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**Review Article**

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**Publication date:**
2020-12

**Permanent link:**
https://doi.org/10.3929/ethz-b-000454411

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**Originally published in:**
Emerging Topics in Life Sciences 4(3), https://doi.org/10.1042/ETLS20190190

**Funding acknowledgement:**
159731 - Structure and Function of the Nuclear Pore Complex (SNF)

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# Membraneless organelles: phasing out of equilibrium

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**ABSTRACT**

Over the past years, liquid-liquid phase separation has emerged as a ubiquitous principle of cellular organisation implicated in many biological processes ranging from gene expression to cell division. The formation of biological condensates, like the nucleolus or stress granules, by liquid-liquid phase separation is at its core a thermodynamic equilibrium process. However, life does not operate at equilibrium, and cells have evolved multiple strategies to keep condensates in a non-equilibrium state. In this review, we discuss how these non-equilibrium drivers counteract solidification and potentially detrimental aggregation, and at the same time enable biological condensates to perform work and control the flux of substrates and information in a spatial and temporal manner.

Cells are amazing mini-reactors, crowded with DNA, RNA, proteins, lipids and metabolites. To avoid chaos and to make intracellular reactions efficient, cells have evolved several strategies to compartmentalise and organize their content. In eukaryotic cells, intracellular membranes form specialised organelles that separate, for example, transcription in the nucleus from translation in the cytosol, or sequester respiratory chain enzymes in mitochondria. In addition, the cytoskeleton provides 'highways' that allow for the directed transport of, e.g., RNA or vesicles to the distal end of cells, and positions membrane-bounded organelles such as the endoplasmic reticulum.
In recent years, it has become clear that there is yet another organising principle: the formation of membraneless organelles by liquid-liquid phase separation (LLPS) (1,2). In brief, LLPS describes the phenomenon that some biomolecules, such as proteins or nucleic acids, undergo dynamic self-association to form condensates within the surrounding cyto- or nucleoplasm. Examples include the nucleolus, nuclear speckles, stress granules or centrosomes. The role of LLPS in organising cellular content is ubiquitous, and critical for many biological processes in all cells, from bacteria to humans. This suggests that the process of biomolecular condensation is evolutionary ancient, and that phase-separated structures might have organized cellular components since the beginning of life (3-5).

LLPS-competent molecules have a certain solubility limit, and if this limit is exceeded, they reversibly condensate. Such behaviour is favoured because separating the cellular milieu into two regions, one of high component concentration and one diluted, minimizes the free energy of the system (6,7). In this respect, LLPS is, at its core, a thermodynamic equilibrium phenomenon. Yet, at equilibrium, the second principle of thermodynamics forbids any form of activity, such as directional molecular processing. Thus, in the absence of external energy input, condensates cannot perform any cellular work. In fact, they might even pose a danger since LLPS-mediated structures are prone to form irreversible, potentially detrimental aggregates (8). However, life does not operate at equilibrium and energy-consuming processes have evolved that keep condensates away from equilibrium. In this review, we discuss how cells exploit such non-equilibrium drivers not only as a protective mechanism but also to perform work, e.g., to regulate the flux of substrates and information, or to control biochemical reactions in a spatial or temporal manner. Thus, these non-equilibrium processes endow condensates with biological functions beyond a mere role in organizing a cell's content.

**Physical principles of condensation**

Interactions between molecules, e.g., between proteins, or proteins and nucleic acids, are characterised by the affinity or 'strength' of individual interactions, and the 'valency', which describes the number of interactions one molecule can undergo with its partners. The formation of many biomolecular condensates by LLPS is based on weak, multivalent interactions: components need to have a valency of at least three to establish a branched, interconnected meshwork (Figure 1) (9,10), and interactions generally have to be weak (in the high nanomolar to low micromolar range) to keep the condensates readily reversible and liquid-like. Interactions can involve globular domains or unstructured protein regions harbouring low amino acid sequence complexity (e.g., unusually rich in glutamine, asparagine, glycine, serine etc.), blocks of

opposite charge, or cation-pi interactions (between aromatic amino acids such as tyrosine and cations like arginine) (1,11). Theoretically, condensates can also form at valencies below three, for example when entropy favours dense packing of large, inert entities accompanied by the un-packing of smaller molecules, a phenomenon referred to as 'depletion interaction' (12). However, since depletion interactions of colloidal particles are not specific, it is unclear whether they play a significant role in biology.

Condensates assemble when the concentration of at least one of their key components crosses its critical saturation limit. The result is the formation of liquid-like 'droplets' where the components can concentrate over their surrounding environment by several orders of magnitude. Following the law of mass action, an "initial equilibrium" that partitions components within the droplet and in the soluble phase outside the droplet is established rapidly, until the soluble pool is again below the solubility limit (7,13). Once an equilibrium is reached, there is no longer any net flux of components into or out of the droplet, although – due to the weak, short-lived nature of the interactions - passive exchange of components between the droplet phase and the soluble phase can still occur.

This "initial equilibrium" state persists if all components keep their original conformations and interactions. However, most biological LLPS systems display additional 'ripening' behaviours (Figure 2): after the two liquid phases are phase-separated, often nucleated by a multitude of sites, the system still tries to minimize the interface between phases, and thus the free energy associated with the surface tension. To do so, smaller droplets can either fuse, or undergo a process called 'Ostwald ripening' where smaller droplets dissolve and their components associate to larger ones until, ideally, a single droplet remains (4,14). Another ripening behaviour is a kinetically slow solidification process that turns liquid-like droplets over time into irreversible aggregates (15,16). While the structural principles underlying this droplet aging remain poorly understood, one possibility is that in the initial liquid-like state the condensate components are not yet in an optimal sterical conformation and only a fraction of all possible inter-molecular interactions are formed. Over time, aligning - or deforming - them into more favourable conformations could then promote the formation of additional and stronger interactions (17). We are only beginning to understand what protein features (18) or factors in the cellular environment could promote these transformations, however, an increase in the percentage of occupied interaction sites would lead to a solidification, which confers rigidity to the denser meshwork limiting exchange of its components. A "final equilibrium" - or minimal free-energy state - is reached when a maximum of interactions is established without generating strain. Solidification has been observed in several biological systems, including centrosomes (19,20), germ granules (21,22), enhancer condensates (23) and heterochromatin (24). For some of these systems, a gel-like material state seems to be beneficial and presumably supports biological function by providing mechanical stability, by increasing component retention times (25) or as a means of celluar adaption (26). But in many cases the benefit of solidification is unclear, and the process might be rather detrimental. In this light, the aging behaviour of phase-separated condensates has gained a lot of attention as it might underly the aggregation phenomena seen in a multitude of age-dependent aggregation diseases, including a large group of neurodegenerative diseases (8,27,28). For example, alpha-synuclein

(29) or TDP-43 (30) and FUS (31), critical drivers of Parkinson's disease or amyotrophic lateral sclerosis (ALS), respectively, can undergo LLPS and over time transform into irreversible aggregates *in vitro.*

**Biological condensates are heterogenous**

Biological condensates, or membraneless organelles, are built from different types of macromolecules, and contain a large number of distinct components. For example, the stress granule proteome was estimated to consist of at least several hundred different proteins and a large number of distinct RNAs (32-35). Among them, 'primary' components (also called 'scaffolds') have the ability to undergo phase separation whereas 'secondary' factors (or 'clients') cannot phase-separate by themselves, but nonetheless can be selectively recruited to condensates via interaction with the primary components, and in consequence enrich significantly in the organelle (2,9). Given the compositional complexity of membraneless organelles such a classification is obviously a simplification. Secondary factors can regulate, enhance or decrease phase separation and thereby dramatically influence the overall condensation result (6,36-38). Importantly, the heterogeneity of condensates can also lead to non-intuitive differences in the behaviour of their constituents. For example, both primary and secondary components can have very different exchange rates, ranging from rapid exchange and liquid-like behaviour to very slow turnover, as if they were 'solid' components of a given organelle (38-40). Likely these behaviours depend on the strength and valency of interactions of a given component with its partners, but potentially also the size and diffusional ability of a component within the LLPS meshwork.

Differences in biochemical component properties can also lead to further un-mixing and formation of 'droplets within droplets'. This can induce the establishment of distinct sub-compartments with different material and thermodynamic characteristics. Gel-like or even fibrillar cores are, for instance, found in the nucleolus (41) or in stress granules (32) and surrounded by more liquid compartments. The inverted situation is found in P-granules, prominent RNA-containing organelles present during development where a shell of gel-like MEG-3 granules forms around, and thereby locally stabilizes a liquid core of phase-separated PGL-3, which by itself is intrinsically labile (39).

**Regulation of biological condensates**

The formation of LLPS condensates is critically regulated by the concentration of its constituents. In a biological context, this is exemplified by P-bodies and stress granules, whose formation depends on the availability of non-translated, ribosome-free mRNA (42). However, changing protein or RNA levels within a cell, either by new synthesis or degradation, is slow and energetically costly. It is therefore of importance that besides changing the component concentration, condensation can also be controlled by changing the strength or valency of interactions, or more generally, by shifting the critical saturation threshold. In cells, this can be triggered by changes in temperature, crowding and also pH, which can occur during heat shock, nutrient starvation or changes in growth state. These altered conditions can directly induce changes in protein conformation to expose interaction sites and to promote the formation of

condensates (43-45). Thus, in particular stress situations the altered cellular environment is ‘sensed’ to shift the condensation threshold and propensity -without altering component concentrations- in order to respond and survive stress situations (46,47).

Furthermore, several energy-consuming, enzymatic processes modulate condensation. Post-translational modifications can change the physical and chemical properties of amino-acids, and thus have the potential to alter interaction strengths (48-51). For example, arginine methylation and phosphorylation systems have been described that prevent or promote condensation, or acutely dissolve existing condensates, thus acting as on-off switches for membraneless organelles. This includes protein kinase A (PKA), which prevents formation of P-bodies when nutrients are abundant by directly phosphorylating the P-body regulator Pat1 (37,52), and the DYRK3 kinase triggers the disassembly of several membraneless organelles during mitosis (53,54). In addition, post-translational modification could also be used to fine-tune cellular condensates, and recruitment of kinases was postulated to control and limit the size of individual condensates (6).

Another critical set of regulators of cellular condensation include protein chaperones and RNA helicases. These enzymes are prominently associated with different membraneless organelles, in particular larger condensates like the nucleolus or stress granules (32,55,56). In general, the main task of these enzymes is to constantly remodel protein-protein, protein-RNA and RNA-RNA interactions, and to shield exposed surfaces (57-60). Heat Shock Proteins such as Hsp70, Hsp90 and Hsp60 (GroEL in bacteria, CCT in eukaryotes) remodel proteins through their ATPase cycle and thus promote folding or disaggregation of condensate components (61,62). Thus, they can ultimately change interaction patterns and efficiently modulate the critical concentration threshold that is required for condensation. As a consequence, members of these protein families were shown to be important regulators of both assembly and disassembly of membraneless organelles. In addition, they can keep condensates in a liquid and reversible state (32,60,62-66), and chaperone malfunction, for example during ageing, has been linked to aggregation diseases and neurodegeneration (28,67).

**How do condensates at equilibrium and non-equilibrium execute function beyond mere assembly?**

Phase separation has now been intimately linked to many important cellular processes, but the holy grail remains to understand the function(s) of biological condensates: do they merely act as glue, sticking together molecules in order to build larger assemblies, or can they accomplish more than that?

In the following, we discuss several types of condensate functions (Figure 3). Some of them, like the spatially localized enrichment of macromolecules in a membraneless organelle, could -in principle- occur at thermodynamic equilibrium. At thermodynamic equilibrium, components can continuously exchange between condensates and the soluble phase, yet by definition, the net fluxes remain zero. However, as discussed, cells employ a whole set of energy-consuming enzymes that keep condensates away from

reaching equilibrium (7,68). For one thing, this can regulate condensate dynamics or prevent the ripening or solidification of condensates avoiding that their components enter into a non-reactive state with no exchange of molecules with the surrounding medium.
However, energy input procures more than the regulation of condensate assembly or reversibility. The injection of energy into condensates by enzymatic processes offsets condensates from equilibrium, yet, at the same time, condensates steadily try to return to their equilibrium state. A crucial consequence is that condensates now exist in a *non-equilibrium steady-state* that is intrinsically different from an equilibrium one: it can generate actual net fluxes, which can be exploited to drive downstream processes. Thus, energy-fuelled condensates can perform biochemical work, transmit directional information, or even power the translocation of macromolecules in a spatial and temporal manner.

**Buffering of concentration changes**

In a simple model, phase-separating molecules have a concentration-dependent solubility limit. Condensates form if the concentration exceeds this solubility limit, and grow until the concentration of the soluble pool is sufficiently reduced (4,7). If the total concentration of a component further increases, a new equilibrium is rapidly reached yet the concentration of the soluble phase remains constant. It is therefore 'buffered' against concentration changes. In consequence, cells could use phase separation to dampen concentration fluctuations and keep the concentration of soluble components constant. However, it has been recently demonstrated that biological, multi-component systems are more complex, and that saturation concentrations and partitioning coefficients depend on the cellular context and interaction networks (36,69,70). Nevertheless, buffering by LLPS could counteract variability or stochasticity in various steps of gene expression reducing the 'noise' in cellular protein levels. Indeed -and despite the fact that phase separation processes in cells are generally kept away from equilibrium-, it was recently demonstrated that membraneless compartments can effectively reduce protein concentration noise in a living system (71).

**Concentration hubs as highly selective reaction centres**

Once formed, condensates act as concentration hubs that sequester both the phase-separating component and other interacting proteins or nucleic acids, away from a homogenous distribution. However, condensates are biochemically selective: they enrich for some proteins while excluding others (19,36,72,73). This has major biological consequences: first, concentrating enzymatic activities and their substrates can influence their enzymatic activity that, by the law of mass action, depends on the concentrations of both enzyme and substrate (74-80).
Second, formation of condensates can control protein or RNA localization in a spatial and temporal manner. As an example, for the diverse set of RNA condensates this could mean that if a transcript enters a specific condensate, it will be selectively modified by enzymes or RNA-binding proteins enriched therein. Condensate formation can therefore speed up and alter RNA modification rates, RNA unfolding, and the remodeling of mRNA-protein (mRNP) complexes. Conversely, selective sequestration into condensates

can also prevent that enzymes and substrates see each other and thus slow down or even completely inhibit enzymatic reactions (81). For instance, mRNAs could be hidden or stored away in cytoplasmic RNP granules preventing their access to the translation machinery (82-84). However, given the constant exchange between the phase-separated condensate and the soluble phase at steady state, complete inhibition will require low exchange rates and high partition coefficients in a condensate.

Phase separation is further important for the formation and function of super-enhancers, where highly dynamic nano-condensates of transcription factors and the mediator complex coalesce on specific DNA sequence elements, and subsequently sequester the transcription machinery to promote transcription (85-92). Thus, phase separation on super enhancers provides an elegant explanation for the highly co-operative processes that are required to bring together many protein factors in order to allow for a highly selective transcriptional activation of developmentally important enhancer and promoter DNA elements.

**Signalling cascades and gradients**

Formation of condensates requires multivalent interactions at component concentrations above a certain threshold. If either the concentration or the interaction strength of primary condensate components is actively and selectively enhanced or diminished, e.g., by post-translational modifications such a phosphorylation, condensates can from or disassemble in a spatially and temporally controlled manner (7,14,68). This can result in spatial condensate gradients or other non-homogenous distributions of condensates that however need to be actively maintained and replenished by energy-consuming processes (93). Cells have adopted this concept to amplify or transmit information.

For example, P-granules in *C. elegans* are not evenly distributed, and they concentrate at the posterior end of the one-cell embryo, which is essential for proper embryonic development. This spatial gradient is achieved by the permanent, phosphorylation-dependent granule dissolution occurring at the anterior pole of the embryo (94-96). Another example is the proposed channelling of nascent mRNA transcripts through a series of condensates formed from transcription initiation or elongation factors, and RNA polymerase II itself. Here, changes in the phosphorylation pattern of RNA polymerase II promote transfer between initiation and elongation condensates, which ultimately enhances and drives directionality of the process (97-99).

Like other concentration hubs, such actively induced condensates can have emergent properties as exemplified by cellular signaling cascades: local phosphorylation-induced phase separation of upstream signalling factors enriches the downstream signaling components beyond a critical threshold to form condensates initiating a signaling cascade (100). Examples include PAR-mediated clustering of factors for early DNA damage response (31,101,102), signaling factor clustering to create asymmetric daughter cells in bacteria (103), clustering of T-cell receptors for nucleation of actin polymerisation (104), or condensation

of centrosome components for microtubule nucleation (19,105). The observed stimulation of signaling activity in such hubs can exceed what is expected merely based on the increase in local concentration, probably by prolonging residence and interaction times in condensates (106).

**ATPases drive and orchestrate substrate flux through membraneless organelles**

While the flow of information is rather abstract, condensates can also orchestrate the directional flux of actual molecules if they receive input from energy-consuming enzymes. For example, mRNAs are sent through a series of condensates, from transcription and splicing to export through the nuclear pore, and ultimately storage or decay in P-bodies and stress granules (107-109). Along their life, mRNA molecules are chaperoned by diverse proteins. In particular, the family of DEAD-box ATPases (DDXs) is not only a chaperone or passive companion, but rather acts as a global regulator of mRNA flux through these condensates. Many DDXs possess low complexity sequences that allow them to undergo phase-separation in the ATP- and RNA-bound form, which results in RNA accumulation in the condensate. DDX ATPase activity triggers condensate dissolution and release of the RNA substrate, that can then transit to another granule. In addition, DDXs can control condensates and RNA accumulation *in trans* by remodeling phase-separated structures (56,66). Mutations that abrogate DDX ATPase activity prevent release of the RNA, and thus halt or diminish the flux of RNA, resulting in increased or even ectopic RNA accumulation and condensate formation (56,63,83,110-112). Since mRNAs and mRNPs can be specifically modified by condensate-enriched enzymes or RNA-binding proteins, the mRNA particle leaving a condensate will be distinct from the one that entered. Such active processes keep the condensates away from equilibrium and can generate directional and irreversible fluxes. Thus, energy-consuming enzymes like DDXs not only regulate condensate formation and turnover – they have the potential to orchestrate and regulate the flux of genetic information in a spatial and temporal manner.

**Concluding remarks**

Slowly, we are beginning to realize how pervasively phase separation is hard-coded in the cellular proteome. A large number of proteins - up to 75%, depending on the species - contain intrinsically disordered sequences. While not all intrinsically disordered protein domains function in condensation, many of them contain sequences of low amino acid complexity and thus have the potential to promote liquid-liquid phase separation. Equally surprisingly, cells keep the majority of their proteins slightly above their intrinsic solubility threshold (16,113). Cells thus operate at the solubility limit, which could explain why upon energy depletion or during stress, the cytoplasm can convert from a fluid state to a more gel-like or solid state (114-117). This transformation is likely influenced by phase separation of some components of the proteome carrying aggregation-prone sequences that are normally kept soluble by energy-consuming processes. While this cytoplasmic transformation might be important for cells to enter a quiescent state or to remain dormant it also poses a potential risk, since phase-separated condensates can mature over time into irreversible, potentially toxic aggregates akin to aggregates observed in age-dependent aggregation diseases. For example, *in vitro* reconstituted condensates of purified proteins like FUS, implicated

in ALS, quickly 'age' into more solid, aggregated forms, whereas FUS solidification is not observed in young and healthy cells (15,25,31).
The implications are two-fold: first, given their potentially detriment, phase separations must provide huge benefits to the cell, otherwise the bulk of low complexity sequences would have been lost over evolution. And second, kinetically unstable condensates pose a major challenge, and cells constantly counteract spontaneous condensation, and once formed, prevent condensate-maturation and ageing to avoid conversion into irreversible aggregates. To keep these processes in check, evolution has created energy-consuming solutions and machineries.

Biomolecular condensates are found in both pro- and eukaryotes, and the cellular usage of LLPS as an organizational principle is thus likely evolutionarily ancient. It is tempting to speculate that molecular condensates appeared even before the beginning of life to allow for the selective concentration of specific macromolecules in the primordial soup (3-5). Turning such condensates into a living system would then necessitate the addition of non-equilibrium drivers, that could utilize free energy from the environment to create ordered structures and ultimately evolve an ability to self-replicate. In this regard, the nucleolus might be the closest remnant of such an ancestral condensate efficiently producing ribosomes around the genetic material coding for ribosomal RNA, which in an early RNA world might have acquired the ability to self-replicate even prior to the evolution of membrane-bounded cells.

Throughout the tree of life biomolecular condensates are widely used in a large number of biological processes, probably because they are an elegant combination of efficiency and flexibility. Interactions within condensates often rely on short, unstructured domains, that unlike complex three-dimensional protein folds can easily change and evolve. Thus, biological condensates might have the ability to readily add new factors or enzymes to their inventory and thereby attain new functionality. Furthermore, membrane-less organelles, in contrast to their membrane-enclosed counterparts, do not need to evolve complex transport machineries to take up new components and they are highly dynamic and can form *de novo*. This enormous flexibility might outweigh the constant energetic demands to keep condensates away from equilibrium and to prevent potential deleterious aggregation. Their dynamics and ease of regulation makes them also particularly suitable to rapidly respond to changes in the environment and to orchestrate changes in metabolism or gene expression during times of stress. Why would cells otherwise want to keep some sticky glue in their belly?

## SUMMARY POINTS

1. Liquid-liquid phase separation is an equilibrium phenomenon which excludes a net flux of macromolecules between condensates and the surrounding environment.

2. Reaching the lowest free energy at equilibrium is potentially detrimental since it can lead to solidification and aggregation of condensates.

3. Cells have evolved energy-consuming mechanisms to keep condensates in a non-equilibrium steady-state. These mechanisms include phosphorylation cycles, protein chaperones and RNA helicases.

4. Beyond keeping condensates in a liquid-like state, non-equilibrium drivers enable condensates to perform work. Cells make us of this to control biochemical reactions and the flux of substrates and information in a spatial and temporal manner.


## ACKNOWLEDGEMENTS

This work was supported by a grant from the Swiss National Fond (SNF 31003A_159731) to KW. The authors thank Vamshidhar Gade and Janka Zsok for critical reading of the manuscript.


## AUTHOR CONTRIBUTIONS

M.H., K.W., and P.R. wrote the manuscript, with critical input from S.H.. S.H. designed and created the figures.

## DECLARATION OF INTERESTS

The authors declare no conflict of interests.

## FIGURE LEGENDS

Figure 1: Valency: molecules with at least three interactions can form phase-separated networks.

Figure 2: Liquid-liquid phase separation is a thermodynamic equilibrium process. In cells, energy input keeps phase separated condensates away from equilibrium, which enables them to perform work and prevents their potentially detrimental solidification.

Figure 3: Examples for equilibrium and non-equilibrium functions of biological condensates. For details, see text.

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Valency

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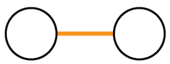

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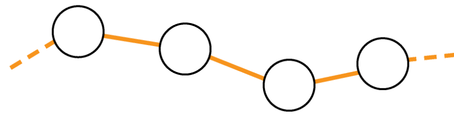

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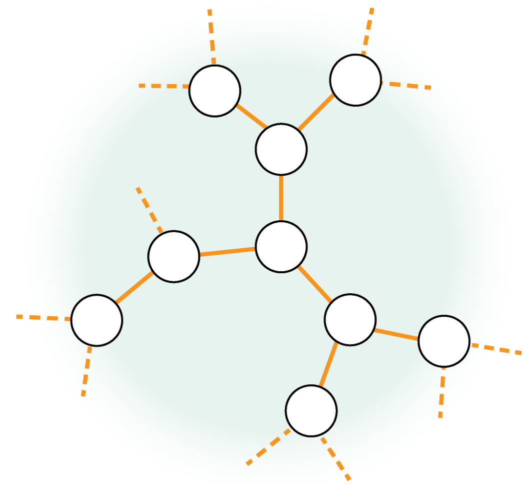

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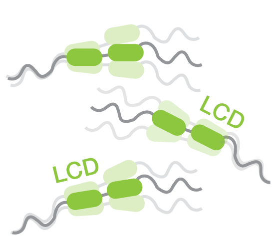


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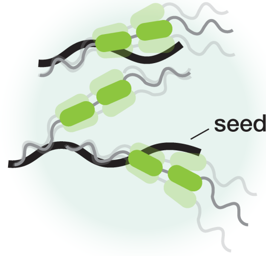


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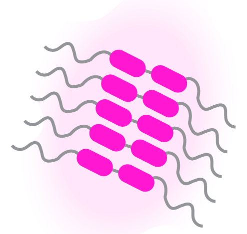


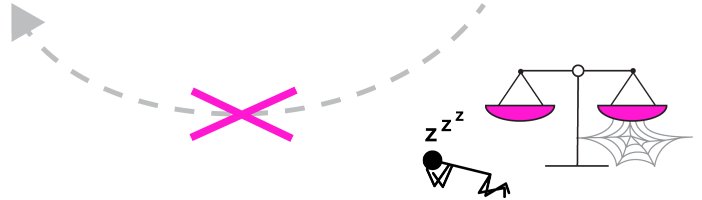

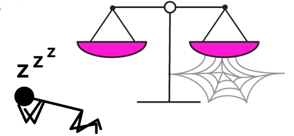


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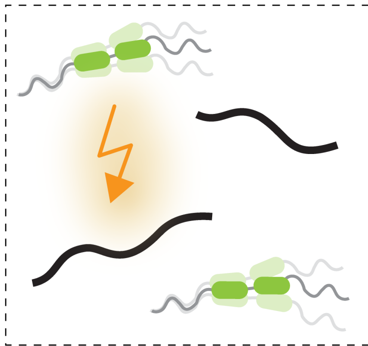

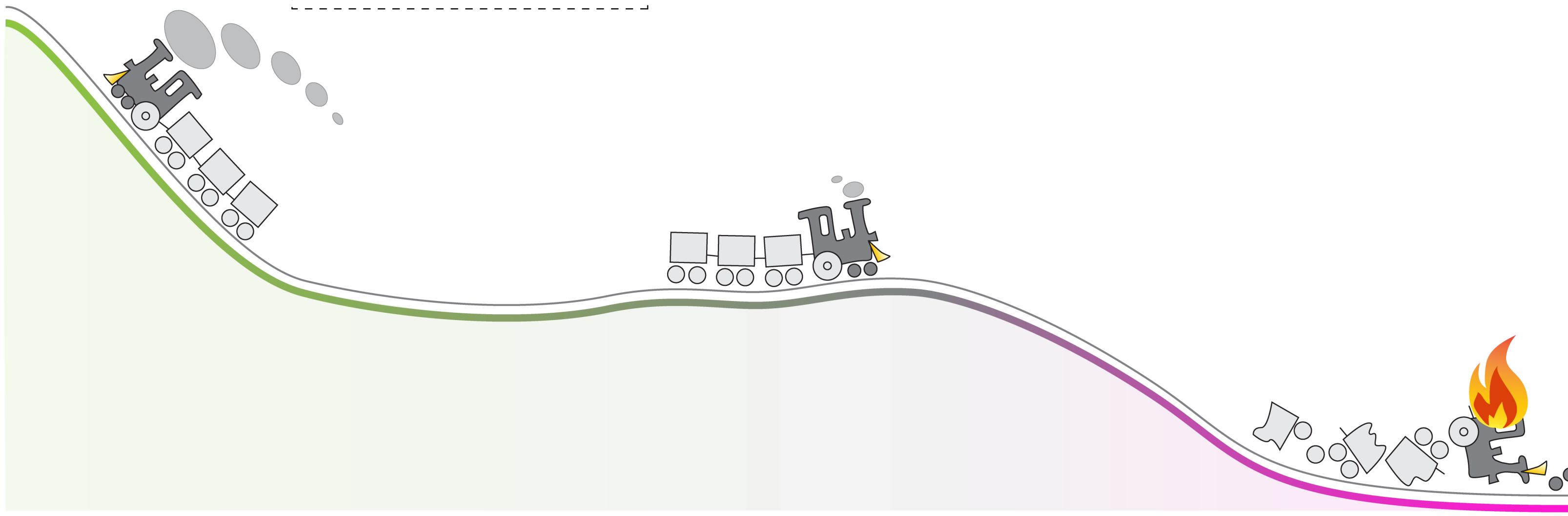

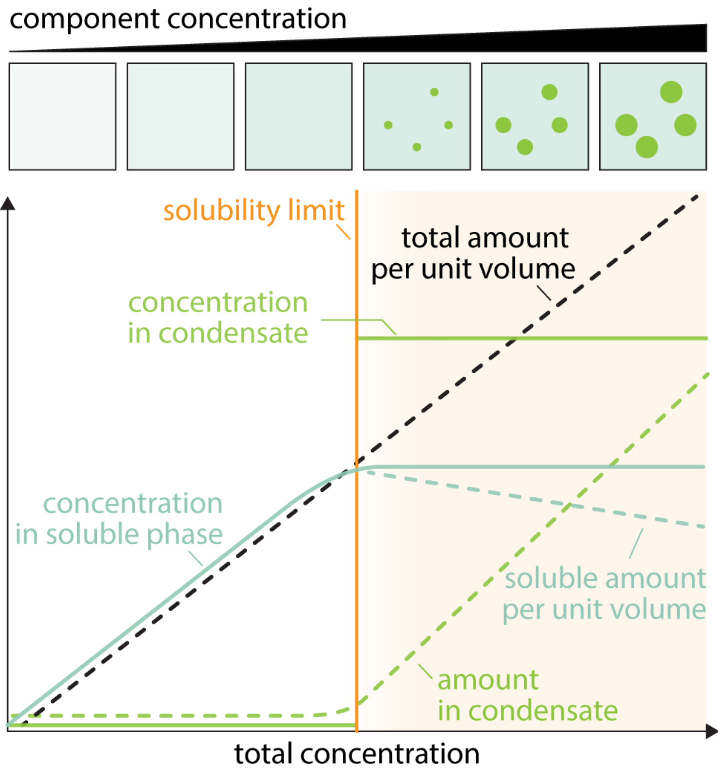
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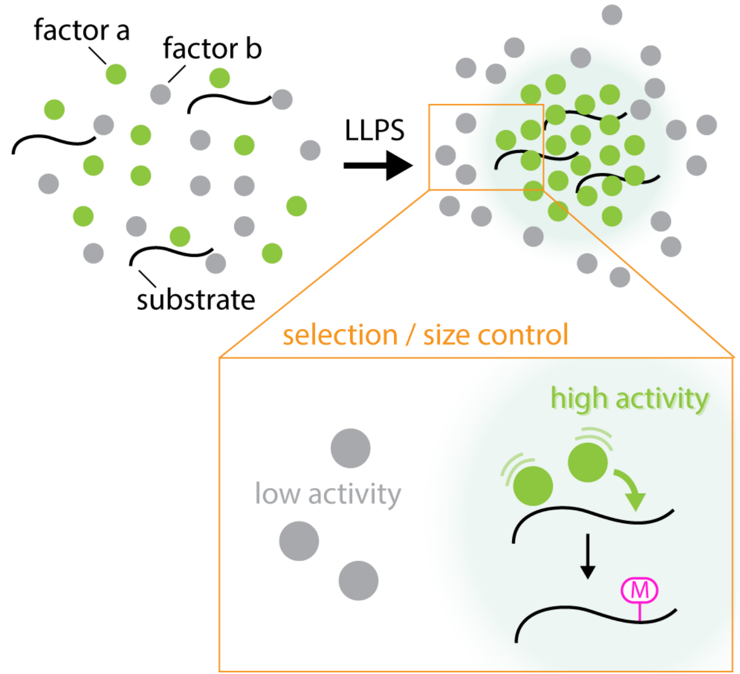
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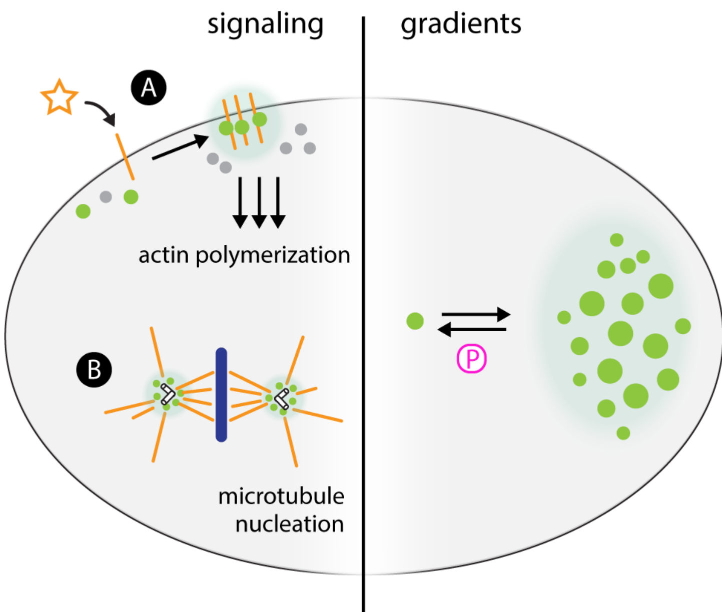
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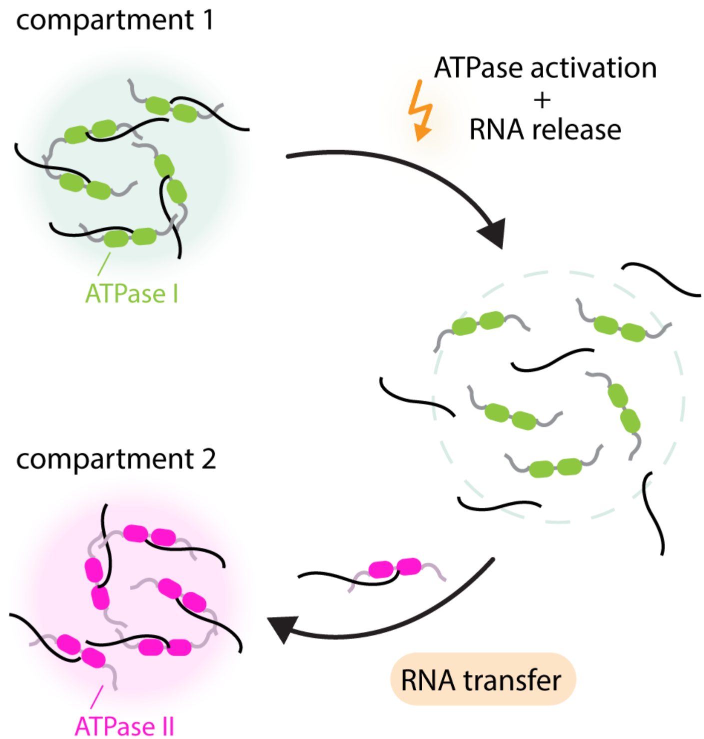
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