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has not yet been applied to all breeding programs, and there are still some major crop species (*e.g.,* soybean, tomato, sunflower) that are recalcitrant to the currently available procedures, or for which present protocols are not efficient enough. The understanding of the maize *in situ* system should help to extend the DH technology to more species or breeding programs by either directly knocking out the functional ortholog of the maize gene, or if needed by transferring the cellular and molecular knowledge acquired on maize. In addition to these applications, the identification of the *NLD/MTL/ZmPLA1* gene offers a unique opportunity to explore the many mysteries of double fertilization in plants.

Where can I find out more?

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Primer

Phase separation in biology

Simon Alberti

Cells have to organize their complex biochemistry to regulate their metabolism and respond to changes in the environment. Traditionally, intracellular organization has been associated with compartments that are surrounded by lipid membranes. However, in recent years, phase transitions have emerged as a novel form of cellular organization. Phase transition is a physical process whereby a substance changes from one physical state to another. Examples are provided by the freezing of water into ice (liquid to solid) or the heating of water to generate water vapor (liquid to gas).

Liquid–liquid phase separation is a special form of a phase transition that is particularly relevant for living organisms. Here, a homogenous solution of molecules spontaneously separates ('demixes') into two coexisting liquid phases, a dense phase that is enriched for these molecules and a phase that is depleted (Figure 1). The interface of these dense droplets forms a boundary that allows the selective passage of some molecules but not others. The presence of this interface makes it possible that liquid droplets can function as compartments. Once formed, liquid droplet compartments can also adopt different physical states; they can, for example, harden into gel- or glass-like states or they can turn into solid crystals. Thus, phase separation allows the formation of a wide range of compartments with different physical properties.

In this Primer, I introduce the emerging topic of biological phase transitions. I further discuss how cells use phase transitions to regulate biological functions and activities in order to increase their fitness and chances of survival.

The colloidal nature of cells

The cellular interior is not a well-mixed collection of molecules. Macromolecules are enriched or depleted in certain areas of the cytoplasm and the distribution of these macromolecules is subject to constant change. Early concepts to explain these density fluctuations proposed a role for local synthesis, anchoring or active transport. Yet these concepts are insufficient to explain the complex organization of cells. We now realize that cells can only be properly understood if we also consider the collective properties of biological macromolecules.

Chemists have known for a long time that macromolecules can have collective properties. The science of macromolecular collectives is called colloidal chemistry. One example of a colloid is Jell-O, which is composed of colored water and gelatin, a large, string-like protein. Gelatin dissolves well in hot water; however, when the water is cooled, the gelatin molecules become insoluble and sticky and then form a cross-linked network. The result of this process is a hydrogel.

The concept of viewing cells as colloidal systems was first proposed in the late 19th century by Edmund Wilson. Wilson pointed out that cells appear as if they are densely packed with macromolecular phases or liquid 'coacervates'. Alexander Oparin later considered these coacervates an essential constituent of the primordial soup. However, these initial concepts of cellular organization were eclipsed by the enormous success of reductionist molecular biology. Very recently, however, we have seen a revival of colloidal biochemistry. Key was the realization that cells contain compartments that lack membranes.

Membrane-less organelles

Membrane-less organelles are micron-sized cellular structures that consist of large collections of macromolecules, such as RNAs and proteins. Modern imaging approaches are revealing an increasing number of these structures (Figure 2). One prominent example is provided by the centrosome, which organizes the microtubule network of cells. Another example is provided by processing bodies or P bodies, which store and degrade RNA. Then there is the example of the nucleolus, an assembly line for ribosomes: it has a fibrillar center, where the ribosomal RNA is produced, and two additional intranucleolar compartments, which

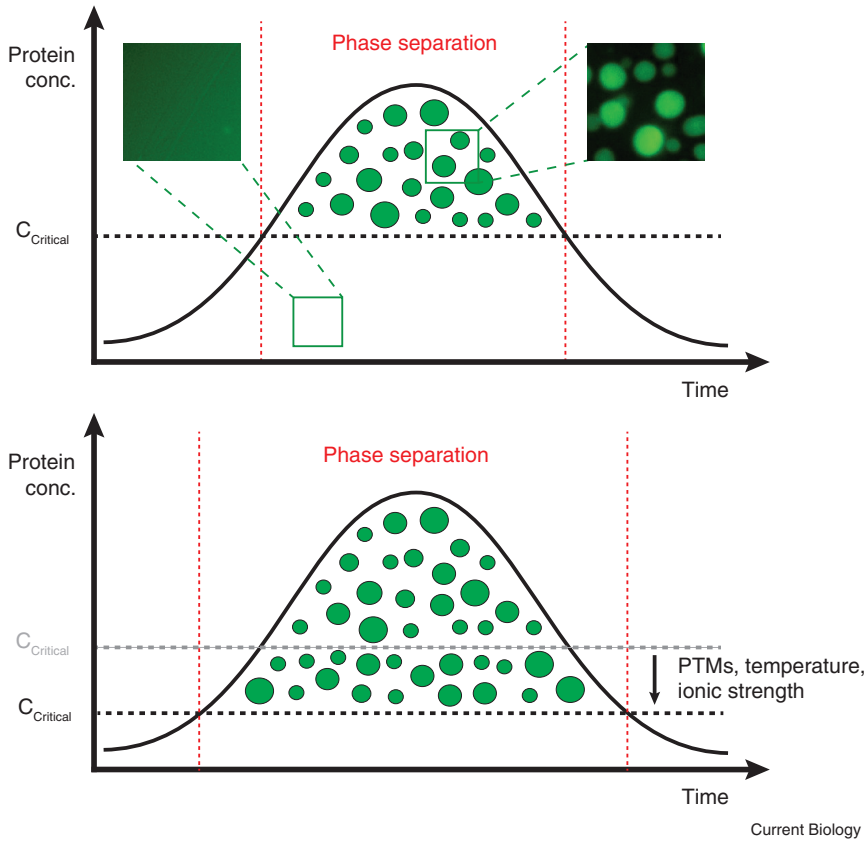

Figure 1. Protein phase separation.
Top: A protein forms dense liquid droplets above the critical concentration ($C_{critical}$) for phase separation. The liquid droplets are stable as long as the total concentration is above the critical threshold. The droplets form a compartment that allows the diffusion of molecules within the compartment and promotes the dynamic exchange of molecules with the dilute surrounding phase. Once the total protein concentration drops below the critical concentration again, the compartments dissolve and the system relapses back to a one-phase state where the molecules are evenly distributed. The insets above show original data from a phase separation experiment with purified GFP-tagged FUS (a prion-like RNA-binding protein). Bottom: Post-translational modifications (PTMs) or changes in temperature or ionic strength can increase the affinity of protein–protein interactions, thus lowering the critical threshold for phase separation and allowing compartment formation at a much lower protein concentration.

assemble ribosomes from molecular components.

These examples illustrate that, despite the absence of membranes, these organelles can be very large in size and have a complex, multilayered organization. Recently, the term 'biomolecular condensate' has been proposed to refer to these different structures. Biomolecular condensates are highly abundant in eukaryotic cells. But how can these condensates concentrate so many molecules in the absence of membranes?

Molecular features driving biological phase transitions

Liquid–liquid phase separation driven by collective protein–protein and protein–nucleic acid interactions is emerging as the key organizing principle of cellular condensates. Using this physical framework, we can now study the assembly, composition and properties of membrane-less organelles. Early concepts to explain the formation of membrane-less organelles invoked a role for strong, stereospecific interactions, but more recent work has emphasized the importance of very weak interactions. It appears that both weak and strong interactions can contribute to condensate formation, but what is most important is that many of these interactions take place at the same time to allow the formation of a network of interactions.

In a key paper in 2012, Mike Rosen and colleagues provided the first experimental evidence that multivalency — the availability of many different binding sites on a molecule — is the crucial parameter that determines whether or not a protein will undergo phase separation. Many proteins, especially those functioning in signaling or gene expression, contain tandem arrays of modular domains. Each of these interaction modules binds a particular ligand, often with relatively weak affinity. The number of these different modules determines the valency of the molecule. Importantly, multivalent proteins have a critical threshold for phase separation that depends on the number of modules and available ligands. Increasing the number of the modules promotes the formation of increasingly larger complexes that manifest macroscopically as phase-separated droplets. Thus, although the composition of cellular bodies appears to be complex, their formation may be governed by very simple rules, such as protein valency and the availability of ligands.

Another feature that strongly affects the phase behavior of proteins is their solubility. Many phase-separating proteins have poor solubility in water. Burying such insoluble proteins in a growing phase-separating structure is energetically more favorable than exposing these proteins to water. As a consequence, solubility changes and phase separation are often coupled. This is particularly relevant for multidomain proteins that are connected by flexible linkers. These linker sequences often have poor solubility and can undergo weak interactions among themselves that can further drive the phase separation process. Thus, linker regions between modular domains can make a strong contribution to the driving force of phase separation.

Besides multidomain proteins, intrinsically disordered proteins (IDPs) have been implicated in promoting phase separation. Unlike globular proteins, these proteins do not have a fixed conformation and they are often characterized by low sequence complexity. This means that these IDPs use only a small subset of the 20 available amino acids. The

exact sequence of IDPs is often not important, but what matters for phase separation are simple charge patterns and the overall sequence composition.

Several different types of IDPs with low sequence complexity have been identified. One type is known as prion-like because proteins with similar amino acid compositions have also been shown to form heritable aggregates, or prions. These proteins are composed of mostly polar amino acids, such as serine, tyrosine, glutamine, asparagine, and glycine. This sequence composition often reduces the solubility of these proteins in water and promotes multivalent interactions. A second class of low-complexity IDPs contains high numbers of positively and/or negatively charged residues arranged in characteristic charge patterns. Such charged IDPs undergo electrostatic interactions that are highly sensitive to the pH and ionic strength of the solution.

Phase separation can occur with one, two or multiple proteins. Phase separation of a single protein is also known as simple coacervation; phase separation that requires complex formation by two or more proteins is known as complex coacervation. One protein that drives compartment formation by complex coacervation is the nephrin intracellular domain (NICD). NICD is a disordered, negatively charged protein that co-assembles with positively charged protein partners to form liquid droplets. Another example of complex coacervation is the interaction of arginine/glycine-rich RNA-binding proteins with RNA. In this case, polyvalent interactions between the positively charged arginine residues and the negatively charged RNA drive the phase separation process. Our increasing understanding of the molecular mechanisms of phase separation now allows us to unravel the principles underlying the formation of cellular bodies.

Principles of condensate formation and growth

The formation of phase-separated compartments is often impeded by a kinetic barrier that has to be overcome by nucleation. How condensates are nucleated in the cellular environment is largely unknown. However, *in vitro* experiments have taught us that different proteins have different critical concentrations at which they undergo phase separation (Figure 1). This suggests that condensate formation could be initiated by the protein with the lowest critical concentration; other proteins with a higher threshold could be recruited into droplets formed by proteins with a lower critical threshold. This further implies that regulation of the protein with the lowest critical concentration could be the rate-limiting step for condensate formation in the cellular environment.

Besides protein nucleators, there also is evidence that polyvalent polymers promote nucleation, as has been described for RNA and a molecule that is chemically related to RNA, called PAR or polyADP-ribose. RNAs have been shown to lower the critical concentration for phase separation of RNA-binding proteins. A seeding role for RNAs has also been suggested by a study showing that transcription is required for the assembly of the nucleolus. Regulation of phase separation by PAR molecules has been reported to occur during the DNA damage response. DNA damage breaks lead to the recruitment of PAR polymerase 1 and the subsequent local formation of elaborate PAR structures in proximity to the DNA lesion. Proteins such as the prion-like protein FUS can subsequently bind to PAR and this increases their local protein concentration above the critical concentration for phase separation, resulting in the formation of a compartment that promotes the recruitment of important DNA repair enzymes. Many PAR polymerases are present in eukaryotic cells and, while we do not know their exact functions, it is conceivable that each one of them promotes the formation of specific nuclear or cytoplasmic bodies.

Cells have to regulate where and when phase separation occurs. In principle, there are four ways in which this can be achieved (Figure 1): cells can regulate the protein concentration, the solubility, the affinity or the valency of a phase-separating protein. Changes in protein concentration could be induced by bursts of

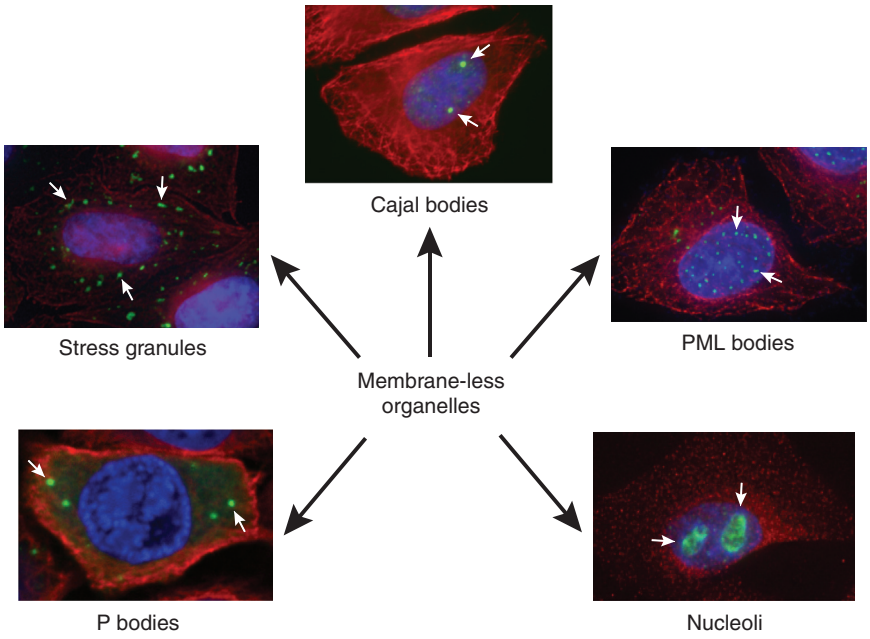

Figure 2. Membrane-less organelles in HeLa cells.
The cells shown express different GFP-tagged marker proteins from bacterial artificial chromosomes (BACs) that localize to different membrane-less organelles. The marker proteins used are: coilin (Cajal bodies), DCP1A (P bodies), MKI67 (nucleoli), G3BP1 (stress granules), and PML (PML bodies). Stress granules were induced with 1 mM arsenate for 30 minutes. The nucleus was stained with DAPI and the red signal is the result of co-staining with an anti-tubulin antibody. Images courtesy of Ina Poser (MPI-CBG).

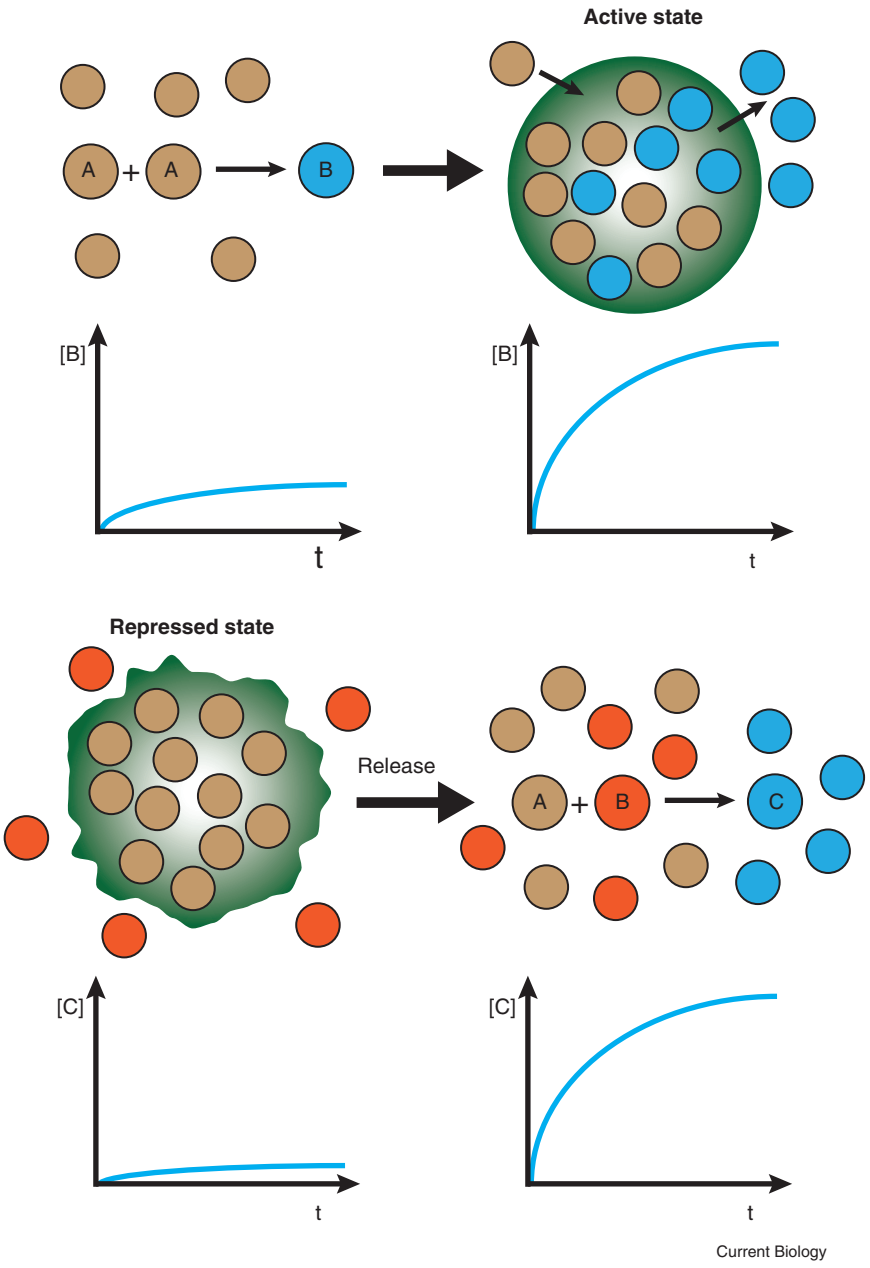

Figure 3. Activation or repression of biological activities by phase separation.
Top: Two proteins A react to form a product B. In the absence of phase separation, the concentration of A is low, thus leading to a low reaction rate. Phase separation allows enrichment of A in a liquid droplet compartment, thus increasing the local concentration of A and accelerating the reaction rate. Protein A could for example be tubulin monomers that are enriched in a droplet compartment to promote nucleation into microtubules (B). Bottom: A factor A is sequestered in a gel-like compartment in a repressed state. Release of A from the compartment allows A to react with B, resulting in the formation of the product C. The factor A could for example be mRNAs that are stored in RNP granules. When the mRNA is released, it is bound by translation factors (B) to synthesize new protein (C).

transcription or the release of a protein from a storage depot. The solubility, valency or affinity of a protein could be regulated through ligand binding or post-translational modifications.

In fact, there now is increasing evidence for regulation of membraneless compartments through post-translational modifications, such as phosphorylation and methylation.

The size of phase-separated bodies often changes with time, and liquid bodies can grow in two different ways. One possibility is Ostwald ripening, which describes the physical phenomenon of diffusion-limited movement of molecules from small to large droplets. Another possibility is growth by coalescence, which is when two small droplets come into close proximity, touch and then fuse into a larger droplet. A prerequisite for coalescence is that the cellular bodies can move freely by Brownian motion, although this is not always possible; for example, many nuclear bodies seem to be anchored to DNA or RNA, which restricts their movement and thus the ability to grow by coalescence. However, there also is evidence for active coalescence in cells. Cytoplasmic stress granules undergo fusion events that are dependent on microtubules and motor proteins. This suggests that in living cells there are important links between phase separation — a thermodynamic equilibrium process — and non-equilibrium phenomena, such as directed transport.

Regulating the material properties of condensates

Phase separation leads to the formation of dense liquid droplets that often exhibit a very high protein concentration. As we know from protein crystallization studies, a high protein concentration can favor nucleation and the formation of a crystal. It is also possible that proteins in the dense liquid phase become kinetically arrested with the droplet adopting a glass- or gel-like state. Recent *in vitro* studies indeed indicate that reconstituted liquid droplets are metastable and convert into a more solid-like state with time. In the case of the prion-like RNA-binding protein FUS, the liquid droplets first harden into gels and then form crystal-like, fibrillar aggregates. This maturation process has been called molecular aging and it seems to affect a large fraction of reconstituted liquid droplets formed by different proteins. This time-dependent change of the material properties is now known as the continuum model of phase separation.

Evidence for molecular aging of compartments in cells is scarce.

This suggests that cells use energy to regulate the material properties of their compartments to keep them in a dynamic, liquid-like state. Moreover, recent studies suggest that phase separation alone is insufficient to explain the distribution of proteins in membrane-less organelles. For example, the assembly of the nucleolus is dependent on active, energy-consuming processes, such as transcription. How condensate formation is regulated by energy-consuming enzymes in living cells will be an important area of research in the future.

Recent studies aided by high-resolution imaging approaches have provided evidence that phase-separated cellular bodies have a very complex internal organization. It appears that many proteins within these bodies localize to their own territories or phases. A recent study focused on the nucleolus to unravel the molecular basis of this multiphase organization. The authors could show that the multilayered organization of the nucleolus is the result of differences in the biophysical properties of the nucleolar phases, most importantly the droplet surface tension. Thus, the multilayered structure of many cellular bodies may be explained by the immiscibility of their different phases.

The functional repertoire of phase transitions

It is now clear that phase separation is a key principle for organizing the intracellular environment. But what are the functions of these cellular bodies? One function may be to concentrate biomolecules in a confined space and thus promote biochemical reactions (Figure 3). Cells seem to use this principle for regulating the formation of cytoskeletal structures. For example, reconstituted pericentriolar material can drive microtubule formation by raising the concentration of tubulin above the critical concentration for nucleation. The nucleation of actin filaments also seems to be promoted by phase separation. A multivalent system of at least three interacting proteins drives the recruitment of the actin nucleator Arp2/3, which subsequently leads to the formation of actin filaments on membranes.

Phase separation has also been shown to organize and facilitate signaling. This has been reported for the T-cell receptor, which can organize into membrane-associated phase-separated clusters. Reconstitution experiments revealed that these clusters promote the recruitment of specific sets of signaling proteins. Importantly, the clusters were enriched for kinases but excluded phosphatases. Thus, phase separation could be an effective way to amplify signals and perform simple forms of computation.

Cellular bodies are particularly abundant in the nucleus. The key biological processes that take place in the nucleus are transcription and splicing, suggesting that phase separation could be used to regulate these processes. Indeed, heterochromatin has recently been shown to be a phase-separated compartment. The presence of a compartment boundary in heterochromatin may be necessary to sterically exclude certain factors, such as RNA polymerases. Furthermore, a phase separation model has been postulated for transcriptional control. Super-enhancer formation by phase separation was proposed to explain the bursting behavior of genes and the simultaneous activation of multiple genes. However, experimental evidence for this model is still lacking.

Although there now is increasing evidence that droplet compartments can promote biochemical reactions, it is also conceivable that phase separation can repress biochemical reactions (Figure 3). One example is provided by ribonucleoprotein (RNP) granules. RNP granules often store mRNAs, which are then transported in a silenced state to diverse locations. By doing so, RNP granules promote the distribution of information in cells. In neurons, this allows the local synthesis of proteins in synapses and in dendrites upon demand.

Are there other functions associated with condensates? Because phase-separated compartments are collectives of many interacting molecules, they can in principle have emergent properties. As an analogy, many water molecules can assemble into a droplet, which displays the emergent property of wetness. Importantly, wetness is a collective property that is absent from individual water molecules. One can envision that such emergent properties are frequently associated with condensates. Condensates could, for example, provide mechanical stability by forming gels, they could generate molecular sieves that filter out molecules, or they could bend membranes to promote membrane fusion events. The theoretical functional repertoire of biomolecular condensates seems vast.

Phase separation and stress adaptation

Phase separation is extremely sensitive to changes in physico-chemical conditions, suggesting that phase separation could play an important role in stress adaptation. Cells put a lot of effort and energy into maintaining stable internal conditions. They have transporters and pumps that keep the osmotic conditions, salt concentration and pH in a range that is favorable for rapid growth. However, when challenged with stress, cells are no longer able to maintain these stable conditions. This could lead to sudden changes in physico-chemical conditions, which could in principle be read out by phase separation. If this phase separation process is coupled to changes in protein function, it could be used to mount an adaptive response.

In agreement with this idea, fluctuations in cytosolic pH promote widespread condensate formation during stress. Recent studies indeed suggest that these condensates have adaptive roles. For example, gel formation by the RNA-binding protein Pab1 has been proposed to regulate the availability of certain stress-protective RNAs. It seems very likely that stress adaptation through protein phase separation is a widespread phenomenon in nature.

The sensitivity of phase-separating proteins to changing physico-chemical conditions also comes with a cost because many phase-separating proteins are associated with age-related diseases. In fact, aberrant phase transitions may be a key driver of aging and disease, given that aging cells often lose control over their internal conditions. Thus, the concept of biological phase transitions may also lead to new insights into diseases and create new opportunities for therapeutic interventions.

Outlook

The framework of biological phase transitions has revolutionized our understanding of cells and organisms. But this emerging field is still young. Questions that we need to address are: what is the molecular grammar of phase separation? How do cells regulate phase separation? How do cells generate compartments with different identities? To what extent are phase transitions able to explain the complexity of cells, and how are phase transitions coupled to active processes? How do cells use phase separation to adapt to different environments, and how do different environments shape the evolution of phase-separating proteins? Can the concept of phase separation help us understand and treat age-related diseases such as neurodegeneration and cancer?

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Distinct locomotor control and awareness in awake sleepwalkers

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Sleepwalkers' complex nocturnal behaviors have inspired fictional characters from Shakespeare's Lady Macbeth to Polidori's Vampyre to Cesare, the homicidal somnambulist in *The Cabinet of Dr Caligari*. Yet although the underlying pathophysiology of sleepwalking, i.e. the partial arousal from slow-wave sleep, is today well-documented, the detailed sensorimotor mechanisms permitting locomotion and further complex behaviors to occur outside of conscious control remain poorly understood [1]. Further, the paroxysmal character, nocturnal pattern, and spontaneous onset have made it nigh on impossible to study somnambulism behaviorally during wakefulness. The goal-directed walking paradigm reported here, based on full-body motion capture and virtual reality feedback, directly addresses this issue and provides unique insights into the functional mechanisms of this common parasomnia: sleepwalkers exhibited improved movement automation and a stronger dissociation between locomotor control and awareness than matched controls when challenged with a cognitive load. Our data therefore suggest that behavioral markers exist in awake sleepwalkers, characterized by their ability to perform complex locomotor actions in the absence of full consciousness. Our findings are important as they firmly link sleepwalking to the neuroscience of motor control and motor awareness and may complement formal diagnosis procedures (normally requiring time, cost-intensive sleep studies and polysomnographic recordings).

Dissociations between automated motor control and awareness, so striking in each sleepwalking episode, have been extensively studied in healthy populations, albeit at much weaker dissociation levels [2]. Generally inspired by the comparator framework [3], such paradigms quantify participants' motor awareness and performance when exposed to different spatiotemporal mismatches concerning auditory or visual feedback about on-going movements [4,5]. Such paradigms have recently been adapted to locomotion, and in combination with dual tasking [6] have illustrated an increased dissociation between locomotor control and awareness under cognitive load [7,8].

To investigate both locomotor control and awareness in sleepwalkers, we asked a group of clinically diagnosed sleepwalkers and a group of age- and gender-matched control participants to move their tracked, virtual body into a virtual target cylinder by performing the corresponding goal-directed movement in the tracking arena (Figure 1A, see Experimental Procedures in Supplemental Information, published with this article online). Feedback of walking trajectories could be veridical or randomly deviated to the left/right by 5°–30° such that participants had to compensate for the deviation in order to reach the target [8]. Participants rated the veracity of the received feedback after each trial (yes/no). The secondary task was articulated backwards counting (steps of 7), a task reliably shown to interfere with locomotor control (independent of errors in arithmetic) [6].

All participants correctly performed the task and accurately identified that the virtual body reflected their own movements in control trials (93 ± 2% μ ± SEM self-attribution, cf. Table S1) and correctly rejected strongly deviated trials (4 ± 2% self-attribution for 30° mismatch, main effect of Deviation: $P < 0.001$), closely replicating previous results [7,8] (Bayes factor analysis BF = 4.58, half-normal prior derived from [8]). As illustrated in Figure 1D, there were no overt differences in locomotor awareness thresholds (controls: 13.4° ± 1.5, sleepwalkers: 13.2° ± 1.7) or walking performance (accuracy, velocity) between the groups in the single task condition.

However, under cognitive load, our study reveals two key findings that link sleepwalking to motor control and motor awareness in wakefulness. The first important finding is the