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It Pays To Be In Phase

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Abstract

To survive, organisms must orchestrate many competing biochemical and regulatory processes in time and space. Recent work has suggested that the underlying chemical properties of some biomolecules allow them to self-organize, and that life may have exploited this property to organize biochemistry in space and time within cells. Such phase separation is ubiquitous, particularly among the many regulatory proteins that harbor prion-like intrinsically disordered domains. And yet, despite evident regulation by post-translational modification and myriad other stimuli, the biological significance of many phase-separated compartments remains uncertain. Many potential functions have been proposed but far fewer have been demonstrated. A burgeoning subfield at the intersection of cell biology and polymer physics has defined the biophysical underpinnings that govern the genesis and stability of these particles. The picture is complex: many assemblies are composed of multiple proteins that each have the capacity to phase separate. Here, we briefly discuss this foundational work and survey recent efforts combining targeted biochemical perturbations and quantitative modeling to specifically address the diverse roles that phase separation processes may play in biology.

Graphical abstract

Introduction

Throughout life, complex biochemical and regulatory transactions must occur within a crowded cellular milieu. One of biology's central puzzles is how these processes – which often involve rare components – are organized in space and time. Emerging evidence suggests that life has taken advantage of many biomolecules' natural tendency toward self-

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association to coordinate such processes. Lipids provide a striking example: their self-organization concentrates hydrophobic molecules in a separated phase. Lipid phase separation underlies many phenomena in biology including compartmentalization of organelles, but also the formation of lipid droplets, rafts, and exosomes^{1–4}. Proteins can also self-organize into liquid droplets and aggregates. Indeed, structural biologists capitalize on this behavior to facilitate protein crystallization. *In vivo*, such phase separation is often driven by intrinsically disordered protein domains – regions that do not adopt a single fixed structure⁵ – and can be promoted by interactions with other molecules⁶. Although it occurs throughout life, this form of phase separation reaches its fullest elaboration in eukarya^{5,7}. Indeed, even among eukaryotes the ubiquity of intrinsically disordered proteins scales with organismal complexity⁵. Yet, for pragmatic reasons, disordered protein regions have often been removed in biochemical studies of protein function. Consequently, the biological significance of many protein-based phase separation phenomena remains unknown.

At the same time, the list of such membraneless ‘organelles’ is ever-growing: germ granules⁸, nucleoli⁹, nuclear speckles¹⁰, P-bodies¹¹, stress granules¹², paraspeckles¹³, the Balbiani body¹⁴, pyrenoids¹⁵, chaperone bodies¹⁶, dynein assembly particles¹⁷, heterochromatin¹⁸, and centrosomes^{19,20} have all been characterized in this way. Although inclusion within a membrane has classically defined cellular compartments, the pervasiveness of protein phase separation suggests that it is also a fundamental organizing principle of cell biology. Here, we discuss some of the defining concepts that have been established in studies to date and survey the biological impact of this form of spatiotemporal organization (overviewed in Fig. 1).

Regulating phase separation

In homogenous *in vitro* preparations, many intrinsically disordered proteins (and others that participate in multivalent interactions) can spontaneously phase separate into liquid droplets. This process is non-linear with respect to protein concentration, meaning that small changes in protein level can exert a large impact on phase separation²¹ (Fig. 2A). Post-transcriptional regulation can also influence phase separation. Differential splicing, which is particularly common for exons that encode disordered protein domains²², can transform the capacity of a protein to phase separate by altering the size or even presence of the disordered region in the protein (e.g. in CPEB/Orb2^{23–25}; RbFox²⁶; hnRNP²⁷; Fig. 2B). In addition, compositional and environmental changes beyond protein abundance can have a large effect on phase separation. Multiple lines of evidence suggest that the threshold for phase separation can be robustly regulated within the cell, implying that this may be important to execute specific biological functions^{15,28, 31–51,28,29, 30–33, 34}.

Regulation by post-translational modification

Post-translational modification can regulate the assembly and composition of phase-separated bodies^{28,31–51}. Much of this insight comes from studies of stress granules. Many of the stresses that initiate eukaryotic stress granule assembly (e.g. hyperosmolarity, heat shock, DNA damage, and oxidative stress) do so through pathways that culminate in the phosphorylation of the translation initiation factor, eIF2 α ^{12,38}. This, in turn, triggers the

prion-like assembly of the disordered TIA-1 and TIAR RNA-binding proteins to seed stress granule formation^{39,40}. Similarly, phosphorylation of the endoribonuclease, G3BP, leads to its recruitment to stress granules, promoting their assembly^{41,42}. Phosphorylation can also drive disorder-to-order transitions that disrupt stress granules, as is seen with the phosphorylation of 4E-BP2⁴³, and Grb7⁴⁴. Phosphorylation and other modifications likewise provide a means of adjusting droplet composition; many of these events modulate the inclusion (or exclusion) of different components (Fig. 1), such as FUS⁴⁵ and RNA polymerase in stress granules⁴⁶.

Regulation of phase separation is not limited to phosphorylation. A myriad of modifications, including O-GlcNAcylation⁴⁷, methylation⁴⁸, and ubiquitination^{49,50}, also impact stress granule composition. Ubiquitination of stress granule components is coincident with stress granule aging (and solidification), accumulation of misfolded protein, and autophagic degradation^{49,50}. Analogous regulation via ubiquitination and autophagic degradation may extend beyond stress granules; polyubiquitin chains themselves can promote the formation of solid fibers susceptible to autophagic degradation^{51,52}.

Post-translational modification likewise regulates the phase separation behavior of germ granules, which include nuage in mammals, polar granules in *Drosophila melanogaster*, and P-granules in *Ceanorhabditis elegans*. P-granules provide a means of cytoplasmic polar partitioning to guide the development of the *C. elegans* embryo⁵³. The formation and dissolution of P-granules is controlled by phosphorylation of the intrinsically disordered proteins MEG-1 and MEG-3⁵⁵, establishing another means through which assembly can be regulated (Fig. 2C). Additionally, arginine methylation of Ddx4, a disordered component that scaffolds P-granules in *D. melanogaster*, hinders its phase transition *in vitro*⁵⁴, providing yet another layer of post-translational regulation. These examples, and many we discuss later in this review (e.g. phosphorylation of pericentrosomal components^{20,55}, SUMOylation of nucleolar PML^{56,57}, and PARYlation of DNA damage and stress granule components^{58–60}) highlight the prodigious capacity of cells to integrate signaling events with reorganization of their proteomes via phase separation.

Intrinsic modulation in response to temperature and pH

Although environmental inputs are frequently coupled to phase separation through post-translational modification, the impact of changes in the cellular environment can also be more direct. Heat stress is well known to cause general protein aggregation^{61,62}. A recent study in yeast suggests that >170 proteins, enriched in RNA-binding proteins, form small aggregates within minutes upon heat stress²⁸. Remarkably, these assemblies are reversible, and dissolve when cells are no longer heat-stressed. Some of these proteins, such as aminoacyl-tRNA synthetases, retain activity in their aggregated state. Others, such as translation initiation factors, are inactive. Indeed, this may help to slow global translation during heat shock²⁸. Such aggregation occurs independently of stress granule formation²⁸, and arises from the proteins' intrinsic sensitivity to aggregation upon small increases in temperature.

Among the most heat sensitive proteins in yeast is poly-A binding protein (Pab1). This RNA binding protein forms aggregates upon heat stress with an efficiency that has been likened to

a molecular thermostat⁶³. In normal growth conditions, Pab1 coats the 5' UTRs of heat shock protein mRNAs, preventing their translation. However, heat induced aggregation sequesters Pab1 away from these mRNAs, allowing for their selective translation during stress. Further support for this hypothesis comes from the observation that disrupting Pab1's ability to phase separate compromises the cell's capability to adapt to heat⁶³. Interestingly, reductions in temperature can also precipitate phase separation. For example, *D. melanogaster* Ddx4 self-assembles to seed P-granules in response to cold shock^{54,64}.

A number of metabolic enzymes can also rapidly aggregate in response to environmental cues. In bacteria, fungi, and insect cells, various metabolic inputs cause cytosine triphosphate synthase to form inactive filaments²⁹. In yeast, nutrient starvation can induce ~20% of cytosolic metabolic enzymes to reorganize into reversible assemblies⁶⁵. One of these enzymes, glutamine synthetase, does so in response to a drop in cytosolic pH caused by the nutrient depletion, thereby attenuating nitrogen metabolism in response to starvation^{65,66}. Similarly, yeast pyruvate kinase forms amyloid-like solid aggregates in response to glucose starvation⁶⁷. Importantly, the aggregates formed by both glutamine synthetase and pyruvate kinase resolve upon the removal of stress. The disordered sequences that govern the behavior seen in pyruvate kinase are also present in other enzymes involved in cell cycle progression, and many demonstrate analogous aggregation behavior⁶⁷.

Regulation by interaction with nucleic acids

RNA plays a key role in regulating many phase separation processes. The key P-granule protein PGL-3 self-assembles in an mRNA-dependent manner (as does MEG-3³⁵), allowing assembly to be manipulated through simple regulation of RNA abundance. During development the RNA binding protein MEX-5 depletes mRNA from the anterior of the embryo, thereby providing spatial regulation of PGL-3 phase separation^{36,68}. *In vitro* droplet preparations of the disordered stress granule component FUS can also be seeded by RNA incorporation⁶⁹, and hydrogel preparations readily incorporate RNA⁷⁰. Interestingly, the binding of different RNA sequences may control the biophysical properties of phase-separated bodies. In *Ashbya gossypii*, differential mRNA interactions with the polyQ rich protein Whi3 drive the formation of droplets that have very different physical properties: Whi3 assemblies that are seeded by *CLN3* are more viscous than those that are seeded by *BNII* transcripts. This difference correlates with the density and number of Whi3 binding sites inherent to the *CLN3* and *BNII* transcripts³⁷ (Fig. 2D).

Within the nucleolus, which itself has liquid-like properties, specific proteins can form sub-compartments composed of amyloid aggregates (termed 'amyloid bodies' or 'detention centers'). Prominent examples include the p53 inhibitor MDM2, which is sent to detention centers during p53 activation³⁰⁻³³, and VHL, which degrades HIF, the transcription factor responsible for regulating the cellular response to hypoxia. Here aggregation is seeded not by general interactions with nucleic acid, but rather by sequence-specific interactions with ribosomal intergenic spacer long non-coding RNAs⁷¹⁻⁷⁴. Yet these interactions also depend upon stress-induced protein conformational changes that expose RNA interacting motifs within the proteins themselves⁷³⁻⁷⁶.

Non-specific interactions with RNA also commonly occur in phase-separated bodies. For example, nearly all transcripts in the cell can be found in purified stress granules⁷⁷ and total mRNA purified from *C. elegans* can promote formation of PGL3 droplets⁷⁸. Moreover, the capacity of nucleic acid to regulate phase separation extends beyond RNA: the Ddx4 assemblies discussed above can be seeded by ssDNA⁵⁴ and post-translational modification with poly(ADP-ribose) has recently been appreciated as an important means of seeding stress granules as well as liquid droplets around sites of DNA damage^{58–60}.

Regulation, but by mechanisms that have yet to be established

In many cases phase separation processes are clearly regulated, but the molecular mechanisms at play are unknown. A striking example comes from photosynthetic metabolism in eukaryotic algae. These organisms partition the critical carbon fixation enzyme Rubisco (Ribulose-1,5-bisphosphate carboxylase) into an organelle called the pyrenoid. Such partitioning protects Rubisco from oxygen and enhances its catalytic efficiency of the reaction. When a structural protein necessary for the coalescence of Rubisco is deleted, Rubisco's CO₂ binding efficiency decreases tenfold⁷⁹. Although less disruptive techniques to specifically ablate phase separation will improve quantitative understanding of this system (e.g. those highlighted in Fig. 4), these data provide a benchmark for evaluating the advantage of the phase-separation on CO₂ fixation. Notably, pyrenoids are remarkably dynamic, fluctuating in size and composition – particularly their Rubisco content – in response to changes in CO₂, light intensity, and developmental cues³⁴. It appears that at least some of this versatility is afforded by the phase-separated nature of the pyrenoid¹⁵.

The very existence of multiple droplets within a cell is puzzling from a simple physical standpoint; phase-separated liquid bodies like stress granules should coalesce into one large droplet through a process known as Ostwald ripening^{80–82}. Yet hundreds of stress granules can coexist within a single mammalian cell, suggesting that there are barriers, whether active or passive, that limit their fusion *in vivo*⁸³. In some cases, the mechanistic bases of these barriers are known. For example, in *Xenopus*, F-actin networks prevent liquid-like nucleolar subcompartments from fusing⁷⁶. But in most other cases the mechanisms remain enigmatic. Theoretical work has proposed that chemically active liquid droplets can generate concentration gradient imbalances along their surface area, creating pressure discontinuities that divide droplets and maintain higher numbers of smaller droplets⁸⁴. Yet this theory remains to be experimentally tested as a governing principle in living cells.

The biological impact of phase separation

The evident regulation of phase separation behavior suggests that it has important biological functions. Indeed, the organization and assembly of several textbook cellular structures – such as centrosomes, nucleoli, and heterochromatin – may depend on phase separation. Below we discuss the many different known and proposed biological outcomes that can be driven by phase separation.

Inactivation

Phase separation can inactivate biomolecules through the formation of solid aggregates (illustrated in Fig. 1 & Fig. 3A and discussed below). Although this has sometimes been linked to pathology (e.g. for FUS in Amyotrophic Lateral Sclerosis), other solid aggregates have adaptive value^{28,85,86}. Inhibition does not require solid aggregation; in some cases, a liquid phase transition is alone sufficient. Stress granules, which sequester RNA, RNA modifying enzymes, and translation machinery, provide a prominent example. Because of this clear functional enrichment, it has been hypothesized that stress granules mainly serve as a means of mRNA triage, sequestering specific RNA sequences^{12,87,88}. Yet, as discussed earlier, nearly all of the cell's transcripts can be found in stress granules⁷⁷. These results suggest that stress granules may serve as a global means of mRNA regulation, rather than a mechanism for sequestering specific sequences.

Stress granules also serve to sequester proliferation and apoptosis signaling components. Incorporation of the RACK1 scaffold into stress granules drives cessation of apoptosis in response to specific stresses including hypoxia and heat shock⁸⁹. Likewise, the growth-associated kinase TORC1 is readily sequestered in stress granules in a phosphorylation-dependent manner, providing a regulatory link between these assemblies, translation, and proliferation⁹⁰.

Compartmentalizing function

Phase separation can also organize active processes, spatially and temporally compartmentalizing specific activities and functions. This has been postulated to prevent the phase-separated constituents from unwanted interactions, and create 'hubs' of metabolic and regulatory activity. Conceptually, such modular organization could facilitate efficient movement and regulation of the associated activity (Fig. 1).

Phase-separated compartmentalization has been proposed to play intriguing roles in cell structure. The *C. elegans* pericentriolar protein SPD-5 can assemble into liquid droplets that recruit microtubule-stabilizing factors and polymerases in a manner sensitive to phosphorylation (e.g. tubulin, PLK-1, SPD-2, SPD-5, and TPXL-1) and nucleate microtubules, effectively creating a liquid droplet centrosome, *in vitro*^{20,55}. FRAP studies of pericentriolar material have established that these factors also exhibit dynamics consistent with liquid-like behavior *in vivo*²⁰. Some other pericentriolar proteins such as BugZ in *Xenopus* demonstrate similar properties¹⁹, raising the exciting prospect that phase separation may be central to the assembly and function of the centrosome. These liquid qualities may enable pericentriolar material to automatically assemble centrosomes around centrioles⁸², and afford centrosomes effective selective permeability of components, strength, and flexibility²⁰.

Recent work has raised the intriguing question of whether heterochromatin is also subject to phase-separated compartmentalization. Heterochromatin protein 1 forms liquid droplets *in vitro* with higher order oligomerization upon phosphorylation^{18,91}. These droplets sequester heterochromatin-interacting molecules, hinting that such phase separation may play an important role in the formation and/or function of these chromatin regions^{18,91}. This

observation is particularly provocative in light of a phase separation based model for an opposing process: super-enhancer mediated transcription upregulation, discussed below⁹². Nevertheless, phase separation may afford heterochromatin a regulated means of nucleation and reinforcement of structure, providing testable hypotheses for several of its unusual behaviors (i.e. distal interactions, sensitivity to temperature, and protein dosage phenomena)¹⁸.

Non-linear behaviors

The advantages of phase separation may extend beyond simple sequestration. This form of assembly, in principle, has the capacity to minimize the barrier of free-diffusion and capitalize on the law of mass action to enhance reaction speed and specificity (Fig. 3B). Indeed, *in vitro* studies lend support to this supposition. A synthetic aqueous two-phase system can concentrate RNA up to 3,000-fold and increase the rate of ribozyme-catalyzed cleavage by 70-fold relative to a homogenous mixture⁹³. Likewise, *Escherichia coli* cell lysates induced to phase separate through coacervation show significantly enhanced rate of mRNA synthesis relative to lysates with dilute, non-phase-separated mixtures⁹⁴. Much progress has been made with *in vitro* reconstitutions of membrane bound signaling modules. Nck, WASP and Nephrin, components of a cascade manifesting in actin polymerization, can cluster *in vitro* to form liquid droplets that exhibit enhanced biochemical activity^{95,96}. More complex *in vitro* systems have yielded similar results: a biochemical reconstitution of a 12-component T-cell receptor activated pathway was capable of inducing the downstream response of actin filament assembly in a manner facilitated by droplet assembly⁹⁷. These examples and many others have motivated the frequent invocation of non-linear improvements in reaction dynamics as a rationale for the existence of phase-separated bodies in biology.

Toward an integrated understanding

In many systems, we now know what is sufficient to drive phase separation *in vitro* and *in vivo*, and are just beginning to understand what aspects of phase separation are biologically important. Although potential benefits are often inferred based on the components involved, our understanding of the advantage of phase separation over homogeneous and diffusion-based distributions is qualitative in most cases. The field is now primed to rigorously investigate these questions.

The importance of modeling

The application of theoretical models has generated some compelling hypotheses regarding the biological significance of phase separation. Indeed, the PGL-3 and MEX-5 interaction underlying P-granule formation and the droplet nature of centrosomes discussed above were both proposed with insight from theoretical models based on experimental data describing cellular concentration and rates of organellar growth^{78,82}. Modeling has also led some to hypothesize a role for liquid phase separation in transcription. Binding of transcriptional activators is a concerted process among transcription factors, transcriptional cofactors, chromatin regulators, enhancer and promoter sequences, and RNA polymerase. ‘Super-enhancers’ are a phenomenon in which hundreds of enhancers cluster to regulate genes that

facilitate cell-type-specific processes. Phase separation-based models have been proposed to explain some puzzling features of super enhancers, including their extreme sensitivity to inhibitors, higher levels of transcription relative to conventional enhancers, transcriptional bursting patterns, and simultaneous activation of multiple proximal genes⁹². Nevertheless, whether super-enhancers are generally phase-separated bodies has yet to be tested experimentally. Conversely, heterochromatin domains may also be phase-separated states, as discussed above^{18,91}. These examples, in which opposing processes (gene activation and silencing) both employ phase separation, highlight the diverse potential biological impacts of such behavior.

From test tubes to cells

Assays borrowed from polymer physics have been critical to understanding the dynamics that modulate the assembly, maintenance, and loss of liquid-like droplets. Yet care must be taken with extrapolation of *in vitro* findings. For instance, 1,6-hexanediol can dissolve stress granules *in vitro* but instead stimulates their formation *in vivo*⁹⁸. Likewise, although protein phase separation *in vivo* is a complex process involving many constituents, *in vitro* studies of protein phase separation have been largely restricted to one or two component systems. Parallels for appreciating the potential complexity of multi-component protein phase separation can be drawn from the field of lipid biology, which has benefited from treating lipid phase separation as a multicomponent phenomenon^{1–4,99,100}. This has led to an in-depth understanding of how individual membrane components contribute to the range of physical properties (e.g. fluidity, rigidity) and diverse phenomena (e.g. rafts, microdomains) of lipid composites beyond those of homogenous assemblies^{1–4,99,100}.

In vitro phase separation of homogenous protein is typically dependent on stochastic nucleation events and blind to other components involved in phase separation. However, in a multi-component context (e.g. the cell), other components completely alter the behavior of phase-separated bodies. For instance, in *Drosophila*, rDNA repeats are important for the formation of the nucleolus. Nucleolar components still phase separate in the absence of rDNA repeats, but with high spatiotemporal variability¹⁰¹. This suggests that the stochastic nucleation-limited phase transition observed in *in vitro* assays is not reflective of the precise and growth-limited phase separation observed in the nucleolus¹⁰¹. Furthermore, some components of the nucleolus, such as Mod and Ns1, are actively incorporated to the nucleolus only after it is formed, in contrast to other components, such as Fibrillin and Rpl135, which passively phase-separate into the compartment¹⁰². That is, the capacity of these two nucleolar components to phase separate, is entirely dependent on other proteins¹⁰². Given the rich knowledge of multicomponent lipid phase separation, this dependency not surprising – and it portends a complex landscape of protein phase separation that is only beginning to be explored.

Yet despite apparent differences on the mesoscopic scale, the chemical footprints of some phase-separated proteins (e.g. hnRNPA2) are very similar *in vivo*, in reconstituted liquid droplets, and in *in vitro* hydrogels¹⁰³. Moreover, even observations made *in vitro* and well above physiological concentrations have provided fruitful inroads to identify basic principles of phase separation and means through which it can be regulated^{85,86}. DNA-PK

phosphorylation of the FUS low complexity region inhibits droplet formation and hydrogel inclusion *in vitro*. Ablation of these phosphosites by alanine substitutions stabilizes them (Fig. 4A), suggesting a means of regulating the physical state of the protein and its assembly⁴⁵. More generally, *in vitro* studies have indicated that the high level of intracellular ATP may act as a biological hydrotrope that encourages protein solubility¹⁰⁴.

Understanding the consequence of spatial organization poses a unique challenge of gathering quantitative data at a subcellular spatial scale. Recent advances such as proximity labeling are starting to be applied to phase-separated bodies, enabling comprehensive interrogation of molecular interactions in the phase-separated state¹⁰⁵. But obtaining accurate spatial interaction maps is only half the battle: it is essential to quantify the output of the phase-separated biological process to relate phase separation to function and define the impact of phase separation on a particular biological process (Fig. 3).

The need for selective perturbation

One of the biggest challenges in the field is determining effective means of selectively disrupting phase separation without perturbing the other functions of the molecule being studied. The capacity to systematically generate partial-loss-of-function mutants of this type would allow researchers to isolate the impact of phase separation *per se*. The most commonly employed perturbation is simple deletion of disordered protein regions. However, intrinsically disordered sequences also regulate protein-protein interactions, half-life, and harbor multiple post-translational modification sites¹⁰⁶, complicating interpretation. Other approaches, such as mutating all amino acids of one type within an intrinsically disordered region, are similarly perturbative.

Genetics provides one fruitful avenue for more targeted perturbation. Disease-associated point mutations in the disordered region of FUS dramatically shift the resulting phase-separated bodies from a liquid-like to solid state both *in vitro* and *in vivo*¹⁰⁷ (Fig. 1 & Fig. 4B). Investigation using similar logic has provided insight into the link between the material properties and function of dynein assembly particles (DynAPs), phase-separated foci where dynein motors are assembled to facilitate ciliary beating¹⁷. Ciliopathy-associated mutations in dynein-specific assembly factors that impair ciliary beating also reduce the dynamicity of the DynAPs (without altering their presence), suggesting that the liquid-like quality of this phase-separated particle is important for its biological function¹⁷.

Evolution provides another potential avenue for selective perturbation. In the case of Pab1, Riback and colleagues were able to identify hydrophobic amino acids under high selective pressure and use this information to design point mutations in Pab1 that disrupt its capacity to phase separate⁶³ (Fig. 4C). These mutants showed a strong fitness defect in stressful environments, specifically linking phase separation to biological phenotype⁶³. Employing similar strategies will likely prove invaluable in the future.

Concluding Remarks

Phase separation is now appreciated as a pervasive form of organization in the cell (Fig. 1). Emerging work suggests that this behavior may facilitate complex organization of

assemblies with different hierarchies of shells and cores. The ensuing biological consequences can be extraordinarily diverse. Insights continue to arise at a furious pace: even RNA, independent of protein, can assemble into liquid-like droplets, opening dimensions of phase separation biology that have yet to be explored¹⁰⁸. As our knowledge grows, the complexity of phase-separated systems is becoming clearer, hinting at characteristics with utility beyond the sum of their parts. Such insight brings with it many questions: How do phase-separated bodies interact with one another? How structured and dynamic are the liquid droplets we observe in cells? How heterogeneous is any given pool of membraneless organelles with respect to composition and function?

And perhaps the most intriguing: what new emergent properties might be afforded by phase separation? The disordered domains that often drive phase separation are frequently described as ‘prion-like’. *Bona fide* prions provide a striking example of the complex behaviors that can be mediated by cooperative protein assembly and aggregation. Prions have the unusual ability to adopt alternate conformations, often with different catalytic capabilities, that can template said conformation in other copies of the same protein. For example, upon conversion to the [*PSI*⁺] prion state, the translation termination factor Sup35 forms amyloid fibrils, resulting in stop codon readthrough events that impart phenotypes adapted to different stresses^{109,110}. Sup35 can also form gels that correlate with survival in response to some stresses¹¹¹. Like other forms of phase separation, this is a self-organizing behavior driven by intermolecular electrostatic and multivalent interactions – often between intrinsically disordered proteins^{112–114}. However, in the case of prions, the self-organization is so robust that it can drive positive-feedback loops that can underlie hysteretic behavior and cytoplasmic inheritance^{112–114} (Fig. 3C). In contrast to prions, many phase-separated bodies break and reform each cell. Yet the similarities between protein domains that drive phase separation and those that drive prion behavior begs the question of whether a similar form of positive feedback might be inherent to other phase separation processes.

Indeed, the integration of phase separation and prion-like behavior can have remarkable ramifications. This is starkly illustrated by the Balbiani body, a ‘super’ organelle found in many animal oocytes that sequesters and allocates other organelles (e.g. endoplasmic reticulum, mitochondria, and the Golgi body) into the developing egg, and is necessary for the polarity of development¹¹⁵. Xvelo, the main protein constituent of the *Xenopus* Balbiani body, has a prion-like domain that is necessary and sufficient to form a physiological, reversible amyloid. Recent evidence suggests that this prion-like nature is required for proper Balbiani function¹⁴. Both loss and mutation of this prion-like domain hinder aspects of Balbiani maintenance including recruitment of mitochondria and assembly¹⁴ (Fig. 4D). Given that eukaryotic proteomes are replete with intrinsically disordered domains that could have a similar capacity for reversible amyloid behavior, the striking emergent properties of this particular example are unlikely to be exceptional.

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Figure 1. Spatiotemporal regulation afforded by phase-separated compartmentalization

A mixture of cellular components, protein and RNA, are shown in a cell along with different schemes of assembly, representing the spectrum of phase-separated phenomena discussed throughout the text – solid aggregation (far left) and four examples of liquid droplets. These phase-separated compartments can form and alter their composition in response to stimuli such as post translational modification, environmental changes, and RNA incorporation, allowing for the activation or inactivation of different components (middle three). Nucleic acids, e.g. mRNA, can seed or inhibit droplet formation (far right).

Figure 2. Regulating phase separation

A) A generic example of phase separation. As either protein concentration or the interaction strength between protein molecules increases, intrinsically disordered proteins (green) approach assembly of a phase-separated droplet, which can also incorporate other components (blue). Many different forms of regulation allow for manipulation of interaction strength, including posttranslational modifications, scaffolding by RNA, differential splicing, and changes in intracellular pH. Assembly can allow the components access to a different set of functions and, consequently, enables access to different biological phenotypes (from Phenotype B to A).

B) Differential splicing: hnRNP isoforms with different de-mixing propensities.

Differential splicing of hnRNP transcripts results in protein isoforms with varying amounts of disorder. Consequently, the assemblies of the hnRNP isoforms exhibit different propensities for phase separation²⁷.

C) Post translational modification: MEG phosphorylation in P-granules.

Phosphorylation of the P-granule components, MEG-1 and MEG-3, imparts negative charge that inhibits their assembly. Consequently, phosphorylation by the kinase MBK-2/DYRK induces disassembly and dephosphorylation by the phosphatase PP2A induces assembly^{35,36}.

D) RNA inclusion: CLN3 and BNI1 mRNA seeding Whi3. Whi3 assemblies are seeded by mRNA through sequence-specific, protein-RNA interaction motifs spaced across the length of an individual transcript. *CLN3* and *BNI1* transcripts both seed Whi3 droplets but *BNI1* droplets are less viscous. This is because *BNI1* is a longer transcript with Whi3 binding sites spaced much farther apart. This allows for regulation of droplet viscosity by modulating the abundance of different transcripts³⁷.

Figure 3. Emergent properties of phase-separated bodies

A. Phase separation can allow for multiple impacts on biochemical activity. For the hypothetical reaction: $A \rightarrow B$, where the substrate A is converted to the product B by two intrinsically disordered proteins capable of forming phase-separated assemblies, we show plots of the reaction rate against the substrate [A].

B. Rate reduction: Sequestration of components via phase separation can result in inactivation that decreases the reaction rate.

C. Rate enhancement: Phase separation can increase the proximity of the proteins responsible for converting A to B, increasing the frequency of interaction and, consequently, reaction rate. Additionally, phase separation can also protect the substrate A from unfavorable competing interactions to increase the reaction rate.

D. Hysteresis: Positive feedback can also operate in the context of phase separation, such as the self-templating formation of prion assemblies, which can give rise to a hysteretic relationship. If the assembled state has an enhanced reaction rate and assembly promotes additional assembly, then the rate of B production relative to [A] could be hysteretic. At one particular [A], there could be either no phase separation (and, thus, a low reaction rate) or complete phase separation as a result of positive feedback (and, thus, a high reaction rate).

Figure 4. Targeted perturbations of IDRs to interrogate function

A) Removal of post-translational modification sites: FUS. If there are characterized PTM sites in the IDR, altering these could offer a specific avenue to disrupt phase separation. For instance, phosphorylation of the low complexity domain of FUS inhibits droplet formation. Mutating two phosphorylation sites to alanine in the IDR prevents the phosphorylation inhibition of droplet dissolution³².

B) Introduction of a disease-associated mutation: FUS. The patient-derived mutation, G156E, in the prion-like domain of FUS alters the material state of FUS phase-separated bodies. The liquid droplets of FUS G156E have a drastically reduced ability to fuse together and mature into striking fibrous ‘starburst’ structures that are significantly less dynamic than the WT FUS assemblies⁹⁶.

C) Mutation of conserved residues: Pab1. Aliphatic residues in the intrinsically disordered P-domain of Pab1 are conserved, suggesting that hydrophobicity is undergoing natural selection. Mutation of these residues abrogates Pab1’s normal ability to demix in response to heat shock, and reduces fitness in response to thermal stress⁵².

D) Mutation of charged amino acids: Xvelo Prion-like domains are replete in charged amino acids, as they interfere with hydrophobic interactions. Mutating as few as 4 charged residues in the prion-like domain of Xvelo increases the dynamicity of the assemblies *in vivo* and eliminates Xvelo’s ability to self-assemble *in vitro*. Furthermore, wild-type Xvelo assemblies are able to bind mitochondria, a critical function of the phase-separated organelle they form. Mutated Xvelo loses this ability, suggesting that this function is dependent on assembly formation¹⁰.