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Review

There Is an Inclusion for That: Material Properties of Protein Granules Provide a Platform for Building Diverse Cellular Functions

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Proteins perform a staggering variety of functions in the cell. Traditionally, protein function was thought to be hard-wired into the folded structure and conformational dynamics of each protein molecule. Recent work describes a new mode of protein functionality driven by the collective behavior of many different proteins; most of which lack a defined structure. These proteins form clusters or granules in which unstructured polypeptides interact transiently. Nonspecific multivalent interactions drive the formation of phase-separated structures resembling aggregates. This type of functional aggregate granule can be thought of as a single supermolecular functional entity that derives function from its unique material properties. In this review we examine the emerging idea of protein granules as a new functional and structural unit of cellular organization.

Trends

Protein granules represent a diverse set of phenomena including clustering of enzymatic factors, liquid–liquid demixing or phase separation, formation of a solid-like aggregate, and amyloid fibril assembly.

Aggregation of proteins containing low-complexity domains and prion-like domains is a platform for building diverse cellular functionality by modulating the contents and stability of the aggregate.

The material properties of granules can be the basis for functions such as filtration and memory storage.

Granules can act as molecular switches and co-occurrence sensors, modulating a vast array of cellular functions.

Aggregation and Granules

Protein folding is driven by the hydrophobic effect, favoring the exposure of hydrophilic amino acids to the aqueous environment, and the burial of hydrophobic residues in the inner core of the protein [1–3]. Folding was historically regarded as the essential basis of function for any polypeptide chain. The phenomenon of protein self-association into aggregates, which becomes thermodynamically favored in the absence of folding, has therefore been thought to preclude protein function.

An alternate perspective on intracellular aggregates emerged when fluorescence imaging enabled the direct observation of protein localization in inclusion structures [4–7]. It thus became apparent that the subcellular organization of aggregates is not random, and that aggregation is therefore a regulated process. This new concept fueled the hypothesis that aggregated proteins might retain some element of identity and prompted the characterization of dozens of inclusion structures, cellular bodies, or granules to add to those that had been previously observed by electron microscopy and other techniques [4,6,8–16]. Several regulatory roles were proposed for the aggregates, including storage, sequestration, partitioning, and degradation of aberrant proteins and RNAs in order to protect the cell [9,17–21].

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Over the past decade, the list of granule structures has grown tremendously, complicating the phenomenology and etymology in the field. This is further compounded by the fact that some aggregate-resembling structures are more accurately described as **liquid droplets** (see Glossary), in that their constituents are highly dynamic and interact transiently with each other. In other words, the biophysical properties of cellular granules occupy a continuum from entirely liquid to completely insoluble. The common features of granule structures are: a lack of membrane surrounding them; a highly context-dependent occurrence; and a transient localization of protein markers to the granules. Attempts to consolidate these structures into a single category has given rise to the term **membraneless organelles**. The rapidly expanding phenomenology of such membraneless organelles suggests spatial **clustering** or granule formation may be the norm rather than the exception for many cellular proteins.

Alongside the expanding phenomenology of aggregate structures and granules, there is a new appreciation of the different ways in which these structures can form. Initially, cellular inclusions were thought of as out of equilibrium kinetically trapped precipitates in which protein aggregation is driven by swapping hydrophobic domains or Beta sheets. In accordance with *in vitro* observations, aggregates grow in size as a function of concentration after a long nucleating lag phase. More recently, it has been proposed, and in some cases demonstrated, that local heterogeneities in protein concentration can exist in a dynamic equilibrium that is consonant with the free energy differences between states [22]. It has been suggested that liquid–liquid phase separation is an example of dynamic equilibrium droplet structures formed in response to phosphorylation of droplet components, which changes their interaction properties. However, it is equally possible that droplet formation is an out of equilibrium steady state driven by the constant consumption of ATP that allows for maintenance of diffusive molecules in an unmixed state [22,23].

Aggregating Terminology

Differences of approach exist as to the terminology that is best suited for writing about protein self-association. First, many of the terms used to describe cell biological entities that look like granules [e.g., inclusion, inclusion body, inclusion structure, aggregate, dynamic droplet, membraneless organelle, (function)osome, and foci] are all to some extent etymologically contaminated by pre-existent notions of the biophysical and functional properties generally attributed to the term. Hence, the term aggregate often carries the connotation of a disordered, dysregulated, and pathological structure that has low internal mobility and excludes water. Second, it is infrequent that a distinction is made about the granule structure and the biophysical processes that may have contributed to its formation.

Since the field is far from settled on a unifying terminology, this review uses several of the above terms interchangeably. Cell biological structures are referred to as clusters, granules, or aggregates, without making assumptions about which biophysical model best describes their formation. The process of granule formation is referred to as aggregation in general, encompassing all known types of self-association. Where possible, a distinction is made between specific types of self-association (e.g., liquid–liquid phase separation or **amyloid** formation). It is important to note that a granule/aggregate may initially form as a result of a **phase transition**, and then mature into a crystal-like aggregate. Often, no single mechanism of formation can fully describe a cell biological granule. For example, synphilin 1 aggregates initially form through a first-order phase transition [24], and only later mature into insoluble, hydrophobically crosslinked aggregates resembling Lewy bodies. Conversely, stress granule (SG) components have been shown to undergo **liquid–liquid phase separation (LLPS)**, but can also form amyloid-like aggregates when overexpressed, mutated, or aged [25,26,89,90]. An important distinction seems to be between structures that are kinetically trapped and therefore stable (many aggregates and amyloids would fit into this category) and granules that

Glossary

Amyloid: ordered crystalline form of protein polymer that exhibits a regular structure usually held together by beta-sheet interactions. Amyloid structures exclude most water molecules and stain with dyes like thioflavin-T.

Amyotrophic lateral sclerosis (ALS): fatal degenerative motor neuron disease, also known as Lou Gehrig's disease. In addition to mutations in superoxide dismutase, mutations in the genes encoding RNA-binding proteins FUS, TDP-43, and several others are common in families with histories of ALS. Aggregates of these proteins are commonly found in the brains of affected individuals.

Clustering: stochastic local enrichment of proteins in a submicron cellular space. Clusters can be homotypic or heterotypic and have a short half life. No assumptions are made about what types of interactions (e.g., phase separation, hydrophobic interactions, and LCD interactions) drive clustering or keep the clustered components together. Typical cellular examples of clustering include Pol II clusters and receptor clusters.

Frontotemporal dementia (FTD): progressive degeneration in the frontotemporal lobe. ALS and FTD show overlapping features, and are now thought to be part of a spectrum disorder.

Hydrogel: Well-hydrated matrix of proteins that are crosslinked, minimizing diffusion. Hydrogels are nevertheless permeable to other molecules.

Intrinsically disordered proteins (IDP): proteins lacking a single defined folded structure. IDPs make up a large part of the proteome, and are especially prevalent among RNA- and DNA-binding proteins. An IDP typically has multiple ID regions.

Liquid crystal: structured fluid in which molecules are disordered (unlike in amyloids of solid crystals) but nevertheless can be induced to rearrange by switch-like mechanisms (e.g., phosphorylation).

Liquid granules or droplets: phase-separated structures that are held together by promiscuous interactions among the low LCDs of the constituents, and likely also by energy produced from continuous ATP hydrolysis. They are thought to

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are in dynamic equilibrium that is constant with free energy differences between two states (LLPS droplets are thought to fit into this category). The latter have rightly gained much attention of late because they are thought to be functional modules. However, it is important to consider that kinetically trapped amyloid-like aggregates such as yeast prions can also serve important cellular functions, and can be disassembled with the input of energy.

From the perspective of cell biology, it is also important to differentiate between the observable (e.g., a granule with a specific marker and with measurable properties) and the biophysical model for how protein self-association might take place in a given context. A specific granule may form as a result of multiple biophysical processes. Granule components may behave as ideal phase-separated liquid droplets when purified *in vitro*, but may nevertheless form crystal-like aggregates *in vivo*, and *vice versa*.

Cluster Function – Cellular Regulation Arising from Disorder

Recent work in the fields studying aggregation and membraneless organelles has led to a new way of talking about the role of granule structures. One of the most important realizations, put forth by Lindquist and colleagues, was that aggregation can modulate the function of potent cellular regulators, for example transcriptional and translational repressors, and that the aggregation state can be heritable [27–30]. The heritability of aggregation states resembles infectious prions [31], and one of the structural features enabling prion-like inheritance (for proteins other than PrP^{Sc}) is often referred to as a **prion-like domain (PrLD)**: a domain that is highly enriched in glutamine or asparagine residues. Another essential discovery was that a surprisingly high percentage of the eukaryotic proteome has a PrLD or a related sequence feature, either a **low complexity domain (LCD)**, or an intrinsically disordered region (IDR). Such proteins have the inherent property of condensing into a liquid-like aggregation state through liquid–liquid demixing [32–34], or forming solid-like amyloid fibrils through cross beta polymerization [35,36]. Liquid–liquid demixing in the cell creates two separate phases: a mixed cytoplasmic (or nucleoplasmic) phase, and a demixed droplet-like phase in which proteins are retained through multivalent interactions between their LCDs. Demixed liquid phases can transition to other phases including **liquid crystal** like (where molecules flow slowly and can rearrange in response to stimuli), **hydrogel** like (where filaments are cross-linked), or crystal like (where regular solid structures emerge) [22,33,37]. The self-association of proteins in liquid-like droplets is sensitive to environmental conditions and can be regulated by simple switch-like mechanisms [25,26,38], whereby information (in the form of DNA, RNA, enzymes, and other functional proteins) can be stored and retrieved at varying times.

Together, these and other findings have upended our understanding of protein function and aggregation. Over the past few years, there have been groundbreaking studies showing that the inherent tendency of many proteins to aggregate underlies a new category of protein structure that is distributive over protein populations rather than protein autonomous. This structural feature is exploited by the cell as a platform for generating a diverse and flexible set of functionalities. Whereas altering the function of a protein requires changing dozens of structural parameters, the function of protein granules can be regulated by modulating two determinants: the sets of proteins that aggregate together, and the stability of the aggregate over time. Aggregation and phase separation are also faster than other modulators. Transcription, translation, degradation, and kinase cascades are time consuming, whereas aggregation can sequester, modulate, and release thousands of different proteins instantaneously.

In this review, we discuss a few cellular functions that exploit protein aggregation, and highlight regulatory features enabled by different modes of protein self-association: spatial clustering, phase separation, and amyloid polymerization (Figure 1). While some aggregate functions, including signal transduction, amplification, and cooperativity, work in a way that is similar to

be well hydrated. Importantly, liquid granules exhibit properties of viscous liquids including diffusion of components within the droplet, as well as in and out of it. They can also be observed to shear and flow within the cytoplasm or nucleoplasm.

Liquid–liquid phase separation (LLPS): process of demixing between two solutes. LLPS has been proposed to occur in the cell and to lead to the formation of different granules, particularly some SGs, P-granules, nucleoli, Nuage bodies, histone locus bodies and many other types of RNP bodies and nuclear bodies. LLPS does not require strong interactions between components of the liquid granule that results.

Low complexity domains (LCDs): protein domains containing arginine–gylcine (RG) and/or arginine–glycine–glycine (RGG) repeats. Can confer affinity for RNA and DNA, and involved in forming multivalent weak interactions required for phase separation.

Membraneless organelles: cellular structures that are not bound by membranes and are held together by protein–protein interactions. Also referred to as granules, dynamic droplets, inclusions, cellular/nuclear bodies, or membraneless droplets. Membraneless organelles are formed by different subtypes of protein aggregation, described below.

Phase transition: processes of nucleation and growth of phase-separated structures, whether resulting from liquid–liquid demixing or liquid to solid transitions. Phase transitions have been proposed to underlie the formation of crystalline aggregates from liquid granules, with implications for cellular pathology.

Prion-like domain (PrLD): another type of protein domain that can drive higher-order assembly. PrLDs are glutamine(Q)/asparagine(N) enriched, charge-depleted motifs that may be considered a subclass of LCD/IDPs.

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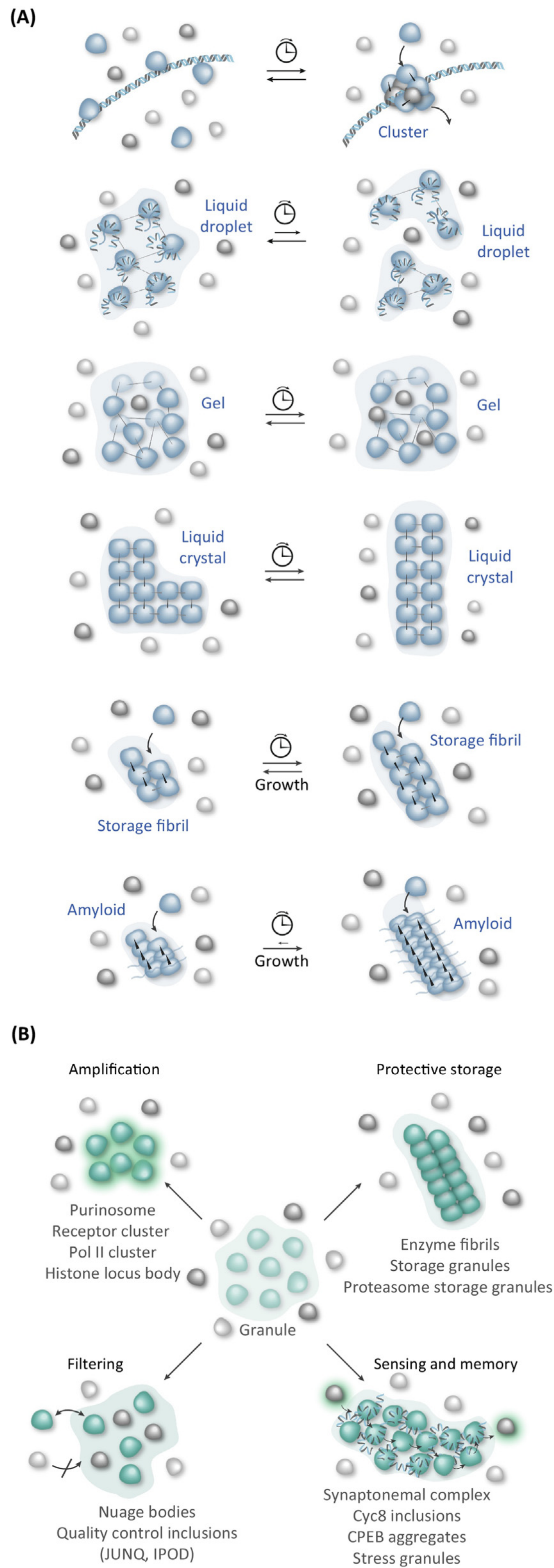

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traditionally studied protein complexes, some newly discovered functions seem to be unique to aggregate structures.

Amplification

One of the key functional features that spatial clustering enables is the ability to create nonlinearities in the associations and activity of enzymatic components by concentrating enzymes in ‘factories’ where local concentrations of reagents and cofactors are high. In many ways this is similar to cooperative binding and kinase signaling cascades in nonaggregating proteins. For example, clustering allows *de novo* purine biosynthetic enzymes to function far from equilibrium in aggregate-like factories that tend to form during stress. Purinosomes or metabolons are membraneless organelles resulting from the clustering of enzymes that cooperate in a pathway [39]. Metabolons increase the metabolic flux through the complex of enzymes, and channel intermediates that have low half-lives in solution [12]. Stimulating purinosome formation has been shown to significantly increase metabolic output [40]. Recently, glycolytic enzymes have been shown to cluster into glycolytic metabolons in the *Caenorhabditis elegans* synapse in response to energy stress [41]. The formation of glycolytic metabolons increases local ATP output. Conversely, failure to form metabolons significantly impairs synaptic recovery and function. Although the precise structural features driving metabolon formation are not completely clear, as with many other clusters subunit phosphorylation has been implicated as a regulatory step and the cluster appears to be nucleated by a scaffold of regulatory proteins.

Similarly, it was recently shown that the transcriptional output of RNA polymerase II (Pol II) directly correlates with the lifetime of Pol II clusters that are formed at transcription initiation sites prior to initiation, implying that Pol II clustering increases the efficiency of transcription [42]. As with metabolons, the model explaining this phenomenon is that the tendency of Pol II to cluster crowds the site of initiation with a regulated dose of rate-limiting factors that must assemble into large complexes before transcription can begin. Clustering, in this case, amplifies the signal to initiate transcription by using available resources more efficiently. The structural basis for clustering is thought to be an LCD in Pol II that consists of a series of 52 repeats of the sequence YSPTSPS [36,42]. Phosphorylation of residues in this sequence completely abolishes clustering and releases the polymerase for initiation. Interestingly, unlike many other phase-separated granules formed by LCD RNA-binding proteins [e.g., fused in sarcoma (FUS), TAR DNA-binding protein (TDP)-43, and heterogeneous nuclear ribonucleoproteins (hnPNPs)], Pol II does not form homotypic droplets, but instead is coordinated into the cluster by additional LCDs.

Pol II spatial clustering is a specific instance of a more general feature of many nuclear enzymes. The nucleus is replete with a diverse set of nuclear bodies, which organize the regulation of gene expression and genome regulation. Nuclear bodies have been directly demonstrated to

Figure 1. Harnessing Aggregation for Generating Cellular Functions. (A) Protein granules that appear in the cell can represent a large variety of aggregate structures, some of which is depicted here. Clustering is usually thought of as a stochastic and transient formation of a complex composed of weakly interacting constituents. Liquid–liquid phase separation results in a liquid-like droplet in which constituents interact through weak low complexity domain interactions and retain high internal mobility and high rates of exchange with the environment outside of the droplet. Granules can also be in the form of a liquid crystal, in which the constituents interact weakly, but have a regular structure with lower internal mobility. Gel-like granules have components that are crosslinked with strong interactions, but are still permeable to other proteins and water. Solid-like (amyloid) fibrils have a defined regular structure, almost no internal mobility nor exchange, and very strong interactions between constituents. Amyloids are usually thought to form from misfolded or unfolded proteins, whereas solid-like fibrils can form from constituents that are folded and functional. (B) The aggregation properties of proteins are exploited by the cell to build a diverse array of functional membraneless compartments. These structures can regulate signal amplification, filtering of cellular components, signal sensing, and memory storage. Some examples are given here.

increase efficiency of enzymatic reactions. For instance, the histone locus body amplifies the activity of the Flice-associated huge protein (FLASH) by concentrating it with cofactors such as the U7 small nuclear RNA [43]. FLASH is only able to efficiently process histone mRNA when concentrated in histone locus bodies together with cofactors.

In a different type of clustering, 2D phase separation of receptors can modulate multiple step functions in order to amplify the ligand signal, as well as the sensitivity to receptor ligand binding. Recent studies have linked receptor clustering to coclustering of actin filaments, which in turn promote receptor aggregation, generating a feed-forward loop. In particular, Su *et al.* [44] recently examined receptor signaling in T cells, which must constantly process and evaluate a massive amount of stimuli. The study examines the behavior of a T-cell receptor (TCR) effector, linker of activated T cells (LAT), and showed that it undergoes 2D phase separation together with other effector molecules, in response to TCR stimulation. The formation of the phase-separated microdomain on the membrane amplifies signaling to the actin cytoskeleton via integration with the actin nucleation factor neural Wiskott–Aldrich syndrome protein (N-WASP), and primes F-actin polymerization into a gel. This in turn alters the shape of the phase-separated microdomain and promotes further clustering in the shape of the actin gel. What is interesting about the material properties of the receptor–effector–cytoskeleton components of this signaling pathway is that they enable the integration of multiple environmental stimuli into a relatively simple set of switches, resulting in a finely tuned response.

Filtering

In addition to enhancing efficiency and amplifying signal, the material properties of phase-separated granules and clusters enable aggregates to function as filters and triage stations in the cell. Since these structures can sequester or remain permeable to specific components, they provide a way to protect vulnerable and reactive species, as well as triage components based on specific characteristics. The nucleus has a high abundance of phase-separated granules, including Cajal bodies, histone locus bodies, speckles, promyelocytic leukemia bodies, nucleoli, Nuage bodies, Pol II clusters, and many other ribonucleoprotein (RNP) granules [42,45]. This is not surprising since a large percentage of RNA- and DNA-binding proteins have LCDs, and because precise compartmentalization is essential to nuclear functions, especially regulation of gene expression. RNP granules regulate RNA transcription and processing, and they can function as molecular switches by modulating local concentrations of active components in response to signals. An interesting property of some RNP granules is the ability to filter out specific types of components. For example, sites of DNA damage prime the formation of phase-separated droplets containing fused in sarcoma (FUS) and other LCD proteins. Recently, it was shown that this is triggered in part by a local increase in the production of a nucleic-acid mimicking molecule poly(ADP-ribose) (PAR), which nucleates liquid–liquid demixing. The droplets formed by PAR have been proposed to protect exposed DNA ends from exonucleases, while concentrating repair enzymes [37,46]. Taking a closer look at DNA-primed phase separation, Nott *et al.* [47] investigated the phase-separation properties of a DEAD-box RNA helicase DDX4, which forms Nuage bodies in germ cells. The study demonstrates that DDX4 droplets are selectively permeable to single-stranded DNA and RNA, while filtering out double-stranded DNA. A follow-up study shows that this selective permeability gives rise to more complex functions such as melting nucleic acid duplexes into single strands in the droplet interior [48].

A particularly interesting example of phase-separation-based filtration has been proposed for nuclear pores. Nuclear pore complexes (NPCs) are multiprotein machines that ensure the selective permeability of the nuclear envelope. Only proteins that are below ~30 kDa or are bound to a shuttling factor are able to pass the NPC. NPCs do not have a catalytic component,

but must rely solely on their structural properties to ensure the selective permeability. It has recently been proposed that, similar to Nuage bodies, NPCs derive their function as filters from disordered multivalent FG dipeptide repeat motifs. Unlike Nuage bodies, NPCs are not aggregates. However, Schmidt and Gorlich [49,50] demonstrated that NPC constituents (Nups) phase separate *in vitro* and *in vivo*, and form gels that have selective permeability for nuclear transport receptors. It appears, therefore, that the inner surface of NPCs acts like a gel phase that is permeable to cargo that is soluble in the gel. Since most cellular proteins have charged residues on their surface, nuclear-targeted proteins (above the exclusion limit) must be coated with sufficient quantities of receptors to occlude the cargo in order to pass through the Nup gel phase.

Triage of Harmful and Useful Aggregation

The phase-separated granules in the nucleoplasm are important for protecting and spatially organizing different types of nucleic acids, whereas the cytoplasm has a parallel system for spatially organizing a diverse set of translation products. Most translation occurs in the cytoplasm, generating folding intermediates that are sensitive to the local environment and to stress. Stress and aging also lead to the misfolding of native cytoplasmic proteins, which must be triaged for refolding, degradation, and protective aggregation by the protein quality control machinery [6,15,17,51–53]. Dysregulated aggregation is potentially toxic to cells in that it can affect the dynamics of protein turnover, interfere with RNA processing in stress granules (SGs), and overburden essential resources [17,54]. Regulated aggregation, by contrast, performs important triage and filtration functions for the cytoplasmic proteome, protecting proteins from damage during stress and partitioning proteins according to their potential usefulness after conditions improve.

Heat stress produces widespread protein misfolding by melting weak interactions between amino acids inside the folded protein. Wallace *et al.* [55] showed that 73% of the yeast protein mass forms reversible aggregates during heat stress, implying that there are mechanisms that protect reusable misfolded proteins from proteasomal degradation, and hand them off to chaperones for refolding after there is no more heat stress. Spokoini *et al.* [9] visualized misfolding-prone proteins in the cytoplasm during heat stress, and showed that they form highly dynamic and reversible foci, termed stress foci (SF) (also referred to as Q bodies in other studies [56]). SFs form minutes after stress induction and display liquid droplet properties in that they are spherical and tend to fuse together. Upon stress reduction, SFs dissipate within an hour, whereas under persistent stress they are delivered via an actin-driven vesicle-sorting-associated mechanism to a site of long-term insoluble protective aggregation, the insoluble protein deposit (IPOD) [9,57]. Hence, SFs can be thought of as a filtration mechanism, adsorbing misfolded proteins, concentrating them with chaperones, and sorting them for either refolding back in to the cytoplasm or protective sequestration in the IPOD. In the IPOD, proteins are thought to be actively induced to aggregate in a single location, in order to protect the rest of the cytoplasm from their harmful effects [15]. Interestingly, SFs associate with lipid droplets, which are lipid biosynthetic granules. Lipid droplets transfer lipids into the SFs in order to increase the solubility of the proteins inside [58]. This association is critical for efficient clearance of SFs after stress is removed. Misfolded proteins that are targeted for degradation are concentrated in the juxta-nuclear quality control compartment (JUNQ) compartment, which is enriched in proteasomes and degradation machinery [6,17,18,30,59–62]. There appear to be many protein quality control compartments for triage, degradation, and storage of stress-induced aggregates. Many of these quality control inclusions (e.g., JUNQ and SFs) have parallel structures across species and cellular compartments [63,64]. What is unclear is whether the function of these structures is fluid, and therefore dynamically regulated (as in the case of SGs), or whether specific quality control structures have discrete functions, as seems to be the case for metabolons and nuclear bodies.

Protective Storage

Protective storage is the sequestration of many proteins, especially metabolic enzymes, into fibrils [65–68], in order to protect the folded functional proteins during stress. As with heat stress, energy stress (sugar starvation) also results in a significant reduction in the protein quality control capacity of the cell, since chaperones and degradation machinery require ATP for function. During starvation the yeast cytoplasm is thought to 'freeze' [65] and many proteins with important metabolic functions such as glutamine synthase are stored in fibrils of folded proteins called storage granules. Other studies have shown that proteasomes, some of the largest and most ATP-expensive molecular machines in the cell, are also sequestered for safe keeping and recycling in proteasome storage granules [66].

Sensing

Inclusions can act as molecular sensors, finely attuned to diverse cellular conditions. For example, since the formation of nucleoli is highly concentration dependent, they can act as sensors of cell size [69,70]. P granules in *C. elegans* [25] exhibit a similar function, whereby they accumulate along a concentration gradient of a nucleation factor, the P granule abnormality protein 3 (PGL-3) RNA, thereby establishing embryo polarity. In another fascinating example of a sensory switch, the synaptonemal complex has been shown to behave like a liquid crystal (where molecules have ordered but weak interactions and therefore are sensitive to stimuli), enabling it to sense and propagate signals along the entire chromosome, and to control the number of crossover instances that a meiotic pair of chromosomes can undergo [71].

SGs represent a class of highly diverse RNP granules, which are the first responders to cell stress [4,8,72]. Over a hundred proteins can localize to SGs and over 12 proteins can nucleate SG when overexpressed. The localization of constituent proteins to SGs is regulated by phosphorylation, mRNA abundance, chaperone function, abundance of constituent proteins, and ATP levels. Initially, SGs were thought to regulate translational arrest, since stalled translational complexes trigger SG formation. More recently, however, SGs have been shown to regulate many other aspects of stress response, cell proliferation, RNA processing, and motility – whereas their formation is not needed for translational arrest [4]. The diversity of potential SG constituent proteins and RNA transcripts make it a hub for sensing the type of stress (based on which SG constituents seed SG formation) and for calibrating the response (by recruiting signaling molecules into the inclusion). Because SGs store genetic information in the form of mRNAs, and determine mRNA release and degradation kinetics, SGs have the potential for short-term or medium-term memory of the stress. Although SGs have not been well studied in the context of memory, there are several important examples of sensory function and memory storage by similar RNP bodies.

Memory

Different RNA silencing foci collectively referred to as synaptic activity-regulated mRNA silencing (SyAS) foci regulate synaptic translation specificity [73–75] by forming in the postsynapse in response to repeated receptor stimulation. While these receptor stimulation memories are thought to be short lived, a model for longer-term information storage exists in yeast. Here, the RNA-binding Whi3 protein forms reversible aggregates via its PrLD and transmits the memory of unsuccessful mating from mother cell to daughter cell [76].

One of the most striking examples of aggregate-driven sensing and memory is the Tup1–Cyc8 transcriptional repressor in yeast [77]. This mechanism was uncovered in a study that characterized the full set of proteins that were SUMOylated in response to osmotic stress. Most of the stress-induced SUMOylation occurred on two proteins comprising a transcriptional repressor, Tup1 and Cyc8. The authors discovered that Cyc8 undergoes rapid reversible aggregation into

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submicron-sized clusters immediately following osmotic stress. This phenomenon, driven by the pol-glutamine PrLD of Cyc8, results in a transient derepression of Cyc8 transcription targets, leading to the expression of hundreds of osmotic stress-response genes. However, within minutes of this clustering event, Cyc8 is SUMOylated on multiple sites adjacent to the PrLD, and the clusters resolve. Abolishing the SUMO sites leads to more persistent aggregates in the nucleus as well as the cytoplasm. Hence, Cyc8 is able to sense osmotic stress by virtue of its aggregation-prone properties, and the derepression is a result of the inclusion formation. What is more, the capacity for information storage and even intergenerational transmission of stress memory is built into this system. Cyc8 is SUMOylated in order to disaggregate the clusters and prevent the cell from spending valuable resources on transcribing the stress-induced genes for longer than necessary to mount the response. However, the PrLD makes Cyc8 able, under certain conditions, to form a prion element, thus permanently aggregating and constitutively derepressing the promoter. Hence, the aggregation property of Cyc8 and the regulation of clustering by SUMOylation enables a multi-tiered response to osmotic stress, as well as the potential for short-term, medium-term, and long-term memory of the stress.

A mechanism that is remarkably similar to the Cyc8 yeast prion works to consolidate memories in the mammalian brain. Memory consolidation requires specific morphological changes at the synapse that are brought about by protein synthesis. Fioriti *et al.* and Drisaldi *et al.* [78,79] have demonstrated how the aggregation properties of cytoplasmic polyadenylation element binding protein (CPEB)3 can regulate synaptic remodeling and memory storage in the mammalian brain. Similarly to Cyc8, the aggregation of CPEB3 is driven by a PrLD and is regulated by SUMOylation at sites close to the aggregation domain. In neuronal synapses, CPEB3 binds to mRNAs required for synapse remodeling. Specific receptor stimulating events trigger CPEB3 SUMOylation, which leads to disaggregation and translation of the mRNAs.

Memory storage and transmission is perhaps the most complex function that aggregates can perform, but also the easiest to assess and conceptualize. Indeed, there are many well-known and recently discovered examples of information storage by amyloid aggregates (e.g., Balbiani bodies and P granules) and other prion-like elements [80]. Yeast prions were initially proposed to serve an adaptive function by Lindquist and colleagues, by virtue of their ability to process environmental cues and translate them into heritable regulatory features that can confer fitness advantages on subpopulations of cells [81–83]. Information transfer via aggregates and RNP granules is not restricted to yeast. *Trypanosoma brucei* parasites have been demonstrated to communicate with each other by secreting RNP granules in exosomes [84]. Mammalian cells are thought to have a similar communication mechanism [85]. Hence, granule formation is a robust platform for storing and transferring information. Aggregation and phase transitions are usually the result of changes in the cellular environment, and therefore a physical record of past events. This record can be kept for the short, medium, or long term depending on the persistence of the aggregate, which itself is the result of the association kinetics of the components and the cellular machinery acting on the aggregate to dissociate it. Cellular machinery can also convert a subset of enzymes and RNA/DNA binding proteins into a self-association-prone state (e.g., via phosphorylation or de-SUMOylation), enabling an infinite set of messages to be retained in the granule.

Concluding Remarks

The case studies illustrating the cellular functions discussed in this review are somewhat fluid. SGs (and related RNP granules), for example, can perform any of the above functions, from amplification and filtering, to sensing and memory. Because so many different inputs can trigger SG formation, they can also be usefully described as co-occurrence detectors, sensing an important combination of events. What is true, however, is that different material properties of inclusions contribute to these different functions. Amplification and enhanced efficiency is

Outstanding Questions

How do we define granule structures?

What are the dynamics of granule formation (e.g., SGs) at the diffraction-limited scale?

What are the biophysical forces that drive transient spatial clustering of enzymes?

How prevalent is the maturation of liquid droplets into insoluble aggregate structures *in vivo*?

Is LLPS a dynamic equilibrium process, or a nonequilibrium steady state driven by continuous energy consumption?

How do cells distinguish between functional and harmful protein aggregation?

Is the reliance on phase-separated structures as units of signaling and memory storage the Achilles' heel of neurons?

largely a matter of assembling the granule with the correct components, and ensuring weak association between constituents for increased mobility and diffusiveness. Sensing, memory, and heredity require a time component for storing and retrieving information, as well as a way for regulating storage and retrieval. Memories that are stored for long periods in amyloid structures like the PSI prion [28] or the Balbiani body [86], can be hereditary. One cell can communicate its memories to another by conveying prion-like aggregates and RNP granules in exosomes [84] and nanotubes [80]. Clearly, aggregation can be a wellspring of highly tuned functions for specialized cells. At the same time, the aggregate or granule as a functional unit for signaling and memory storage, seems to be the Achilles' heel of certain neurons that succumb to unwanted aggregation of pathogenic proteins in stress granules, resulting in pathologies such as the **amyotrophic lateral sclerosis–frontotemporal dementia (ALS–FTD)** spectrum of diseases [87,89,90]. A better understanding of how aggregates function may therefore lead to breakthroughs in understanding neurodegenerative disease. The potential for designing function using granules as a platform may also be exploitable for designing artificial functions. Fittingly, a recent study by the Brangwynne group designed synthetic cytoplasmic droplets that could be triggered to form conditionally [38].

The next goal of research focused on membraneless organelles is to integrate biochemical phenomena relating to how granules operate as functional entities, with cell biological information relating to the dynamics and mechanism of granule formation (see Outstanding Questions). This will inevitably raise the question of how we think about scale as well as function. The current definition of an SG (or any other granule) is a micron-scale microscopically visible structure containing certain markers, such as TIA-1 in the case of SGs. However, it is increasingly clear that aggregates need not be visible with conventional microscopy in order to carry out function. Pol II clusters [42], for example, are nanometer scale and below the diffraction limit. At the other extreme, *Tardigrade* water bears induce aggregation at the whole organism level [88] by expressing tardigrade-specific **intrinsically disordered proteins (IDPs)** in response to stress and desiccation. Thus, the continued study of aggregation and granules has the potential to not only tell us about the results of cell stress, but open a new window of how cells and organisms cope with the ups and downs of life.

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