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It's Raining Liquids: RNA Tunes Viscoelasticity and Dynamics of Membraneless Organelles

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Paradigm-shifting advances have revealed that diverse membraneless organelles originate via liquid-liquid phase separation, but how their distinct structural and functional milieux are specified is not understood. Recent work elucidates that RNA governs the biophysical characteristics of liquid droplets formed by RNA-binding proteins with low-complexity domains and can decelerate pathogenic fibrillogenesis.

To function effectively, eukaryotic cells enforce a co-operative division of labor by partitioning their contents into numerous specialized microreactors termed organelles. Non-membrane-bound organelles such as ribonucleoprotein (RNP) granules differ from classical membrane-delimited compartments in that they behave like liquid droplets that rapidly assemble and disassemble in response to changes in the cellular environment. Membraneless organelles include nucleoli, Cajal bodies, gems, paraspeckles, and PML bodies in the nucleus, as well as processing (P) bodies and stress granules in the cytoplasm (Zhu and Brangwynne, 2015). These dynamic structures experience free diffusion in their interior and rapid exchange with their exterior environment. Dysregulation of RNP granule dynamics is a key pathological step in several devastating neurodegenerative diseases (Li et al., 2013). However, how their assembly and disassembly is spatially and temporally controlled has remained largely unknown. Groundbreaking studies by Gladfelter and colleagues shed light on the mechanism by which RNA specifies how a single RNA-binding protein (RBP) can assemble into different cellular compartments with distinct biophysical and functional properties (Zhang et al., 2015).

Emerging evidence suggests that the assembly of RNP granules is driven by liquid-liquid phase separation (LLPS), which rapidly increases the local concentration of protein (Zhu and Brangwynne, 2015). Known triggers for LLPS include (1) increasing protein concentration, (2) lowering salt concentration, (3) decreasing temperature, (4) RNA, and (5) crowding agents (Figure 1). Therefore, several different factors can drive formation of the liquid separated phase (Figure 1). For example, many RNP granule proteins harbor a prion-like domain (PrLD) (Li et al., 2013), which is a type of intrinsically disordered region (IDR) or low-complexity domain (LCD). PrLDs and other LCDs can self-associate into oligomeric structures maintained by non-specific weak interactions between multiple adhesive elements (Li et al., 2013). Another common property of RNP granule proteins is RNA-recognition motif (RRM) domains, which can provide additional multivalent interactions through RNA binding. In the case of the P granule protein, Ddx4, LLPS is driven by electrostatic interactions enabled by patterns of alternating charged residues (Nott et al., 2015). Thus, granules can be maintained by multiple, distinct interactions, which can generate structures with diverse biophysical properties. For example, P-bodies are more liquid-like compartments, whereas stress granules are more gel-like phases in yeast (Kroschwald et al., 2015). Yet, precisely how distinct, emergent biophysical properties stem from these multiple, weak interactions is unclear. Using a RBP, Whi3, which has dual functions in different phase-separated compartments, Zhang et al. (2015) show that RNA is critical in defining the biophysical properties and thus the localization and function of granules. These findings suggest that mRNA encodes not only genetic information but also architectural determinants for various membraneless organelles.

Similar to many other liquid droplet-forming proteins, Whi3 has an IDR and a RRM (Zhang et al., 2015). Whi3 assembly is essential for organizing both cyclin transcripts (*CLN3*) at sites of nuclear division and formin transcripts (*BNI1*) localized at distal sites of polarity or new branch sites of the filamentous fungus *Ashbya Gossypii* (Zhang et al., 2015). Moreover, the Whi3 homolog in yeast harbors predicted prion domains and can form super-assemblies that are retained by mother cells and function in cellular memory (Caudron and Barral, 2013). Using advanced microscopic techniques, Zhang et al. (2015) showed that Whi3 phase separates into liquid-like droplets in vivo and in vitro with biophysical properties that were tunable by changing the concentration or identity of the mRNA binding partner, i.e., *CLN3* or *BNI1*. Importantly, RNA binding to Whi3 retarded the maturation or aging of the liquid droplets to a more fibrillar solid-like state that is potentially pathogenic (Zhang et al., 2015).

Several other recently published papers, including two that appear in this issue of *Molecular Cell*, demonstrate a variety of other IDR-containing proteins form liquid droplets via LLPS (Burke et al., 2015; Lin et al., 2015; Molliex et al., 2015; Nott et al., 2015; Patel et al., 2015). These proteins range from engineered proteins containing IDR and RRMs (Lin et al., 2015) to human RNP granule proteins (i.e., FUS and hnRNPA1), which form pathological inclusions in fatal neurodegenerative disorders such as amyotrophic lateral sclerosis and multisystem proteinopathy (Burke et al.,

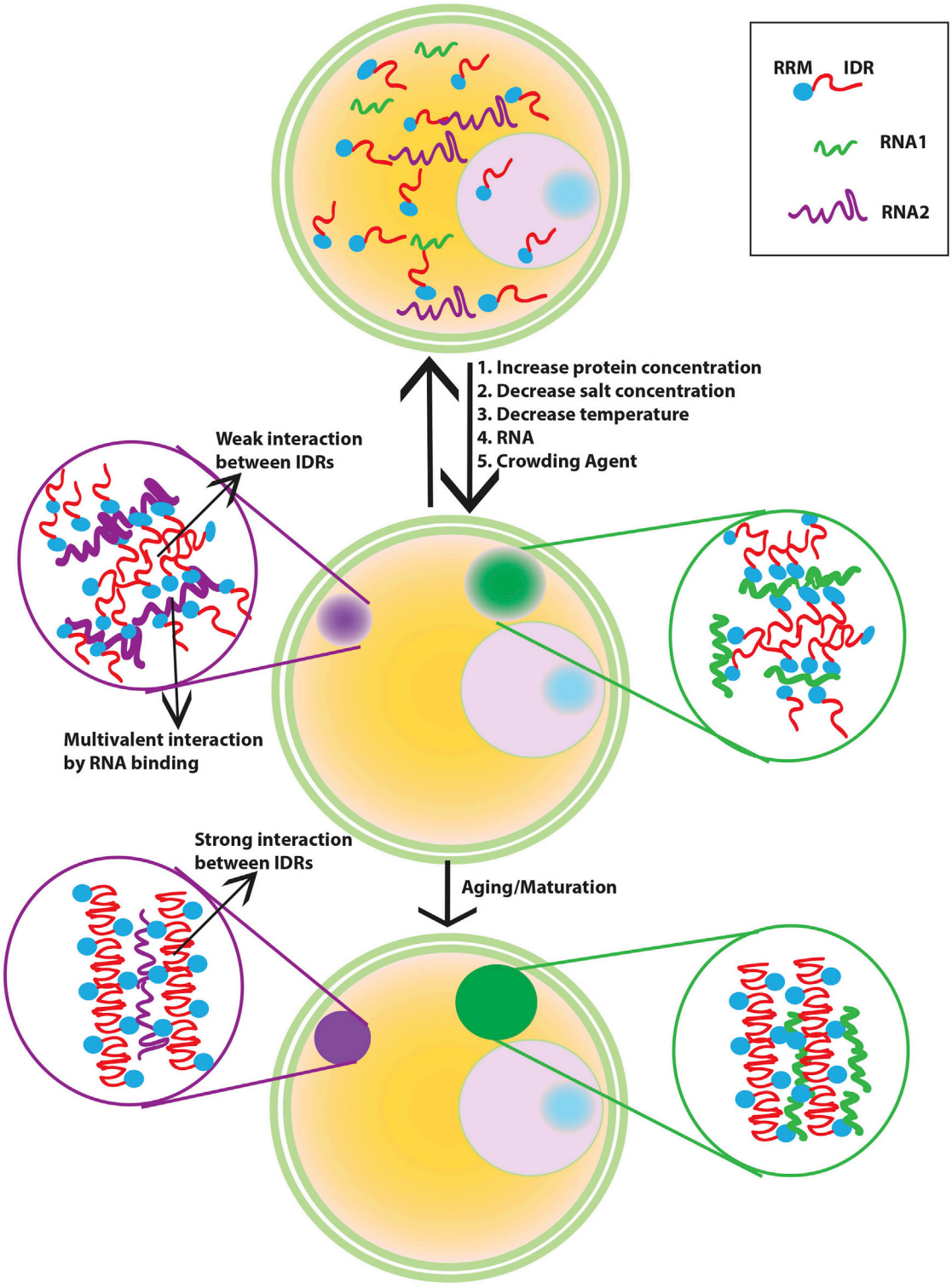

Figure 1. Model of RNP Granule Formation and Regulation

RNP granule proteins often contain an IDR and a RRM. RNP granules can be formed by liquid-liquid phase separation, which rapidly increases the local concentration of IDR-containing proteins. Freshly formed RNP granules behave like liquid droplets that have weak interactions between molecules. Different RNAs participating in the separated liquid phase govern the biophysical properties and localization of the granules. Over time, the separated liquid phase matures to a more solid-like phase with strong interactions between molecules. Fibrillar structures are often observed within matured liquid droplets.

2015; Li et al., 2013; Molliex et al., 2015; Patel et al., 2015). Liquid droplets formed by these proteins exhibit classic liquid behaviors including fusion, dripping, and wetting. Moreover, a comprehensive NMR study on the structure of liquid phase separated FUS LC domain shows that the FUS LC domain in liquid droplets remains disordered and is structurally very similar to the dispersed monomer, confirming the dynamic nature of the protein liquid droplets (Burke et al., 2015). However, not all droplets formed by IDR-containing proteins behave like classic liquids. For example, less concentrated Whi3 (Zhang et al., 2015), TDP-43 (Molliex et al., 2015) and three engineered proteins (i.e., SNAP-PTB-Lsm4$_{IDR}$, SNAP-PTB-Tia1$_{IDR}$, and SNAP-PTB-hnRNPA1$_{IDR}$) (Lin et al., 2015) form smaller droplets with more stable microstructure, which attach to each other in strings without fusion. Moreover, liquid droplets or more stable hydrogel structures can recruit other IDR-containing proteins into the phase-separated structure through heterotypic interactions (Kato et al., 2012; Lin et al., 2015). Thus, RNP granules in vivo that contain multiple IDR-containing proteins and RNAs are likely more complex and can access physical states that deviate from classic liquid. For example, stress granules in yeast are more solid-like than stress granules in humans, and these more static structures might provide extra protection for yeast under extreme stress (Kroschwald et al., 2015). This finding suggests that the more solid-like phases formed by RNP proteins, such as hydrogels, might also represent functional and beneficial states of RNP granules (Kato et al., 2012). Indeed, RNP granules likely adopt a spectrum of different functional states by regulated adjustment of interactions controlling the biophysical properties of the separated phases (Figure 1).

One factor that controls the biophysical properties of the separated liquid phase is the RNA content (Figure 1). By strategically choosing two distinct mRNA targets of Whi3, Zhang et al. (2015) establish that different RNAs can impart different properties on liquid RNP droplets, including altered viscosity, fusion kinetics, and exchange rates with components with bulk solution. Typically, increased RNA content increased Whi3 droplet viscosity (Zhang et al., 2015). By contrast, the P granule protein, Laf1, forms liquid droplets whose viscosity is decreased by RNA (Elbaum-Garfinkle et al., 2015). Thus, RNA can tune liquid droplet viscoelasticity and dynamics in different ways. Several other papers show that addition of RNA can shift the phase boundary and facilitate formation of liquid RNP droplets (Burke et al., 2015; Lin et al., 2015; Molliex et al., 2015). Nonspecific RNA preparations that do not induce phase separation in Whi3 (Zhang et al., 2015) are able to induce phase separation of the more promiscuous RNA binders: TDP43, FUS, and hnRNPA1 (Burke et al., 2015; Molliex et al., 2015). Importantly, RNA can promote or inhibit the assembly of liquid droplets. While initial addition of RNA promotes formation of liquid droplets, higher concentrations of RNA actually inhibit liquid droplet formation (Burke et al., 2015; Zhang et al., 2015). Further study is necessary to understand the mechanism by which RNA exerts these differential effects on liquid phases. Some features to be considered include RNA sequence, secondary structure, length, charge distribution, and pattern of RBP binding sites along the mRNA. The results will not only be critical for understanding the biogenesis of membraneless organelles but will also provide valuable insights for designing therapeutic RNA to combat neurodegenerative diseases whose development is linked to aberrant accumulation of RNP granules (Li et al., 2013; Lin et al., 2015; Molliex et al., 2015). For example, RNAs might be designed to prevent or even reverse the maturation of FUS, TDP-43, or hnRNPA1 granules toward intractable solid-like states comprised of pathogenic fibrils.

Surprisingly, multiple studies suggest that disease-causing mutations in RNP proteins do not change the biophysical properties of the liquid droplets (Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015). Rather, disease-causing mutations promote maturation of liquid droplets to a more solid-like state with decreased molecular mobility comprised of potentially pathogenic amyloid-like fibrils (Figure 1). Indeed, it is suggested that maturation of liquid droplets to a solid-like state is a pathological step and high local IDR concentration in liquid separated phases can accelerate fibrilization and promote droplet maturation (Lin et al., 2015; Molliex et al., 2015; Patel et al., 2015). Thus, methods that promote LLPS at lower protein concentration, such as specific RNAs (Zhang et al., 2015), might be exploited to maintain RNP granule function while avoiding aberrant phase transitions to a solid state. Thus, RNAs could play therapeutic roles in regulating RNP granules and preventing disease development. We suspect that protein disaggregases might also be harnessed to reverse aberrant phase transitions and dissolve deleterious fibrils (Jackrel and Shorter, 2015).

On the other hand, it is important to note that phase separation is neither sufficient nor strictly required for fibrillization. For example, a hexapeptide deletion mutant of hnRNPA1 that does not fibrillize in vitro still phase separates into liquid droplets (Lin et al., 2015; Molliex et al., 2015). Moreover, hnRNPA1 can fibrillize without phase separation (although with decelerated kinetics). Thus, liquid droplet maturation and fibrillization are mechanistically distinct and separable processes. Further work is needed to determine whether solid-like mature droplets are physiologically relevant or whether they are invariably pathological. It will be important to dissect the interactions mediating fibrillization and droplet maturation and to determine their respective toxicities. We suspect that RBPs with LC domains can assemble into distinct fibril structures or "strains." Some fibril strains are likely toxic, whereas others are likely benign and even functional (Li et al., 2013). Finally, is the liquid phase invariably beneficial, or could inappropriate or excessive liquid states also contribute to disease pathogenesis?

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Endless Quarrels at the End of Genes

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Two recent papers in *Molecular Cell*, Fong et al. (2015) and Zhang et al. (2015), reopen the debate between the contribution of the allosteric versus the torpedo model of transcription termination.

The process of transcription termination must be as precise and robust as its initiation. Dismantling a stable and processive transcription elongation complex probably requires as much informational energy as its assembly. In eukaryotes, the 3′ end of an mRNA is defined by the position where both cleavage of the nascent transcript and the addition of a poly(A) tail occur. A large, multicomponent complex (in mammals called CPSF-CstF-CFI-CFII) is the main actor of this processing step, which occurs when specific signals are encountered on the nascent RNA. Termination of transcription occurs at a variable position after the site of polyadenylation and depends on the same signals and the same complex. Whether cleavage of the nascent transcript and transcription termination are causally linked has been the subject of intense investigation and debate for the last 15 years or so. In theory, 3′-processing of the RNA might occur co-transcriptionally and be required for termination to occur; alternatively, the two processes might be independent form each other although triggered by the same signal. As with every debate, founding facts support both hypotheses. The first hypothesis is supported by the fact that it has been difficult to conclusively separate the two events in vivo, and mutants that affect cleavage and polyadenylation also impact termination (Luo et al., 2006; Schaughency et al., 2014). In this "torpedo" model (Figure 1A), cleavage is proposed to provide an entry point for a 5′-3′ exonuclease, Xrn2 in mammals (West et al., 2004) and Rat1 in yeast (Kim et al., 2004), that catches up with the polymerase by degrading the still nascent 3′ RNA fragment and elicits termination. However, the impact of Xrn2 depletion on termination in mammals has been challenged by subsequent genome-wide studies (Nojima et al., 2015 and references therein), and in vitro studies have provided contradictory results on the capability of purified Rat1 and its exonucleolytic function to induce termination (Dengl and Cramer, 2009; Park et al., 2015; Pearson and Moore, 2013).

Early support for the independence of termination from cleavage comes from the analyses of Miller's spreads in *Drosophila*, revealing that at most genes termination can occur without apparent cleavage (Osheim et al., 2002). This alternative "allosteric" model (Figure 1B) posits that when polyadenylation/termination signals are transcribed, a conformational change occurs in the elongation complex that commits the polymerase to termination.

Two recent papers in *Molecular Cell* have stirred up the debate. In the first, Zhang and colleagues (Zhang et al., 2015) have developed an in vitro assay to decrypt the relationships between cleavage and termination. They assembled elongation complexes on synthetic DNA templates and used a ChIP-like strategy to assess the impact of polyadenylation signals (PAS) on the distribution of polymerases in the template and termination. Because the DNA contains biotin hooks, the assembled elongation complexes can be purified and their stability and functionality can be assessed under different conditions. This experimental setup recapitulates both PAS-dependent cleavage of the RNA and termination of transcription. Importantly, efficient transcription termination could still be observed under conditions in which cleavage was inhibited by omitting from the reaction creatine phosphate, which is required for the cleavage reaction in vitro. Because the loss of polymerases is evaluated relative to polymerases that have seen a non-functional termination signal, these experiments strongly suggest that elongation complexes that have transcribed a PAS are qualitatively different. The authors conclude that these elongation complexes have