Cellular sensing by phase separation: Using the process, not just the products

Published, Papers in Press, March 15, 2019, DOI 10.1074/jbc.TM118.001191

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Edited by Paul E. Fraser

Phase separation creates two distinct liquid phases from a single mixed liquid phase, like oil droplets separating from water. Considerable attention has focused on how the products of phase separation—the resulting condensates—might act as biological compartments, bioreactors, filters, and membraneless organelles in cells. Here, we expand this perspective, reviewing recent results showing how cells instead use the process of phase separation to sense intracellular and extracellular changes. We review case studies in phase separation-based sensing and discuss key features, such as extraordinary sensitivity, which make the process of phase separation ideally suited to meet a range of sensory challenges cells encounter.

Phase separation is a process through which a single phase composed of mutually soluble components demixes into two or more distinct phases (Fig. 1*A*), as with oil and water. In biology, liquid–liquid phase separation has emerged as a means to form coherent structures with a range of potential functions (reviewed in Refs. 1–4). Structures resulting from phase separation have been given various names, including membraneless organelles or biomolecular condensates, reflecting the breadth of scenarios in which they occur and the potential functions they may fulfill. The nucleolus provides a canonical example of a membraneless organelle, compartmentalizing key steps in ribosome production within the nucleus without membrane boundaries. The vertebrate nucleolus displays liquid-like behaviors (5, 6), and its structure arises in part from phase separation (5, 7).

Efforts to determine cellular functions for phase separation have focused primarily on its products: the resulting condensates, their material states such as liquid or gel, and the composition and dynamics of the cellular bodies hypothesized to form by phase separation. In this view, phase separation is a means to generate condensates, which are the functional entities: compartments, filters, depots, reaction vessels, factories, force generators, regulators of cell signaling, and more (8–21).

However, the existence of a large fluid organelle does not imply that it formed by phase separation or even that phase separation occurred during organelle assembly. There are many processes by which large liquid-like structures may form: phase separation; coalescence of smaller structures; transport processes, including those involving active transporters such as pumps and insertases or local synthesis; permeation involving docking and regulated transport between structures, liquefaction (melting or dissolution of a solid structure), or other processes (Fig. 1*B*).

In certain cases, such as coalescence and permeation, phase separation may generate the subunits being assembled, but the assembly process is distinct from phase separation. The distinction is critical: the hypothesis that, for example, a membraneless organelle forms by phase separation is biologically and physically quite distinct from the hypothesis that this organelle forms by coalescence of phase-separated subassemblies. By analogy, imagine a child displaying a castle she has just constructed out of stackable plastic bricks. To tell her that the castle was formed by injection molding (the process used to make the bricks) rather than by her painstaking assembly process would be an obvious and grave error.

The same distinctions between processes of formation and the resulting product matter in biology. For example, lipid droplets are fluid organelles. Their constituents, lipids, spontaneously phase-separate in the cytosol—they are literally oil in water. However, lipid droplets do not form by such spontaneous processes; instead, they bud from the endoplasmic reticulum. New molecules may also later be added to lipid droplets via coalescence, permeation, and local production at the surface (22). Lipid droplets are separate phases but do not form by phase separation.

Each alternative formation process presents distinct features, such as kinetics, energy requirements, and mechanisms for regulation, yet they result in the same product: in this case, a fluid organelle. That multiple processes can result in the same product is familiar: there are alternative recipes for the same dish, different manufacturing processes for the same car, and different approaches to write the same document. Although some processes may yield a subtly different outcome (a tastier dish, a more coherent letter), other alternative processes may differ only in their efficiency, speed, reliability, yield, cost, compactness of encoding, and so on, resulting in effectively indistinguishable products. When alternative processes can yield the same product, two questions arise. Are there scenarios in which one process, such as phase separation, might be favored over alternative processes? And are there situations in which the

This article is part of the thematic series, Phase separation of RNA-binding proteins in physiology and disease. The authors declare that they have no conflicts of interest with the contents of this article.

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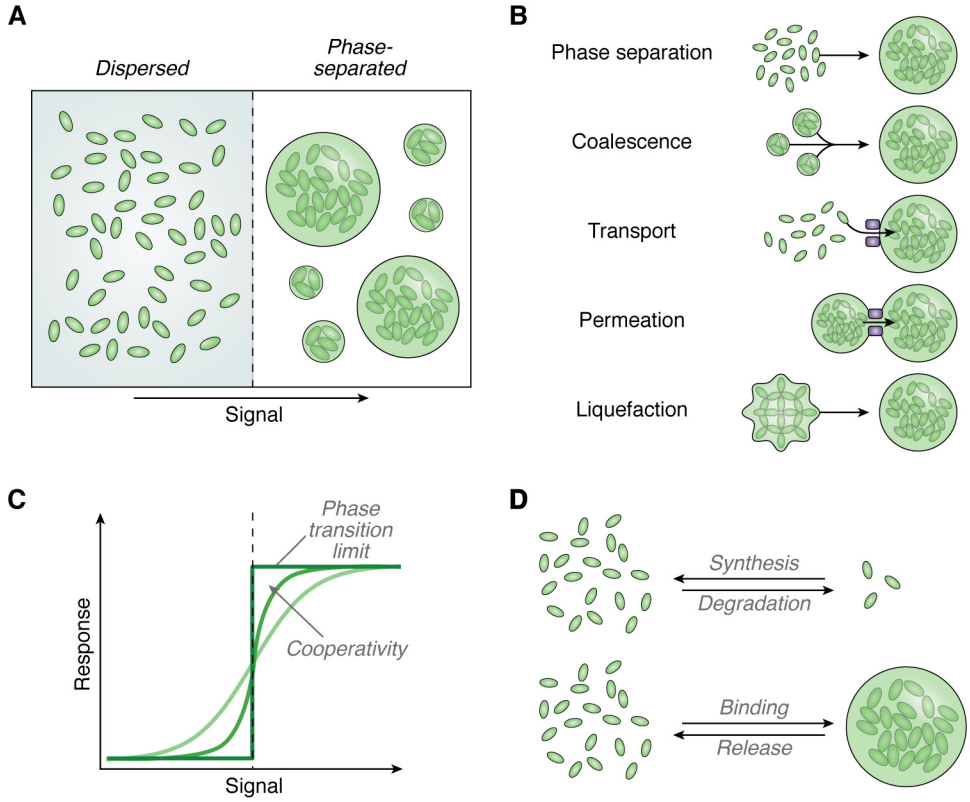

Figure 1. Distinguishing the process and the products of phase separation. *A*, phase separation of a single-phase solution into dense (droplet) and dilute (surrounding medium) phases. *B*, mechanisms for forming large fluid structures. This list is not exhaustive. Some mechanisms involve pre-existing phase-separated subunits, whereas others do not involve phase separation at all. *C*, phase boundaries represent a sharp thermodynamic transition, making them well-suited for sensing small changes in important conditions. *D*, efficiency and kinetics of large-state changes can differ markedly depending on implementation. *Top*, synthesis and degradation processes (*e.g.* by changes in mRNA or protein synthesis and turnover) take minutes to hours and substantial energy expenditure. *Bottom*, phase separation processes (*e.g.* phase separation of cGAS upon binding DNA) rearrange matter in place, allowing rapid changes on a system-wide scale in seconds, in many cases spontaneously.

process may be as important, or even more important, for biological function than the resulting product? Cellular sensing of internal and external variables provides a set of biological scenarios where, recent work suggests, both questions may be answered affirmatively.

In this minireview, we address why and how biological systems exploit the cooperativity and efficiency of the process of phase separation, and phase transitions more broadly, for cellular sensing. Phase transition refers to any transition between one phase of matter to another, for example from liquid water to solid ice. Liquid–liquid phase separation, or phase separation for short, specifically refers to transition of a single liquid phase to two or more distinct liquid phases. Both phase transitions and phase separation have the shared feature of extraordinary cooperativity that allows system-wide changes in response to small changes in the environment and the ability to rearrange matter in place, in many cases without energy expenditure. Cells can exploit these features for specific biological functions (23). We review three recent case studies that exemplify this in the context of cellular sensing: 1) poly(A)-binding protein (Pab1) in sensing thermal stress (24); 2) Sup35 in sensing change in intracellular pH during starvation (25); and 3) cGMP–AMP synthase (cGAS)[2] in sensing cytosolic DNA (13). In all three case studies, the process of phase separation plays critical sensing roles; functions played by the product of phase separation remain either enigmatic or, in the case of cGAS, are involved in the response pathway downstream of sensing. Before reviewing each case study in detail, we first discuss features of phase separation in more detail and how these features make phase separation ideally suited to solve a range of sensory challenges.

Phase separation in environmental sensors

To survive and thrive, all organisms must sense features of their environment and internal state—particularly when conditions take a turn for the worse. For primordial environmental conditions such as temperature, oxygen concentration, and nutrient availability, individual cells in an organism retain the capacity to sense stressful changes (26–29). We know this largely because cellular responses to such stresses are universally conserved. The stress-induced formation of cytoplasmic clusters of RNA and protein appears to be universal in eukaryotes (30–35).

Temperature provides an instructive example of how small changes in a physical environmental parameter can lead to dramatic biological consequences. How eukaryotes sense temperature at the molecular level has remained surprisingly unclear (36). A major challenge is to explain how small changes on the temperature scale—such as the two or three degrees, a mere 1%

[2] The abbreviations used are: cGAS, cGMP–AMP synthase; cGAMP, cyclic GMP-AMP; PrD, prion domain.

in absolute terms—are converted into dramatic system-wide changes. For example, eggs of the red-eared slider turtle incubated at 26 °C produce all males, at 31 °C produce all females, and at 29.2 °C produce an equal male/female ratio (37). Such temperature-dependent sex determination is common, yet the mechanism behind this extraordinary sensitivity remains unknown. More prosaically but no less consequentially, the mechanism by which a few degrees' increase in temperature produces a thousand-fold induction of heat-shock genes also remains incomplete (27).

In contrast, we are constantly confronted with dramatic system-wide behavior sensitive to a fraction of a degree: the freezing of water into ice and its vaporization into steam. Melting, freezing, vaporization, separation, and other phase boundaries (Fig. 1*A*) mark transitions in which individual molecules cooperate to change their state in response to a small change in the relevant variable, such as temperature or pH or the concentration of a ligand. Hypersensitive behavior is expected at a phase boundary, providing a tantalizing class of potential solutions to otherwise tricky problems in sensory biology.

Unlike the two-dimensional phase diagram shown in Fig. 1*C*, a phase diagram for a biomolecule may have multiple dimensions, each of which can be modulated to regulate phase behavior. For example, the intracellular environment of yeast undergoes dynamic changes when the cell encounters stress: the cellular ATP level drops (38); the intracellular pH drops by 0.5–1 pH unit (39–41); the cellular volume shrinks and the intracellular environment becomes more crowded (42); and the cytoplasm transitions from viscous fluid to a more glass-like state (39, 42). These parameters—concentrations of specific mRNAs, ATP, protons, and crowders—have been demonstrated to affect the phase boundaries of proteins that undergo phase separation (24, 43–48). Post-translational modification following stress can also trigger or modulate phase separation (10, 11, 49). Other changes, such as production of cytoprotective metabolites like glycerol and trehalose (50) and production of molecular chaperones, are also likely to affect the phase boundary and contribute to the accuracy and adaptability of the sensing system through signal integration. Understanding the phase response of proteins to both changes in concentrations of cellular components and intensive system properties such as temperature is of great interest; some factors, such as the volume fraction of components, are under cellular control, whereas others are products of the environment (23). Ultimately, these intracellular environmental changes and resulting phase behaviors appear likely to contribute to major stress-induced functional changes: global translational attenuation (30, 51), arrest of the cell cycle (52), and induction of a transcriptional program.

Below, we review three specific case studies in detail. All three case studies highlight the two properties of phase separation most relevant to sensing. First, phase separations are highly cooperative, enabling switch-like responses to small changes (Fig. 1*C*). Second, phase changes rearrange existing cellular matter without the need for creation or destruction, raising the possibility that such processes can execute changes with less expenditure of time and energy than processes involving synthesis and degradation (Fig. 1*D*). If, as in the figure, only monomers or only demixed molecules are active, regulating activity can be achieved in seconds, in some cases spontaneously, without any need for *de novo* synthesis of mRNA or protein molecules, which take minutes and substantial energy. Similar logic underlies the utility of post-translational control in rapid cellular responses (53), and indeed, protein phase separation is a mode of post-translational control. Phase separation in general, and environmentally sensitive spontaneous phase separation in particular, may thus provide an ideal mechanism for mounting an immediate response to an abrupt environmental insult which, only on a longer time scale, would be accompanied by changes in transcription, translation, and turnover.

Case study 1: temperature sensing by phase separation of poly(A)-binding protein

Temperature presents a universal challenge to all living organisms, which typically inhabit a narrow thermal range and can survive only brief excursions outside this range. All cellular life induces production of so-called heat-shock proteins in response to a nonlethal rise in temperature. All eukaryotes form stress granules, cytosolic clusters of RNA and protein, at the upper extreme of survivable heat shock. Severe heat stress causes proteostasis catastrophe and accumulation of misfolded proteins, which lead to induction of the heat-shock response and other protein quality control processes such as endoplasmic reticulum-associated protein degradation (30, 54–56). Despite these well-studied responses to thermal stress, how temperature is mechanistically sensed in eukaryotes remains largely unknown.

A study by Riback, Katanski *et al.* (24) revealed that poly(A)-binding protein (Pab1 in yeast; PABPC1 in humans), a highly conserved RNA-binding protein component of stress granules, undergoes phase separation to form a hydrogel in response to physiological thermal stress both *in vivo* and *in vitro* (Fig. 2). In yeast, Pab1's phase separation is tuned to occur at the organism's heat-shock temperature by modulatory hydrophobic residues in its proline-rich domain. Phase separation is mediated by its RNA-binding domains, and Pab1 releases RNA during phase separation.

The ability of Pab1 to autonomously sense a mere 3% change in absolute temperature, from robust growth (30 °C/303 K) to stress (40 °C/313 K), makes Pab1's phase separation one of the most thermosensitive biomolecular processes yet found (24). The standard way to characterize temperature sensitivity in biology is the Q_{10} value, the ratio of any two biological properties of a system at temperatures 10° apart (36). Typical biological reactions have Q_{10} values of roughly 2–3, meaning a 2–3-fold change over a 10° range (57). In contrast, the rate of radial growth of Pab1 phase-separated assemblies has a Q_{10} of 350 at 36 °C (24). Pab1's assembly rate is smoothly graded as a function of temperature, indicating that Pab1 senses the magnitude of thermal stress as well as its presence or absence. Pab1's phase separation is also highly sensitive to pH, a physiologically relevant feature because heat shock is accompanied by a pH drop (40), and other stresses, such as energy depletion, involve only a pH change (39). Interestingly, the magnitude of cytosolic acidification correlates with the severity of heat stress (58). Thus, Pab1 may be able to integrate both thermal and intracellular pH

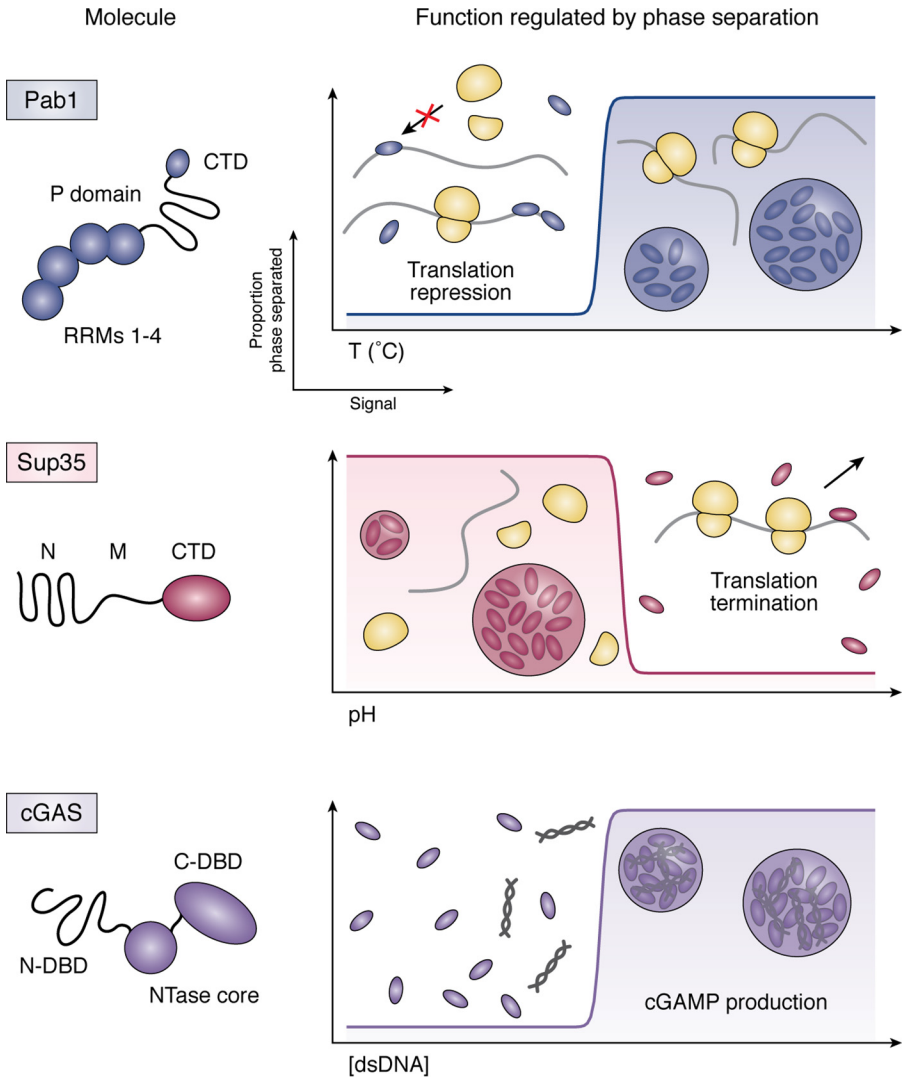

Figure 2. Proposed functions of phase separation–based sensory systems. The following abbreviations are used: *CTD*, C-terminal domain; *P domain*, proline-rich domain; *RRM*, RNA recognition motif; *N*, N-terminal prion domain; *M*, middle domain; *DBD*, DNA-binding domain; *NTase core*, nucleotidyltransferase domain.

information to accurately sense both the presence and magnitude of thermal stress. Crucially, preventing Pab1's stress-triggered phase separation compromises growth during stress (24), indicating that phase separation is adaptive.

Although the function remains speculative, Pab1's phase separation may regulate translation, translationally repressing heat-shock mRNAs by binding their A-rich 5′ UTRs before stress and following recovery, but derepressing these mRNAs upon releasing RNA during stress-triggered phase separation (24). In this mechanism, the sensitivity to temperature and pH, which is required for sensing, is provided by the process of phase separation. Further studies are needed to establish the function(s) of Pab1's phase separation.

Case study 2: starvation sensing by phase separation of Sup35

As sessile organisms, yeast cells depend on their current environment for nutrients; when nutrients run out, growth stops, and when nutrients become plentiful, growth must rapidly restart. Correspondingly, cells rapidly arrest translation during starvation (36) and resume translation during refeeding. How do cells regulate translational activities during stress? And more broadly, how do cells sense and adapt to a changing environment?

Sup35 is a translation terminator factor in yeast and is also one of the classic yeast prions (59, 60). Inheritance of phenotypic variations through prions has been studied extensively as an evolved adaptation mechanism in fungal species (61–66). For example, prion formation of Sup35 leads to translation read-through, which has been hypothesized to provide cells a means to expose hidden genetic variations, some of which might have adaptive value (63). Like other prions, Sup35 has a disordered, low-complexity domain enriched in polar and aromatic amino acids (67). This prion domain (PrD) mediates formation of fibrillar, amyloid-like Sup35 prion conformation (68, 69), but a recent study by Franzmann *et al.* (25) uncovered its additional role in mediating phase separation of Sup35.

The PrD of Sup35 can mediate phase separation of Sup35 into nonfibrillar structures in energy-depleted yeast cells by sensing the intracellular pH (Fig. 2) (25), which drops during starvation and other stresses. Sup35 consists of the N-terminal

PrD (N), a charged middle domain (M), and a C-terminal GTPase domain. The GTPase domain is essential for soluble Sup35's function as a translation terminator. Sensing of pH is mediated by the charged M domain; removal of the negative charges from the M domain abolishes the pH-dependent phase behavior of Sup35. Yeast cells expressing Sup35 without NM domains recover growth and translational activity more slowly after starvation compared with WT. Whether these differences come from loss of phase separation or loss of another NM domain activity remains open. In energy-depleted yeast cells, Sup35 readily dissolves upon re-addition of glucose in an Hsp104-independent manner (25). This pH-dependent, reversible phase separation of Sup35 is likely to provide a fast and efficient mechanism to sense energy depletion and possibly other stresses that trigger reduction in the intracellular pH.

What is the fitness benefit of Sup35 phase separation *versus* prion formation? Franzmann *et al.* (25) showed that a strain carrying Sup35 prions recovers more slowly from the stationary phase compared with a strain without Sup35 prions. Are phase separation and prion formation two distinct evolved mechanisms to sense and/or react to different forms and/or severity of stress? Are phase separation and prion formation mutually exclusive? More studies are necessary to address these questions and delineate distinct functions between the two processes.

Case study 3: cytosolic DNA sensing by phase separation of cGMP–AMP synthase (cGAS)

In eukaryotes, cellular DNA resides in the nucleus, and introduction of cytosolic DNA upon microbial or viral infection or after severe genomic damage triggers the innate immune response. The enzyme cGAS, a DNA-binding enzyme that converts GTP and ATP into cGAMP (70), is responsible for the detection of this aberrant cytosolic DNA. cGAMP activates the adaptor protein STING, which induces type I interferons and other cytokines (71).

Du and Chen (13) discovered that DNA sensing by cGAS involves phase separation. The N terminus of cGAS is disordered and positively charged. The C terminus contains a structured nucleotidyltransferase domain. Both termini bind indiscriminately and cooperatively to DNA. Longer DNA, which allows more multivalent DNA– cGAS interaction than shorter DNA, promotes cGAS phase separation better than shorter DNA. In buffer with physiological concentrations of salt and zinc, even nanomolar concentrations of cGAS are capable of phase-separating in response to similar concentrations of DNA. ATP and GTP partition into cGAS droplets, and the cGAMP synthesis activity of cGAS increases upon phase separation.

Phase separation of cGAS illustrates how the process and product of phase separation may play separate but coupled roles: phase separation provides the sensitivity and conditional behavior, whereas the resulting compartment accelerates specific biochemical reactions. When the cytosolic DNA concentration exceeds the critical concentration, which depends on both the length of the cytosolic DNA and cytosolic zinc concentration, cGAS phase-separates and sequesters the cytosolic DNA into a confined space (13). The resultant cGAS droplet acts as a microreactor for synthesizing cGAMP for downstream signaling.

Whether the product can be spontaneously reversed or requires additional factors is unclear. The authors noticed that the fluorescence recovery after photobleaching (FRAP) recovery rate of cGAS droplets decreased with increasing time, suggesting that the cGAS droplets gradually transition into a gel-like state. Further studies on how cells regulate both the formation and dissolution of cGAS droplets by, for example, changing cytosolic zinc concentration or molecular chaperones are necessary.

Direct *versus* indirect sensing

The case studies presented here are paradigms for sensing achieved via phase behavior, yet they have important differences that typify the diversity of mechanisms by which phase separation can achieve threshold detection and adaptation. Some proteins, such as Pab1 and cGAS, may directly sense a signal (heat or DNA) and undergo a phase separation as a result. In other cases, a molecule might undergo phase separation in response to a downstream signal or secondary messenger; this appears to be the case for Sup35 and in some scenarios for Pab1, in which the signal being sensed is a decrease in the intracellular pH in response to energy depletion.

This observation opens the possibility that previous results, although not identified as sensing by phase separation, may fall into one of these categories. For example, the RNA-binding protein Whi3, which regulates cell-cycle progression, has been implicated in the process in which yeast cells resume budding after nonproductive mating attempts (72). The authors note that Whi3 forms "super-assemblies" in such cells. Given that the protein contains a domain known to contribute to phase separation in other systems and that a homologous RNA-binding protein has been shown to phase-separate *in vitro* (47), it is plausible that Whi3 acts as a phase-separating sensor for the cellular state of unproductive mating. Notably, the molecular chaperone Ssa1 was shown to interact with the assembled form of Whi3, providing a plausible mechanism for resetting the sensing system (adaptation). Further research is needed to determine whether the protein actually undergoes phase separation and, if so, what signal directly triggers the change.

Components of a larger sensing system may display phase behavior that can confer threshold detection indirectly. A recent study in budding yeast by Simpson-Lavy *et al.* (73) may represent such a case in a glucose-sensing system. The study demonstrates that glucose-dependent release of Std1 from its binding partner Sip1 leads to the formation of Std1 cytoplasmic focus, which sequesters the catalytic component of AMP-activated protein kinase (SNF1 in yeast; AMPK in human) from the nucleus to switch the mode of metabolism from respiration to fermentation. Std1 has an asparagine-rich disordered region, which is both necessary and sufficient for the cytoplasmic focus formation *in vivo*, and displays a relatively fast FRAP. The authors propose that, in the presence of glucose, activated Vhs1 kinase phosphorylates Sip1 to release Std1. More experiments need to be done to determine whether Std1 forms cytosolic focus via phase separation, aggregation, or a combination of processes described in Fig. 1*B*.

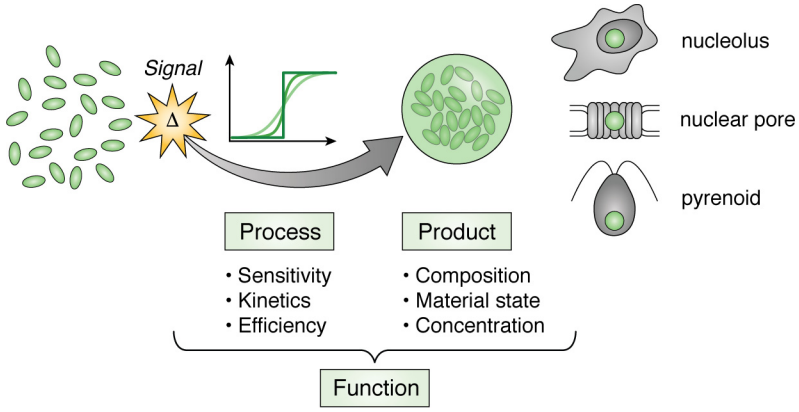

Figure 3. Distinct features of the process and the products of phase separation. Both may carry out functions, and specific functions (such as sensing, signal transduction, and isolation of events in time) may rely mainly on features of the process, whereas other specific functions (such as colocalization, filtration, and isolation in space) depend primarily on the products.

A similar process was also recently reported for another important stress-associated transcription factor in yeast (74). Snf5, a component of the SWI/SNF complex responsible for the expression of many glucose-repressed genes, has a poly-Q stretch that the authors find is crucial for transcriptional activation. Strikingly, although removal of the domain represses transcriptional output, replacement of the domain with an exogenous domain that aggregates in a pH-sensitive manner partially rescues this phenotype. The authors note that the poly-Q stretch is in close proximity to several histidines, and it may act as a pH-sensor that responds to starvation-associated acidification. However, more *in vitro* data are needed to determine whether the protein or complex truly undergoes phase separation in a pH-dependent fashion.

Finally, we have proposed that temperature and pH sensing may be carried out by proteins having phase diagrams like Pab1, with state changes depending on both variables, for induction of the transcriptional response regulated by heat-shock factor 1 (Hsf1) (24). Hsf1 is constitutively bound by molecular chaperones, and stress produces as-yet-unidentified molecular species (speculated to be unfolded proteins), which titrate away these chaperones, activating Hsf1. Phase-separating proteins, such as Pab1, can substitute for unfolded proteins in this model, potentially linking phase separation to transcriptional induction. Sensing of pH would, as above, represent indirect sensing of stresses that compromise ATP production, membrane integrity, or other aspects of pH homeostasis.

Autonomous *versus* facilitated reversal

Many studies of conditional phase separation have focused on whether the process is reversible, generally in the context of returning the environment to its initial state and asking whether demixed molecules disperse. Although this is, of course, a point of curiosity, reversibility has deeper significance in regulation, taking on a different meaning if dispersal is spontaneous or, alternatively, if it depends on factors that are induced by the conditional signal.

Some phase-separated structures, like Sup35, are autonomously dispersed when the signal returns below the threshold. Others, like Pab1, require molecular chaperones for facilitated dispersal (30). The type of stress can also dictate whether a protein phase-separates into a spontaneously reversible structure as illustrated by poly(U)-binding (Pub1); heat-induced Pub1 droplets require Hsp104, whereas pH-induced Pub1 droplets spontaneously dissolve upon reversing pH (75). What are the benefits and costs of autonomous *versus* facilitated reversal? One benefit of autonomous reversal may be that a cell can immediately resume growth when the environment returns to favorable conditions, such as with nutrient withdrawal and replenishment. Interestingly, a list of metabolic enzymes has been reported to form reversible cytoplasmic foci or filaments upon stress (76–80), and at least some of these metabolic enzymes have been shown to undergo autonomous reversal (78, 79). Reversal indicates (senses) that the stress is over.

In contrast, facilitated reversal by signal-induced factors may be useful for programming a timed or graded response. In the case of chaperones induced by stress, the dispersal of Pab1, Pub1, and other such proteins reveals that the cell has obtained sufficient free levels of chaperones to effect dispersal. In other words, facilitated reversal indicates the completion of the stress response rather than the end of the stress.

Nucleation and growth *versus* spinodal decomposition processes

Phase separation can occur by two mechanisms: nucleation and growth and by spinodal decomposition (81). In nucleation and growth, an energetically unfavorable nucleation step must be first accomplished, followed by spontaneous growth of the dense phase of nuclei that have formed. In spinodal decomposition, the nucleation step is itself spontaneous, such that nuclei appear throughout the solution, and the entire system spontaneously and simultaneously separates. Pab1 shows clear signs of being in the nucleation and growth regime under physiological conditions, preferentially forming new assemblies on top of existing assemblies (24). Nucleation and growth offer biological systems the opportunity to regulate each step separately and to control the location where phase-separated structures form by controlling the location of nucleus formation. By contrast, spinodal decomposition might help ensure a synchronized, system-wide switch as soon as a biological condition is reached.

These alternative processes of phase separation itself thus open possibilities for alternative biological control mechanisms.

Concluding remarks

Given the universal need for sensing in biology, we expect sensory phase separation to be exploited widely. Although our case studies are eukaryotic, a few examples of phase separation in bacteria have been reported recently (82, 83). The field is wide open and filled with opportunities to discover more examples of sensory phase separation in different cell types and in different contexts, to dissect out the underlying molecular mechanism of sensory phase separation, to investigate how multiple sensory inputs are simultaneously integrated or processed separately, and to illuminate the costs and benefits of these molecular sensory strategies.

We end by emphasizing that the process of phase separation itself has features distinct from its products that make it uniquely well-suited to certain biological functions (Fig. 3). A prominent body of existing work on the products of phase separation exists, such as the nucleolus where key steps in ribosome assembly are compartmentalized (5, 6), the pyrenoid where CO_2-fixing enzymes are defended by colocalized radical scavengers (14), and the nuclear pore where selective transport (filtration) regulates access to and from the nucleus (84, 85). As in the case studies highlighted here, we anticipate many more studies uncovering functional roles exploiting the unusual sensitivity, efficiency, kinetics, and other temporal features that characterize the process of phase separation.

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