly, monovalent RNA binding proteins, such as YB-1, can bind to RNA and compete for RNA–RNA interactions that would otherwise promote RNP granule assembly (Boundedjah et al. 2014). The family of DEAD/DEVH-box “RNA helicases,” which are ATP-dependent RNA-binding proteins, are found in essentially every RNP granule (Gruidl et al. 1996; Hubstenberger et al. 2013, 2017; Calo et al. 2015; Nott et al. 2015; Jain et al. 2016) and play two roles in modulating RNP granule formation. Monovalent DEAD-box proteins such as eIF4A or DDX19A can limit RNP granule formation by competing for RNA–RNA interactions, including those that occur in *trans* (Tauber et al. 2020). Conversely, DEAD-box proteins that have additional multimerization domains can promote RNP granule formation, presumably by forming protein–protein interactions that cross-link RNAs together into higher-order structures (Hondele et al. 2019). RNP granule formation is also modulated by the local concentration of RNA. For example, depletion of cytosolic mRNAs by RNase L induction alters stress granule assembly (Burke et al. 2019). Conversely, RNP granules can be nucleated by high local concentration of RNA, such as those produced by rRNA transcription leading to the nucleolus, or translational shutoff increasing the pool of nontranslating RNA in the cytoplasm, which induces SG formation (Falahati et al. 2016; Protter and Parker 2016).

RECRUITMENT OF RNPs TO RNP GRANULES

A key step in partitioning RNPs into RNP granules is the interaction of RNPs with the surface of an RNP granule.

Figure 3. The surface of RNP granules are dynamic and recruit mRNPs. Formation of a transient surface interaction (“docking”) can lead to more stable interactions (“locking”). Translating mRNPs only interact transiently with P-bodies (PBs) and stress granules (SGs), whereas nontranslating mRNPs may form transient or stable interactions.

ule and/or the subsequent stable interaction of the RNP with the RNP granule. Although limited information is available about this process, we consider what is known about mRNP partitioning into stress granules and/or P-bodies to begin to address the mechanisms that modulate RNPs interacting with RNP granules.

The imaging of single mRNAs has revealed that mRNPs interact with SGs and PBs in mammalian cells in two manners (Fig. 3). Both stable and dynamic interactions of RNPs have been observed for PBs (Pitchiaya et al. 2019). For both PBs and SGs, one population of mRNAs is relatively stable in these RNP granules and can be rigidly positioned within the granule (Moon et al. 2019). A second population of RNPs transiently interacts with the surface of these granules, showing rapid exchange with the bulk cytosol with an average dwell time on the granule surface of ~10 sec (Moon et al. 2019). Stable associations are increased with granule size and mRNA length and decreased by ribosome association with the mRNA (Moon et al. 2019). These observations suggested a “dock and lock” model wherein RNPs can form transient protein or RNA-based interactions with the surface of an RNP granule, and that surface association can lead to formation of more interactions, leading to a stable association of the RNP with the granule (Moon et al. 2019). Interestingly, surface binding leading to more stable associations through the creation of a high local concentration can also occur on the surface of RNA condensates created from an RNA homopolymer (Tauber et al. 2020), suggesting that surface interactions of RNP granules will be critical in controlling the entry and egress of RNPs.

FACTORS AFFECT RNPs PARTITIONING INTO STRESS GRANULES AND P-BODIES

Examination of the composition of both SGs and PBs has revealed that there can be substantial differences in the partitioning of individual mRNAs into these assemblies. On a correlative level, both assemblies are biased toward longer, poorly translated mRNAs (Hubstenberger et al. 2017; Khong et al. 2017; Namkoong et al. 2018; Matheny et al. 2019). Similar results are seen for the collection of mRNAs enriched in *C. elegans* P-granules (Lee et al. 2020). In addition to these correlations, further experiments have revealed both positive and negative components of RNPs that affects their assembly into these complexes.

Three observations argue that the engagement of an 80S ribosome limits the ability of an mRNP to stably associate with a SG or PB. First, trapping mRNAs in polysomes with translation elongation inhibitors prevents SG, PB, and P-granule formation (Kedersha et al. 2000; Brengues et al. 2005; Lee et al. 2020), demonstrating that these assemblies require mRNAs dissociated from ribosomes for their stable formation. Second, mRNAs with long coding regions are delayed in their accumulation in stress granules after translation inhibition in a ribosome-dependent manner (Khong and Parker 2018). Finally, simultaneous monitoring of translation status and interaction with the RNP granules of single mRNPs revealed that mRNAs engaged with ribosomes can only interact with SGs or PBs in the dynamic mode and are unable to enter the stable state (Moon et al. 2019). Thus, mRNAs engaged with ribosomes can interact with SGs

and PBs but are unable to form a stable interaction. This suggests that the energetic cost of partitioning a ribosome into an RNP granule is significant, perhaps because of continued ribosome elongation, an absence of stabilizing interactions between the ribosome and other RNP granule components, or an altered geometry to the mRNA owing to elongating ribosomes (Adivarahan et al. 2018; Khong and Parker 2018).

Individual RNPs can be targeted to RNP granules by the combined action of multiple positive elements. For example, insertion of the *NORAD* lncRNA, which is highly enriched in SGs (Khong et al. 2017), into a reporter efficiently targets that reporter RNA into SGs (Matheny et al. 2020). Moreover, deletion analysis of *NORAD* demonstrates that multiple regions of *NORAD* act in an additive manner to promote SG accumulation of the RNA (Matheny et al. 2020). The number of binding sites for known SG RBPs within the region of *NORAD* inserted into the reporter correlates with increased SG enrichment, whereas control RNAs of the same length, but without known sites for RBPs, showed lesser accumulation in SGs (Matheny et al. 2020). This suggests that the SG accumulation of *NORAD* may be largely dictated by the presence of multiple binding sites for SG proteins and raises the possibility that nonspecific RNA–RNA interactions contribute less to the SG accumulation of *NORAD* RNA.

More direct evidence that some RNA-binding proteins can target RNPs into SGs has come from examining how artificial tethering of proteins to reporter RNAs affects their accumulation in SGs. For example, the tethering of G3BP1 or TIA-1 to a luciferase reporter mRNA increases its accumulation in stress granules in a dose-dependent manner (Matheny et al. 2020). This provides direct evidence that proteins bound to an RNA can dictate its accumulation in an RNP granule. This is consistent with the observations that in *C. elegans*, the P granule protein MEG-3 is required for proper mRNA localization and P-granule formation in germ cells (Lee et al. 2020), and that in neurons, trafficking of specific mRNAs in neuronal transport granules is dependent on sequence motifs for the binding of heterogeneous nuclear ribonucleoprotein (hnRNP) A2 and A3 (Shan et al. 2000). Similarly, sequence elements in the 3' UTR of *Drosophila* germ granule RNAs are necessary and sufficient for localization in maternal mRNA granules (Rangan et al. 2009).

The observation that *NORAD* contains many elements promoting SG accumulation and the observation that efficient SG targeting by tethered proteins requires 25 sites of tethering suggest a model wherein the accumulation of a given RNP into a SG is due to the summation of many interactions (Matheny et al. 2020). This view is consistent with the strong bias toward longer RNAs in SGs, which by their length would simply have more sites for positive interactions. Interestingly, this summative model predicts that any given protein would have small effects on the set of RNAs that accumulate in a SG. This provides an explanation for the surprising observation that U-2 OS cells lacking G3BP1 and G3BP2, two abundant and important proteins for SG formation, form SGs with essentially the

same transcriptome as wild-type cells (Matheny et al. 2020).

HOW DO CELLS SORT RNPs BETWEEN MULTIPLE GRANULES?

The combination of translation status and sets of specific interactions allows cells to form multiple types of RNP granules with distinct RNA and protein compositions in the same compartment. For example, P-bodies in unstressed cells are biased toward poorly translated mRNAs and have a distinctly different mRNA composition than stress granules (Fig. 4). (Hubstenberger et al. 2017; Matheny et al. 2019). However, during stress conditions, when most mRNAs cease translation, the transcriptome of P-bodies and stress granules is very similar (Matheny et al. 2019). During such stress conditions, individual mRNPs then can be understood as partitioning into stress granules or P-bodies based on their RNA binding proteins. Because proteins found in P-bodies or stress granules preferentially interact with themselves (Jonas and Izaurralde 2013; Youn et al. 2018), this would allow two distinct forms of mRNPs that partition into stress granules or P-bodies, respectively. Such distinct mRNPs could be dictated by the status of the poly(A) tail because deadenylated mRNAs are preferentially bound by specific components of P-bodies (Bouveret et al. 2000; Tharun et al. 2000), which would then target deadenylated molecules to P-bodies, consistent with P-bodies failing to stain for poly(A)⁺ mRNA by oligo(dT) FISH, whereas SGs do (Kedersha et al. 2000; Cougot et al. 2004). Similarly, specific RNA–RNA interactions can target the *bicoid* or *oskar* RNAs to anterior or posterior RNP granules during *Drosophila* oogenesis (Ferrandon et al. 1997; Jambor et al. 2011).

Partitioning of RNPs into different RNP granules may also be controlled by RNA structures that form to limit any RNA–RNA interactions between components. For example, it is suggested in the filamentous fungi, *Ashbya gossypii*, that the tertiary structure of the *CLN3* mRNA, which associates with the Whi3 protein and forms RNP granules, limits the *CLN3* mRNA's possible interactions with other mRNAs that also interact with Whi3, thereby giving rise to distinct RNP granules (Langdon et al. 2018).

CONCLUSION

The combination of available protein and RNA-based interactions determines whether an RNP granule will form and whether an individual RNP will partition into the granule. The diversity of interactions allows for redundancy in RNP granule formation as well as many layers of potential regulation. Future work to understand how the availability of these interactions is regulated will be important for understanding how aberrant granules form in neurological disease.

Figure 4. P-bodies and stress granules are composed of nontranslating mRNPs. During nonstressed conditions (*left*), only P-bodies are present, and nontranslating, deadenylated mRNAs partition into P-bodies. During stress (*right*), both P-bodies and stress granules are present in cytosol, and mRNAs may partition into either stress granules or P-bodies, likely dictated by their poly(A) status.

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