Assembling the right type of switch: Protein condensation to signal cell death

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Abstract

Protein phase transitions are particularly amenable for cell signalling as these highly cooperative processes allow cells to make binary decisions in response to relatively small intracellular changes. The different processes of condensate formation and the distinct material properties of the resulting condensates provide a dictionary to modulate a range of decisions on cell fate. We argue that, on the one hand, the reversibility of liquid demixing offers a chance to arrest cell growth under specific circumstances. On the other hand, the transition to amyloids is better suited for terminal decisions such as those leading to apoptosis and necrosis. Here, we review recent examples of both scenarios, highlighting how mutations in signalling proteins affect the formation of biomolecular condensates with drastic effects on cell survival.

Keywords: LLPS, Amyloid, Cell death, RNA-binding proteins, Deep mutagenesis.

From water to inorganic polymers and solvents, the ability of molecules to adopt different material proper- ties has fascinated scientists for more than a century [1]. Macromolecules, such as proteins, are not different in this sense [2]. Their transition from a soluble to a solid amyloid state is, for example, key to the onset of a range of neurodegenerative diseases and currently the target of several therapeutic strategies [3]. While, at least in vitro, the process of amyloid formation is relatively well understood, more recently, proteins were shown to undergo another type of phase transition, where they demix in two separate liquid phases, a light one and a dense one [4]. This process of liquideliquid phase separation (LLPS) has been observed for a wide range of proteins with different sequence compositions [5] and, in contrast to amyloid formation, is reversible. Condensates that consist of a separate liquid phase inside the cell include the nucleolus and stress granules [6,7], among many others, making LLPS a recognized mechanism of spatiotemporal organization of the cytoplasm [8]. However, observing liquid demixing *in vitro*, or under specific conditions, is not enough to imply that LLPS is essential for a specific protein function. With a rapidly expanding set of examples of liquid demixing proteins, the distinction between ‘does this happen?’ and ‘does this happen for a reason?’ has never been more important. Nevertheless, in a few cases, a direct causative link between phase transitions and protein function has been demonstrated [9,10]. This is the case of the low-complexity domain of DYRK3 and a number of other signalling proteins [10-12]. Indeed, several features of phase transitions and more specifically of LLPS are particularly amenable for the process of cell signalling:

1) Phase transitions are frequently, although not always, initiated by intrinsically disordered regions (IDRs). These regions can be easily modulated as they are enriched for Post-Translational Modification (PTM) sites. IDRs tend to contain multiple binding motifs and adapt to different binding partners. In addition, their exposure to the solvent makes them particularly accessible for interaction [13].
2) The condensates that form upon LLPS act as a reservoir of highly concentrated molecules and therefore can regulate signalling cascades in at least two ways: (i) by temporarily sequestering specific molecules and (ii) by concentrating binding partners [14].
3) LLPS is a thermodynamically driven process that occurs rapidly, in response to even tiny environmental changes in solution conditions (pH, temperature) or protein concentration [15]. There is no need for ATP consumption and for time-consuming processes such as protein synthesis and protein degradation.
4) LLPSs, but also other phase transitions such as amyloid formation, are extraordinarily cooperative pro- cesses that allow for efficient switching of an entire system, therefore providing a mechanism for binary decision-making when certain concentration thresholds are reached [16].

Choosing the right type of switch

In this review, we highlight how the commitment to a specific decision over cell survival can depend on the properties of the condensates formed upon phase transition of proteins in upstream signalling cascades. In the case of LLPS, condensation can be immediately reversed in response to changes in the cellular environment. For example, condensation of the yeast prion Sup35 in response to energy depletion and decreased intracellular pH reverts immediately upon addition of glucose [9]. This allows translation to rapidly start again, as soon as starvation has subsided. On the other hand, other types of transitions, such as liquid-to-solid or amyloid formation, are suited for more terminal types of decision due to the irreversibility of their nature (Figure 1). This is the case of the RIPK1/ RIPK3 complex that adopts an amyloid form to signal programmed necrosis [17] or of the inflammasome adaptor proteins apoptosis-associated speck-like protein (ASC) and mitochondrial antiviral signalling protein (MAVS) [18] which similarly undergo a prion-like polymerization.

Irreversible phase transitions to signal terminal decisions

Homotypic ASC assembly into amyloid-like fibrils is the signal required to activate caspase-1, providing a compelling example of prion polymerization as a mechanism of signal transduction with lethal consequences, leading to apoptosis in the context of innate immunity and inflammation [18,19]. Tumornecrosis-factor (TNF)-induced programmed necrosis is also mediated by the formation of a very stable type of complex, the necrosome, which instead consists of heterotypic fibrillar structures [17,20]. These are formed upon interaction of the active RIPK1 and RIPK3 kinases and display all the properties of classical insoluble amyloids, such as the ability to bind Thioflavin-T and a cross-beta X-ray diffraction pattern. The cooperativity of the amyloid process and its irreversibility make this transition suited for determining necrosis. Amyloid formation amplifies the signal by further potentiating kinase activation (Figure 1). Mutations in the aggregating domains of RIPK1 and RIPK3 both interfere with amyloid formation and impair RIPK3 kinase activation [17].

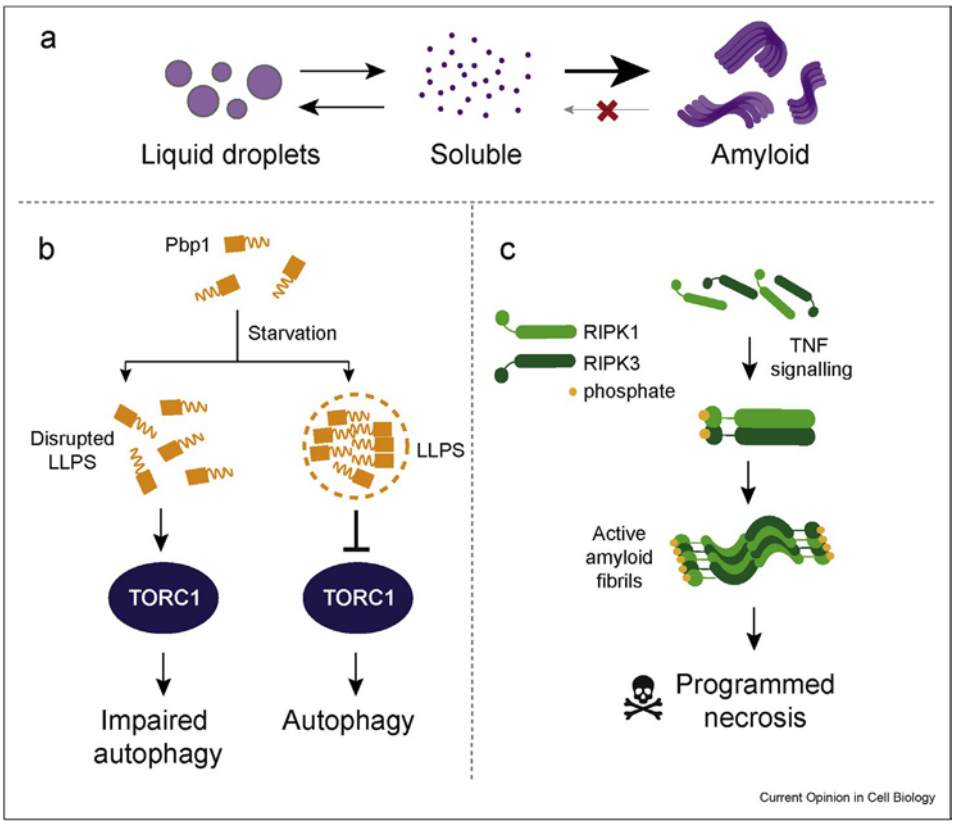

Figure 1: Different scenarios in the assembly of biomolecular condensates. (a) Through different processes, proteins can form condensates that display distinct material properties. On the one hand, proteins can undergo liquid demixing to form condensates that exhibit liquid-like properties (left). These condensates are dynamic and reversible. On the other hand, proteins can assemble in nondynamic, highly ordered fibrillar structures, such as amyloids, which can exclusively be disassembled by the action of specific chaperones. (b) Example of a reversible phase transition. During starvation, when yeast needs to switch from aerobic to respiratory growth, Pbp1 forms reversible condensates that inhibit TORC1 signalling. Inhibition of TORC1 promotes autophagy in cells and ultimately promotes cell survival. When LLPS is altered by mutations in Pbp1, autophagy is impaired, which compromises cell viability. (c) Example of an irreversible phase transition. Under necrotic stimuli, TNF-alpha signalling can induce NF-kB activation, apoptosis or programmed necrosis. In the last of these scenarios, when caspases are inhibited or under viral infections, the kinases RIPK1 and RIPK3 dimerize and form heterotypic amyloid fibrils. These amyloid fibrils constitute the core of the RIPK1/RIPK3 necrosome, a stable condensate that promotes cell death by necrosis.

Self-assembly with terminal consequence is also at the basis of ‘heterokaryon incompatibility’, that is, programmed cell death upon cell fusion of fungi. In this case, the transition to amyloid of the prion domain in heterokaryon incompatibility protein S (HET-S) acts as the switch for signal transduction, leading to activation of its HeLo domain with pore-forming ability [21,22]. The conservation of similar domains in signalling proteins across kingdoms strongly suggests that amyloid self-assembly is a common and effective strategy for the delivery of biological information [22].

In other circumstances, the type of programmed cell death can be decided on the basis of the physical state acquired by a specific protein. For example, while soluble mutant huntingtin exon (Httex1) is a known trigger of apoptosis, its transition to a solid phase

was shown to instead deactivate apoptosis and shift the signal to cell quiescence and a delayed death by necrosis [23]. Here, a question still stands regarding the mechanisms by which different material properties acquired by the very same sequence encode the activation of radically different pathways.

Disruption of signalling: when LLPS goes wrong

When it comes to cell division, reversible LLPS of signalling proteins has also been shown to play a critical role. In the process of spindle assembly, the effective condensation of SPD5 in *C. elegans* [24,25] and BugZ in *D. melanogaster* [26,27] promotes stabilization of micro- tubules via recruitment of high concentrations of the Aurora A kinase into droplets and consequent Aurora activation. Several interactors of Aurora, such as the proteins of the chromosomal passenger complex, were also shown to undergo LLPS [28]. Overall, proteins in the spindle assembly checkpoint pathway are essential and highly conserved [28] and so is their ability to condensate, suggesting a functional role of LLPS in this signalling pathway. Derangement of the spindle assembly checkpoint causes a premature or inappropriate entry in mitosis that results in cell death or aneuploidy [24].

Reversible polymerization and LLPS are also hypothesized to drive puncta formation by the Dishevelled proteins which in Drosophila function as propagators of signal to β-catenin effectors in the Wnt pathway [29]. Recently, Dvl puncta formation was shown to depend on reversible lysine acetylation of Dvl [30]. Mutations that disrupt Dvl puncta formation also alter Dvl-mediated signalling with consequences for cell survival and proliferation [30]. This calls for approaches able to systematically elucidate the effect of Dvl mutations on puncta formation, on the dynamics of the puncta formed and on the resulting signalling activity.

Stress granules (SGs), which assemble upon stalled translation during stress conditions, are one of the most well-characterized examples of reversible LLPS in the cytoplasm [7]. Ageing, mutations or persistent stress [31] are thought to impact SG dynamics and increase the likelihood of liquid-to-solid transitions [7]. SG dissolution is promoted by DYRK3, which allows release of mTORC1 for downstream signalling, allowing protein synthesis to start [11]. If this happens in inappropriate times, for example, when resources are still inadequate, it may lead to serious imbalances incompatible with cell survival. An additional function of mTORCs is inhibiting autophagy [32]. When mTORC1 is dysregulated by upstream alteration of the dynamics of SGs, this may prevent autophagy with consequent accumulation of cell debris, organelles and misfolded proteins. However, the link between autophagy and LLPS involves more than just DYRK3 [32]. Pbp1, the yeast orthologue of mammalian ataxin-2, is a LLPS protein that inhibits activation of TORC1 during respiratory growth (Figure 1). Mutations in methionine residues that alter the LLPS propensity of Pbp1 were shown to lead to hyperactivated TORC1, inhibition of autophagy and cell death [33].

Phase transitions have implications that go beyond the fate of an individual cell. Indeed, upon infection and consequent release of DNA to the cytosol, LLPS was shown

to play a role in innate immune signalling. cGAS, an enzyme that transforms ATP and GTP in cGAMP, is able to undergo LLPS upon binding to cytosolic DNA. The condensates formed by cGAS provide a way to boost its enzymatic activity and produce cGAMP which in turn activates STING [34]. It is the adaptor protein STING that then induces the production of cytokines. The process of cGAS LLPS is therefore a way for the cell to transform a linear signal such as an increasing concentration of cytosolic DNA into a binary response that is the activation of STING, driving production of cytokines for secretion [34].

Phase transitions and RNA processing

Signalling cascades ultimately deliver a message to the RNA processing machinery, with consequences on transcription, splicing, RNA modification and translation. Many of the proteins in charge of RNA processing have the ability to shuttle between the nucleus and the cytoplasm and to form ribonucleoprotein (RNP) gran- ules by LLPS. Dysregulation of these liquid condensates can be deleterious in several ways: (1) by alteration of nucleocytoplasmic transport, (2) by sequestration or displacement of specific molecules or (3) by a gain-of- toxic-function mechanism, where the LLPS proteins themselves acquire a toxic conformation. The following examples provide a brief overview of how alteration of the material properties of condensates that form downstream of signalling pathways can result in cell death.

Ribosomal rRNAs, produced in the nucleolus, are shuttled to the cytoplasm thanks to the activity of nucleophosmin (NPM1). NPM1 and rRNAs cophase separate in RNP granules and travel to the cytoplasm, enabling ribosome biogenesis. Apart from rRNA, NPM1 can bind to a vast set of arginine-rich protein motifs [35]. Disease-related expansions in C9orf72 produce arginine-rich repeats that localise to the nucleolus where they disrupt NPM1/rRNA condensates, bind NPM1 and sequester rRNAs in irreversible puncta. As a result, NPM1 is found in a dispersed form in the nucleoplasm [36], and its shuttling function is impaired due to the unavailability of rRNAs, eventually causing ribosomes to stop working.

RNA-binding proteins (RBPs) that act downstream of signalling pathways frequently display IDRs [37]. Mutations in IDRs can alter drastically the material properties of the condensates formed by RBPs and impair or limit their physiological function [38e40]. FMRP and CAPRIN1, two such RBPs that form condensates through their IDRs, regulate translation by promoting mRNA degradation. *In vitro*, upon a series of phosphorylation events, these proteins can give rise to a range of condensates with distinct composition and material properties that reflect in different deadenylation and translation rates [41]. Disruption in the balance among these condensates can therefore affect RNA processing and translation.

Fused in sarcoma (FUS), another disordered RBP, is a DNA- and RNA-binding protein involved in DNA repair and RNA biogenesis. FUS can be found in liquid condensates at DNA damage sites in the nucleus or in cytoplasmic RNP granules, suggesting that a precise regulation of its localisation is needed for correct function. PTMs, as phosphorylation, can quickly modulate the spatiotemporal organization of FUS in the

cell, and phosphomimetic mutations act as modulators of FUS LLPS ability [42,43]. Most of the disease-causing mutations Amytrophic lateral sclerosis (ALS) in FUS are found in its Nuclear localization signal (NLS) and IDR. These mutations cause a liquid-to-solid transition of FUS condensates, which results in persistent foci formation in the cytoplasm [38,44]. Overall, the mechanism by which alteration of FUS phase transitions can lead to toxicity has not been fully elucidated yet. Two nonexcluding theories have been proposed. One suggests a loss of function: FUS cannot shuttle as it should, causing inefficient DNA damage repair and transcription. The other theory proposes a gain-of-toxic-function mechanism: high concentrations of cytoplasmic FUS might be toxic for the cell, but here, there is no consensus on whether toxicity comes from the increased cytoplasmic concentration or from the formation of irreversible foci that can be toxic. Recent studies in other disordered RBPs, such as the Tar DNA-binding protein TDP-43, also point at gain-of-toxic-function mechanisms. Upon mutation, TDP-43 can also form different types of condensates. However, in this case, cell death better relates to the liquid condensates that TDP-43 forms at the nuclear periphery [39].

Deep mutagenesis of signalling proteins

Albeit not comprehensive, this summary shows how functional or dysfunctional phase transitions of signal- ling proteins can affect cell survival. Regardless of the specific protein(s) involved, this makes the case for the possibility of designing selection assays coupled to deep mutagenesis (deep mutational scanning [45]) to exhaustively investigate the impact of mutations in signalling proteins that can undergo phase transitions (Figure 2). With this perspective in mind, it should be possible to design DNA libraries encompassing many or all possible mutations in, for example, Pbp1 and quantify the effect of each of these mutations on cell division by deep sequencing before and after a selection step in which cells are allowed to compete. If mutations that affect the process of Pbp1 LLPS cause loss function and decrease fitness [33], this will reflect on the representation of cells containing those specific Pbp1 variants, which can be accurately quantified by deep sequencing [46].

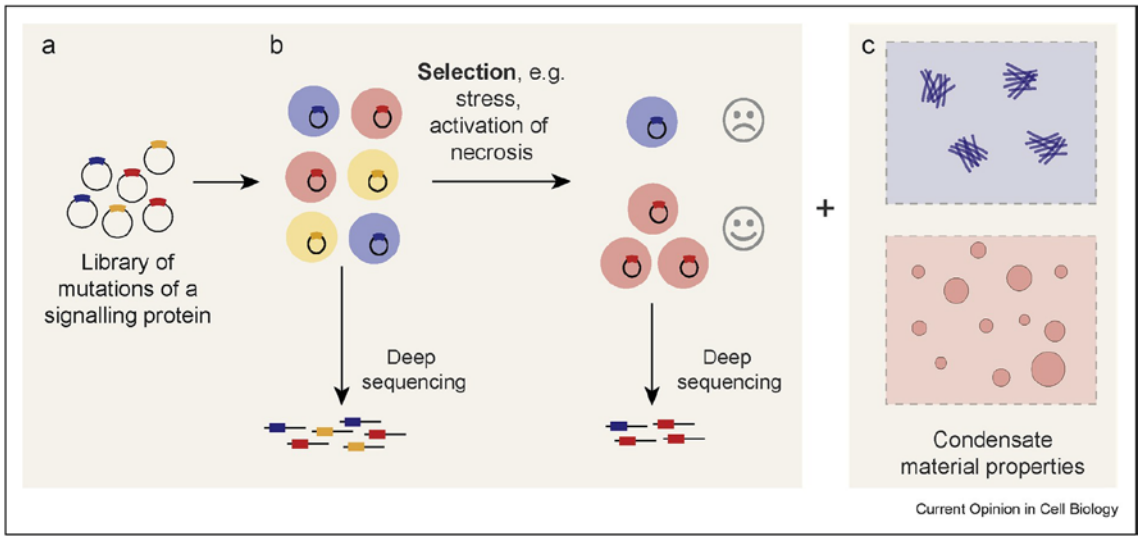

Figure 2. Deep mutational scanning pipeline. (a) A mutational library of a signalling protein is built and transformed/transfected into an appropriate cell system for selection. Protein variants will have different effects on condensation, signalling and ultimately on cell viability. (b) Under selection, beneficial variants will therefore be progressively more represented, while detrimental ones will be depleted. Deep

sequencing of the variants before and after selection results in a quantitative score for thousands of different protein sequences. (c) Coupling these scores with the assessment of material properties using techniques such as FRAP or FCS provides an insight into how condensate properties relate to signalling activity and cell viability.

Since deep mutational scanning provides a systematic strategy to link thousands of genotypes to phenotypes, we foresee its potential in deciphering how cell fitness is affected upon mutations in signalling proteins. In addition, if coupled to biophysical techniques able to report on the material properties of the condensates formed or on their internal diffusivity, such as Fluorescence recovering after photobleaching (FRAP) and Fluorescence correlation spectroscopy (FCS) [47,48], this approach will allow to link condensate properties to the efficiency of a specific signalling pathway and its downstream impact [39]. Besides their impact on phase transitions, mutations can also affect protein stability, protein-protein and protein-RNA interactions. Although this represents a caveat in establishing a causative link between genotype, phase transitions and outcome of signalling, such effects can partially be ruled out by small-scale control experiments. While acknowledging this limitation, the perspective that can be gained by quantitatively assessing the outcome of thousands of sequence changes is by far more comprehensive than what can be inferred from studying just a handful of cherry-picked mutations and especially appealing for signalling proteins containing IDRs that are extremely difficult to study by other means.

Concluding remarks

The spectrum of properties of condensates formed by signalling proteins provides a dictionary for a range of different outcomes in terms of cell survival. On the one hand, the cooperativity and irreversibility of amyloid formation are better suited for terminal decisions, such as those leading to necrosis or apoptosis. On the other hand, inappropriate or dysregulated LLPS of signalling proteins can become deleterious and lead to cell death. Finally, cell survival can also be affected by gain-of-toxic- function mechanisms or dysfunction of LLPS proteins involved in RNA processing, downstream of signalling pathways. This summary also highlights the need for massively parallel approaches able to quantitatively measure the effect of mutations in signalling proteins on both cell survival and protein condensation.

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* of special interest

** of outstanding interest

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