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Protein Condensation, Cellular Organization, and Spatiotemporal Regulation of Cytoplasmic Properties

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The cytoplasm is an aqueous, highly crowded solution of active macromolecules. Its properties influence the behavior of proteins, including their folding, motion, and interactions. In particular, proteins in the cytoplasm can interact to form phase-separated assemblies, so-called biomolecular condensates. The interplay between cytoplasmic properties and protein condensation is critical in a number of functional contexts and is the subject of this review. The authors first describe how cytoplasmic properties can affect protein behavior, in particular condensate formation, and then describe the functional implications of this interplay in three cellular contexts, which exemplify how protein self-organization can be adapted to support certain physiological phenotypes. The authors then describe the formation of RNA-protein condensates in highly polarized cells such as neurons, where condensates play a critical role in the regulation of local protein synthesis, and describe how different stressors trigger extensive reorganization of the cytoplasm, both through signaling pathways and through direct stress-induced changes in cytoplasmic properties. Finally, the authors describe changes in protein behavior and cytoplasmic properties that may occur in extremophiles, in particular organisms that have adapted to inhabit environments of extreme temperature, and discuss the implications and functional importance of these changes.

1. Introduction

The cytoplasm of cells is a complex aqueous solution of macromolecules, small organic molecules, and ions, amongst other components. The properties of this solution are determined by its constituents, and the solvent in turn determines the behavior of these constituents. Understanding this interaction is key to understanding cellular organization and function. Specifically, it is necessary for a full understanding of protein behavior in different cellular contexts.

Within the cytoplasm, a protein chain self-organizes into a distinct fold, which is its energetically most favorable 3D conformation.[1] These folds are determined by both protein sequence and solvent properties, and determine protein function.[2] Therefore, the diversity of protein folds in cells allows them to carry out a range of functions, such as catalysis of different reactions. Functional versatility within polypeptide chains is further increased through the presence of multiple independently folding sequences, defined as domains, each associated with distinct functions, such as dimerization or responsiveness to a regulator. However, it is now recognized that some proteins do not have defined conformations, or feature domains that are intrinsically disordered (intrinsically disordered regions, IDRs), and that these confer function by mediating context-dependent protein–protein interactions.[3]

The concept of self-organization of proteins is also applied to assemblies of multiple proteins, in which case it refers to formation of dynamic multi-component structures,[4] including certain oligomeric complexes, filaments, and phase-separated assemblies. In phase-separated assemblies, of which a range exist, IDR-containing proteins form a dense phase within the cytoplasm, commonly referred to as biomolecular condensates.[5] As for folds, the formation of these assemblies depends both on interactions between the phase-separating proteins, which can be mediated by their IDRs, and between these proteins and the surrounding solution. Within the cytoplasm, these phase-separated "droplets" commonly contain RNA, in which case they are referred to as ribonucleoprotein (RNP) granules. They can be considered to have "emergent properties:" certain characteristics can be ascribed to assemblies that are not properties of individual constituent proteins.[6] For instance, biomolecular condensates can display liquid-like properties such as fusion and wetting, which led to their identification as phase-separated droplets.[7] These properties allow them to function as dynamic membraneless organelles, or compartments. This compartmentalization is commonly referred to as occurring on the "mesoscale," which is defined as that range of lengths larger than the size of individual molecular machinery such as ribosomes, but smaller than that of the whole cell.[8]

The sensitivity of protein folds and macromolecular interactions to local environmental conditions confers both regulatory potential and risk of loss of function in "extreme" conditions. This regulatory potential is exemplified by and has been

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extensively described for RNPs: their (dis)assembly can be regulated, which allows them to locally integrate extracellular and intracellular cues to regulate protein synthesis. Therefore, they play a role in the organization of cells into distinct subcellular domains, or parts of the cell with functional specialization, such as neuronal processes. In this case, specific RNPs respond to local cues to regulate translation of a subset of mRNAs. However, RNA-binding proteins (RBPs) are also involved in concerted changes in translation of many transcripts in parallel during stress responses, via "stress granule" formation. More generally, stressors can modulate the properties of the cytoplasm to alter behavior of all cellular proteins at once, either as a direct consequence of the stressor (such as osmotic shock) or via signaling pathways (such as in cells' entry into a dormant state). Therefore, organisms that inhabit ecological niches in which extreme conditions occur must adapt their cellular organization and protein properties to allow cellular processes to continue.

In this review, we will consider how proteins behave within the cytoplasmic environment, and how this in turn organizes the cell in a way that is sufficiently versatile to meet the functional requirements of a range of cell types under different conditions. First, we describe how the properties of the cytoplasm define the behavior of proteins in general. We show the regulatory potential of proteins' dynamic condensation under physiological conditions, using the key example of neuronal RNPs and local protein synthesis (LPS). We then consider how the interaction between cytoplasmic properties and protein behavior in stress responses facilitates cell survival, using three relevant examples. We finally consider how protein properties are changed in cells and organisms that exist in functional or ecological niches that mean they are subjected to "thermodynamic extremes," using the example of extreme temperature. We conclude by summarizing open questions in these interconnected fields.

2. The Properties of the Cytoplasm Determine Protein Behavior

2.1. The Cytoplasm Is a Complex Solution

The cytoplasm is water-based, which determines which protein conformations are favorable. Protein folding is largely mediated by the "hydrophobic effect:" water molecules form lattices around non-polar and aromatic side chains, which is entropically unfavorable, and so the protein chain folds into a structure with a hydrophobic core.[9,10] More generally, the entropic cost of folding is offset by many interactions between amino acid residues, and between residues and the solvent, and folds are therefore dependent on protein sequence as well as the properties of the surrounding solution.[2]

The cytoplasm is also a crowded solution, meaning it contains a high concentration of macromolecules, which again affects protein folding. Crowding results in a volume exclusion effect: 20–30% of a cell's interior volume is occupied by macromolecules, and this volume fraction is therefore not accessible.[11] It has been argued that in such a crowded solution, the most favored state of the system becomes that with minimum excluded volume, as this state increases the volume available to the solute molecules.[11] Therefore, crowding can stabilize protein folds and compact conformations in vivo. However, models based on hard sphere theory that explicitly consider the small solvent molecules, instead predict that larger molecules are weaker crowders at equivalent packing fraction:[12] in this scenario, the introduction of a large solute displaces fewer solvent molecules if there are large crowders, reducing the excess chemical potential.[13] Furthermore, larger crowders reduce the steric penalty for protein surface exposure in this model, and therefore reduce the driving force for protein folding.[13] However, volume decrease on mixing for protein-water mixtures could compensate for these predicted reductions in crowding in practice, and this is a point for future investigation.[13]

The nature and activity of proteins means that the cytoplasm is not a solution of densely packed spheres, resulting in complex behavior. The presence of high concentrations of macromolecules also has non-steric effects on protein behavior, due to contributions to chemical potential from crowder-solute interactions, which must be considered to explain the distinct effects of different crowders on different proteins' stabilities.[13,14] Furthermore, the cytoplasm is not at equilibrium, due to the continuous expenditure of energy at the molecular level by the metabolically active cell. Enzymes have been described as a form of active matter: they can consume energy to generate mechanical forces that can influence their motion, and this is true for soluble enzymes as well as motor proteins.[15] This was first reported for jack bean urease, for instance, the diffusion of which is increased by up to 28% in the presence of its substrate in vitro.[16] Understanding how this behavior drives collective non-equilibrium dynamics in large interacting systems, and therefore how it affects mesoscale cellular organization, is a key challenge in the field of active living matter.[17]

The above-described cytoplasmic properties have wide-ranging implications for the behavior of proteins, with functional consequences. These are not limited to protein folding, but also include effects on motion. Furthermore, effects arise at larger scales: molecular interactions are affected, and this enables the formation of protein-based compartments within the cytoplasm.

2.2. Steric Effects and Energy Expenditure Influence Protein Motion in the Cytoplasm

At the scale of individual proteins, macromolecular crowding sterically affects the motion of proteins within the cytoplasm. At short length scales (<100 nm), Brownian motion has been observed for the inert protein GFP within the mammalian cytoplasm, using fluorescence-fluctuation analysis.[18] However, due to the excluded volume effect, apparent diffusion coefficients decrease when diffusion is measured across larger distances.[11,19] This can be explained by considering large particles in the cell to be obstacles (**Figure 1**a): the higher their concentration, the more difficult it becomes for a protein to find a path around them, given the limited separation distance between macromolecules at any point.[19] This can be experimentally shown by fluorescence correlation spectroscopy in human cell lines: measurements of the mobility of GFP monomers and

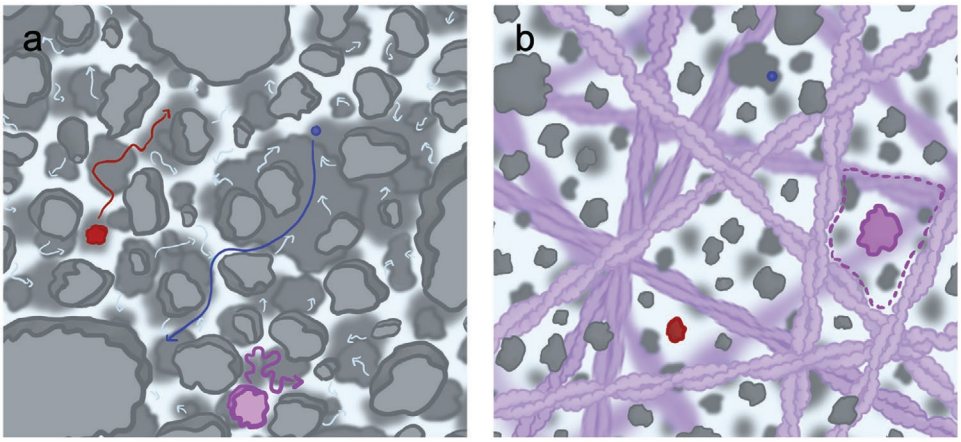

Figure 1. Molecular crowding and confinement have size-dependent steric effects on diffusion. a) A highly crowded solution has a reduced accessible volume, but also acts as a "molecular sieve" or size-dependent obstacle course. Here, the lilac molecule is slowed down more than the red one, which is in turn slower than the blue molecule. b) The presence of immobile structures such as networks of protein filaments (purple) results in size-dependent molecular confinement. Here, only the lilac molecule is significantly confined.

multimers correspond to simple diffusion in a porous medium consisting of randomly distributed obstacles.[20] In bacterial cells such as *Escherichia coli* (*E. coli*), which are very crowded, the cytoplasm has been proposed to behave as a glass-forming liquid approaching the glass transition for particles larger than circa 30 nm in size.[21] This means it has features such as dynamic heterogeneity, where particles locally become trapped by their neighbors and so display limited diffusion.[21,22] As a consequence, diffusion-limited processes are slower in crowded than in dilute solutions. For example, *E. coli* protein synthesis rates may be limited by diffusion of tRNA-translation elongation factor complexes.[23]

Crowding-associated "molecular confinement" can further reduce diffusion coefficients. This confinement has been defined as volume exclusion of a soluble molecule by a fixed boundary (as opposed to molecular crowding, which is considered volume exclusion of one soluble molecule by another).[24] In eukaryotic cells, the presence of the cytoskeleton contributes significantly to reduced diffusion,[25] with the filaments creating a porous network (Figure 1b). This again increases effective path lengths for proteins, which can reduce diffusion coefficients several-fold.[26] Overall, this confinement mostly affects larger particles: in human cell lines, the presence of structures like cytoskeletal elements and organelles affects the behavior of particles larger than circa 100 nm in size.[27] As a consequence, the apparent viscosity of the eukaryotic cytoplasm is dependent on the size of the diffusing particle of interest: for a particle of less than 100 nm in size, it is a colloidal suspension, but for larger particles, it behaves like a gel, i.e., as a mostly fixed matrix with a certain pore size that is permeated by an aqueous solution.[27]

Some of the effects of crowding and confinement on diffusion are ameliorated by motion associated with cellular energy expenditure. In prokaryotic cells, metabolism-associated conformational changes in proteins can contribute to cytoplasmic "fluidization" by promoting local remodeling of "cages" formed by large molecules in the cell.[21] Modeling studies support this notion that small conformational change-associated volume changes can accelerate cytoplasmic dynamics.[28] In eukaryotic cells, the cytoplasm is also "mixed" by non-equilibrium processes.[29] Active processes like stochastic motor activity, actin network contraction, and filament network remodeling result in significant random force fluctuations in the cytoplasm.[30] These can in turn drive motion that qualitatively resembles thermal diffusion of particles, but is faster, and has been termed "active diffusion."[30] Quantification of these random forces has shown that they substantially enhance the intracellular motion of both small and large particles in eukaryotic cells.[31]

In some instances, aligned active processes or external forces can stimulate directional flow within the cytoplasm, which can further redistribute proteins.[32] This "cytoplasmic streaming" or cyclosis is particularly prominent in large cells as a consequence of cargo motion by active transport, where many filaments align directionally.[33] Such drag-induced flow has been proposed to contribute to transport of non-membrane materials (slow transport) in axons.[34] However, other active processes can also induce flow in the cytoplasm, such as the formation of hydrostatic pressure gradients or cell shape deformation in motile cells. This can occur through the action of cytoskeletal filaments, or in some instances by polarized distribution of ion channels.[32,35–38] Temperature gradients may also result in convection of cytoplasmic fluid.[39] In addition, the charge of molecules and other non-covalent interactions may result in vectorial flow.[40–42]

2.3. Non-Specific Interactions Are Promoted in the Cytoplasm

Crowding not only affects diffusion, but also affects protein interactions. The excluded volume effect increases protein association equilibrium constants, as protein association increases the volume freely accessible to solute molecules, similarly to protein folding[11] (again, though not if macromolecules are treated as hard spheres displacing small solvent molecules[13]). However, crowding also has non-steric effects on macromolecular interactions. As proteins are not hard spheres, but have surface properties such as charge, a protein will not experience the crowded cytoplasm as an inert solvent: proteins "stick" to

each other through "soft" electrostatic, hydrodynamic, and hydrophobic interactions with neighbors, which depend on shape and surface properties.[14] (In hydrodynamic interactions, protein motions are coupled to each other by the small flows generated by their motions.) These interactions can transiently occur between a protein and other molecules in its environment, and are referred to as "quinary structure" (when favorable to the cell).[43] Like protein folds, quinary structure represents a form of "evolved protein organization,"[14] and the importance of these surface interactions was initially suggested based on the evolutionary conservation of protein isoelectric points.[43]

Non-specific interactions between cytoplasmic proteins further slow diffusion in a protein species-specific manner, which has been investigated computationally. In one study that combined modeling with simulations, a comparison was made between two crystallin proteins, which are small globular proteins that make up the lens of the eye. This study showed that crowding reduced the diffusion of the two proteins to different degrees, which can be explained by differences in attractive surface patch–patch and anisotropic (directional) interactions.[44] Models of macromolecular motion in the *E. coli* cytoplasm better recapitulate experimental data when these interactions are included, rather than only excluded volume effects.[45] However, it has also been argued that hydrodynamic interactions may dominate over non-specific attractive interactions in the effect of crowding on protein motion.[46]

Crowding-promoted soft interactions can also affect protein folding and conformation. While repulsive interactions stabilize folds, similarly to the excluded volume effect, attractive non-specific interactions can decrease structural stability by favoring exposure of interacting residues,[47] as can be monitored by proton exchange NMR.[48] For instance, introduction of a positively charged surface residue in a surface loop of the *E. coli* "protein G" reduces its stability in vivo, but not in vitro, and is therefore likely due to non-specific attractive interactions.[49] As the authors of this study note, this has implications for charge-altering post-translational modifications (PTMs) of proteins in signaling.[49] Within the cellular context, where adsorbing surfaces are abundant, the effect of crowding on protein adsorption to surfaces can furthermore combine with the excluded volume effect to promote protein fibrillation.[50,51]

2.4. Intrinsically Disordered Regions Mediate Phase-Separation in the Cytoplasm

The promotion of protein–protein interactions in the cytoplasm also has profound implications for the behavior of proteins containing IDRs. Though IDRs cannot engage in highly specific interactions based on large and unique surfaces to form structurally defined protein complexes (though some adopt structures upon interaction[52] or are buried in the cores of complexes such as the ribosome[53]), their side chains can interact with other molecules, including the solvent as well as other IDRs. They commonly contain multiple short sequence motifs that facilitate protein–protein interactions, including "low-complexity" (LC) sequences that contain comparatively few different types of amino acids, and may for instance be enriched in glycine and arginine.[54] Therefore, one IDR can engage multiple other IDRs at once via its different motifs, which is known as multivalency, and this enables phase separation or biomolecular condensation of IDR-containing proteins.[5] Such condensation involves the spontaneous separation of a homogenous solution of molecules into two coexisting phases, of which one is enriched and one depleted in these molecules.[6]

Biomolecular condensation can be driven by different types of intermolecular interactions, depending both on solute and solvent properties. It can be associative or segregative, meaning it can be mediated by attractive interactions between condensing molecules (such as charge-based ones, in which case it is known as complex coacervation), or by repulsive interactions between the solvent and the condensing molecules (c.f. the hydrophobic effect).[55] In the case of protein condensation mediated by IDRs, interactions can include hydrogen bonds, π–π stacking, hydrophobic interactions, electrostatic interactions, and cation–π interactions.[56] These interactions and therefore condensation are strongly enhanced by the cytoplasm's crowdedness.[57] This occurs due to the excluded volume effect increasing the effective concentration of condensing molecules, but may also partially be due to reduced solubility of IDRs in crowded solutions.[55]

As a phase transition phenomenon, condensation can occur rapidly upon a change in local environmental parameters or in the concentrations of the condensing proteins. Due to the multivalent nature of IDR-based interactions, molecules can be cooperatively recruited to the separated phase, and so demixing (condensation) is "switch-like," meaning it for instance readily occurs above a threshold or "critical" concentration.[58] This also means that formation of condensates occurs in nucleation and growth phases, with nucleation being more efficient in the presence of a compatible seed.[59]

Condensate phase state can change, which again also depends on properties of the local environment. Condensates can reversibly cycle between liquid and hydrogel states, but aging of the gel state can cause them to become irreversibly gelated, as has been shown in vitro. This has been demonstrated for the protein FUS and for its LC domain in isolation: both can reversibly form hydrogels that consist of "loose fibrils" in vitro, but gel irreversibly with time, a transition that is associated with formation of a more condensed network of fibrils.[60,61] Structural studies have been done on reversible and irreversible fibrils formed by the FUS LC domain, which indicate that both form largely through hydrophilic bonds, but that irreversible fibrils may feature larger and more stable fibril cores.[62,63] These changes can be influenced by the local environment. For instance, gelling is promoted by crowding in vitro,[64] and irreversible gelling transitions can occur upon temperature cycling[60,61] and flow-induced shear.[65]

As condensation is switch-like and sensitive to local cytoplasmic properties as well as to the features of condensing proteins, condensate phase transitions can be rapidly triggered by signaling cues. As condensation can rely on electrostatic complementarity, it is often sensitive to local changes in pH,[66] and to PTMs of binding partners that alter their charge.[67] This sensitivity allows condensates to carry out a range of functions, from the molecular to the cellular scale.[68] These functions may include modulation of reaction rates,[69] cytoskeletal filament nucleation,[70] (in)activation of component molecules, sensor

activity in regulatory pathways (e.g., where granule formation depends on pH), and localization (e.g., to allow a group of functionally related molecules to be co-transported).[71,72]

3. Dynamic Protein Behavior Is Locally Functionalized: The Case of RNP Granule Condensation Regulating Protein Synthesis

The effect of cytoplasmic properties on the behavior of proteins has implications for cellular organization at different length scales. This is particularly the case for self-organization of proteins into functional compartments by biomolecular condensation. A key example is the formation of RNA-containing compartments that regulate protein synthesis, which can spatiotemporally localize protein activity.

3.1. RNA and Protein Co-Condense into RNP Granules

Biomolecular condensation can involve different types of macromolecules, as long as they can interact: in particular, many cytoplasmic condensates consist of both protein and RNA molecules,[5] which affects their formation and properties. Many RBPs are likely capable of the multivalent interactions required for phase separation and can co-partition with RNA into condensates: it has been estimated that up to half of eukaryotic RBPs contain IDRs,[73] and these IDRs can promiscuously enhance RNA-protein condensation mediated by interactions between RNA and structured RNA-binding domains (RBDs; e.g., RRM domains).[74] The resulting condensates are referred to as RNP granules.

RNP condensation is mediated by a range of intermolecular interactions, including but not limited to those between IDRs and between RBDs and RNA (**Figure 2**). Under some conditions, RNA–RNA interactions can also promote condensation.[75–77] Furthermore, as RNA has a negatively charged backbone, RNP condensation can occur through complex coacervation; this may enable its regulation by modulation of protein charge by phosphorylation, as phosphate groups are negatively charged.[78]

Given the role of RNA in RNP condensation, its levels can affect RNP nucleation. RNA can act as a buffer for condensation of RBPs with "prion-like" (LC) domains, with low RNA levels promoting condensation and high RNA levels inhibiting it.[79] A recent modeling study suggests that RNA can enhance the nucleation phase of condensation by reducing the number of competing nucleation centers.[80] Nucleation can be facilitated by different types of RNA, and some long non-coding RNAs are thought to specifically function as scaffolds for condensate assembly.[81]

As an increase in RNA levels can mediate RNP disassembly, these condensates can undergo what is known as a re-entrant phase transition. This means that as RNA concentrations increase, condensates assemble and then disassemble, so that two types of dispersed state exist: in the low-RNA dispersed state, proteins are mostly unbound, but in the high-RNA dispersed state, small protein-RNA complexes exist.[82] For complex coacervation-driven condensates, this represents a charge

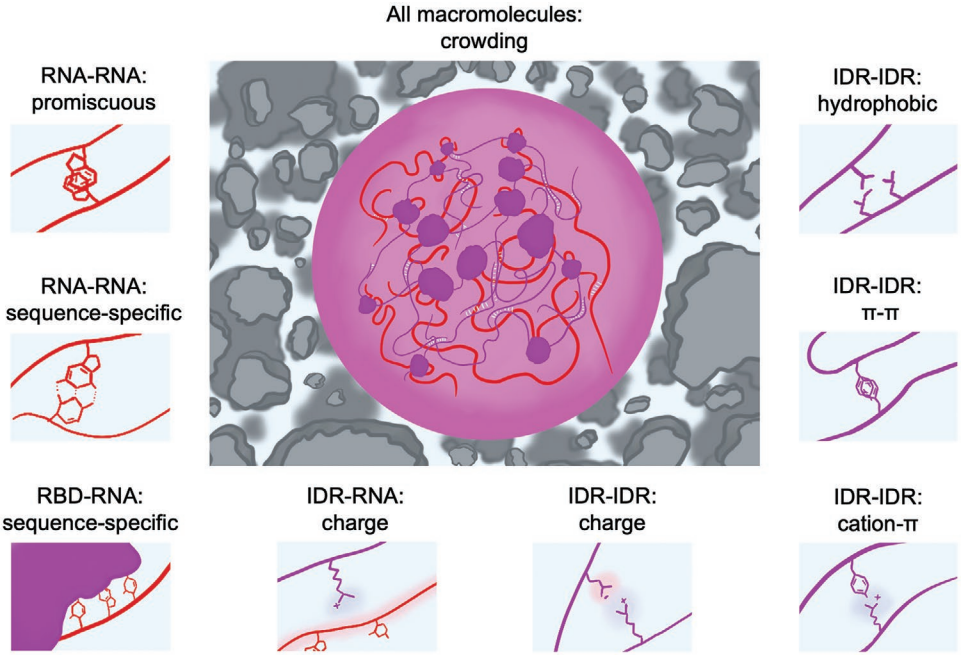

Figure 2. Intermolecular interactions facilitate co-condensation of RNA (red) and RNA-binding proteins (purple) into ribonucleoprotein granules. These interactions involve the intrinsically disordered regions (IDRs) and RNA-binding domains (RBDs) of the proteins, and the negatively charged backbone as well as nitrogenous bases of the RNA. The interactions can be of multiple types, including hydrogen bonding (base-pairing), charge-based bonding, base stacking, and solvent effects (hydrophobic interactions). The properties of non-condensing molecules are also relevant: crowding by other macromolecules (grey) lowers the critical concentrations for phase separation, and the solvent water molecules (not shown) determine whether partition of hydrophobic residues etc. is favorable.

inversion mechanism: initial condensation is driven by short-range attractions between RNA and arginine-rich IDRs leading to charge neutrality, and the re-entrant phase transition is driven by excess RNA binding resulting in long-range charge repulsion following loss of charge neutrality.[83] This can drive the formation of complex topologies, as the re-entrant phase transition can be nucleated within droplets: for instance, in one in vitro charge-driven RNA-IDR condensing system, high RNA levels induced vacuole formation within droplets (and eventual droplet disassembly).[84] Multilayer condensates that are organizationally similar to re-entrant systems are found in cells and are functionally significant, for instance for multi-step assembly of ribosomes in the nucleolus,[85] so whether re-entrant phase separation occurs during complex condensate assembly in vivo is an area of future study.[82]

3.2. Condensation into RNPs Regulates mRNA Translation and Localization

RBPs can regulate the localization and translation rates of the mRNAs they bind, which makes RNP granule formation an important form of post-transcriptional regulation of gene expression. These effects arise through RBPs modulating mRNAs' interactions with other proteins, either acting as a linker or preventing interactions.

Characterization of different RNP granules has shown them to be associated with different levels of translational activity, with some containing masked and inactive mRNAs, some being associated with stalled polysomes, and some potentially engaging in translation.[86] This level of translational activity may be linked to RNP phase state: as an extreme example, translational repressors induce co-assembly of RNPs into large viscoelastic solids in arrested *Caenorhabditis elegans* oocytes, which likely contributes to silencing of interacting mRNAs.[87] As a consequence, regulation of RNP phase state or (dis) assembly provides a dynamic way of regulation translation rates. Alternatively, regulation of mRNA translation may occur through colocalization of the target mRNA with other gene expression-associated factors, such as with ribosomes,[88] or with the RNA-induced silencing complex, which targets specific mRNAs for degradation or translational repression via complementary microRNAs.[89]

RNPs' response to stimuli can potentially alter their state on longer timescales, conferring a form of local memory of stimuli, or hysteresis. Based on optogenetic experiments with artificial protein condensates containing RBP IDRs, liquid droplets have been proposed to be a form of spatial memory within cells.[90] In this study, application of shallow gradients of light stimulation that dissolved droplets resulted in sharp boundaries in droplet behavior, as larger droplets formed in stimulus-free regions due to the influx of monomers from stimulated regions. These larger droplets persisted in these regions after stimulus removal, as they are more stable than smaller droplets and would even be expected to coalesce and ripen with time in the absence of a stimulus dissolving them. Therefore, the switch-like behavior of condensates can potentially binarily define the identity of cellular regions in response to complex applied stimuli.[90]

RNP granule formation also allows coupled transport and silencing of functionally related mRNA molecules, through binding by the same set of RBPs.[91] Specificity of regulation is conferred by the properties of the mRNA molecules, which all ultimately derive from their sequence. RBPs (*trans*-acting factors) preferentially bind to specific (*cis*-acting) sequences in the mRNA, or certain secondary structures (i.e., in the case of RNA, 3D structures or conformations), which restricts their activity to a subset of target mRNAs.[92] These sequences are known as zipcodes. For instance, *β-actin* mRNA is localized via a 54-nucleotide zipcode,[93] which contains a six-nucleotide motif that can be tightly bound by the RBP ZBP-1.[94] ZBP-1 can then facilitate stimulus-dependent interaction with a kinesin motor protein in dendrites.[95] Alternatively, RBPs may mediate "hitchhiking" of mRNAs on motile organelles via condensation, for instance through condensation-mediated interaction with annexin A11 to be tethered to lysosomes.[96]

3.3. Neuronal RNPs Support Neuronal Domain Autonomy

As (dis)assembly and transport of RNPs enables mRNA localization and regulation of translation, it enables spatiotemporal localization of protein synthesis, which can be critical to cellular organization. First, it can ensure proteins are able to reach distal domains, if diffusion of centrally synthesized proteins is an insufficiently efficient delivery mechanism. This can occur if diffusion is highly limited due to cell-specific constraints, such as due to cytoskeletal structures in muscle cells,[97] or if protein half-lives are short compared with time required to diffuse across cellular length scales, such as in very long axons. Second, localization of translation allows a protein product's function to be spatiotemporally restricted. Local cue-dependent activation of mRNA translation can then enable localized remodeling of the proteome, which can support or establish cellular polarization. Condensation is a suitable form of regulation for this process. First, condensates' composition is largely flexible, both in identity of macromolecules and in quantities, allowing co-regulation of different mRNA species by multiple RBPs. Second, condensates' assembly is rapid and reversible, as a consequence of their structural dependence on many relatively weak interactions and their sensitivity to the local environment's properties,[98] and therefore dynamically regulatable.

3.3.1. Local Protein Synthesis Supports Polarized Cell Organization

RBP-regulated LPS has an important role in highly polarized cells featuring distinct domains. This has been studied extensively in neuronal axons and dendrites, given their lengths (some animal axons are well over 10 m long[99]). In axons, LPS can support key housekeeping functions. For instance, mitochondrial proteins and translational machinery components are synthesized under basal conditions (without any stimulation) in distal axons.[100] However, LPS also confers autonomy in signal integration and response. For instance, cue-dependent LPS in axons and dendrites aids in the establishment of synaptic connections and the storage of information.[101]

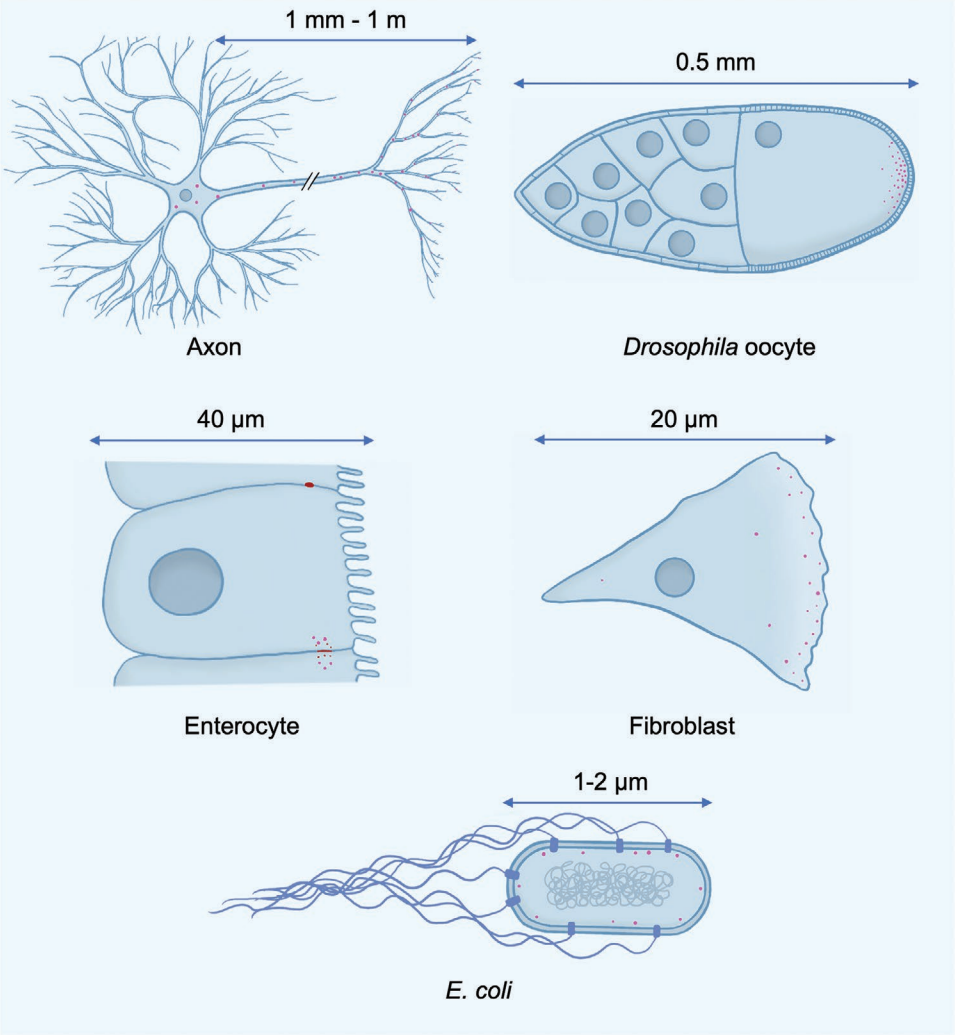

Figure 3. Localization of mRNA-containing granules (lilac) localizes protein activity to establish functional asymmetry in cells of different sizes. Cell sizes and hence the dimensions across which asymmetry must be established are indicated.

Though mRNA localization and LPS have traditionally been studied on larger spatial scales where system polarity is evident, specifically in the contexts of neurons and embryonic development, it should be noted that RNA localization is functionally important in a range of other cellular contexts (**Figure 3**).[102,103] Other cells also have clear axes of polarity, such as migrating cells, in which LPS of β-actin and cytoskeleton-associated proteins occurs near their leading edge.[104–106] Furthermore, mRNA localization is increasingly recognized to also occur in non-eukaryotic cells, though the smaller size of these systems has made it more challenging to study the process.[107] For instance, the mRNA encoding the membrane protein lactose permease (LacY) localizes to the vicinity of the cell membrane in *E. coli*.[108]

3.3.2. Dynamic Condensation Regulates LPS

The sensitivity of RBPs' ability to condense to PTMs means that local activation of signaling pathways can readily regulate LPS, as has been demonstrated in neurons. In the dendrites of cultured neurons and acute brain slices, *β-actin* mRNA is transported in RNPs containing multiple copies of the mRNA, but these largely disassemble upon depolarization with KCl.[109] Similarly, desumoylation of the RBP CPEB3 in response to neuronal stimulation causes loss of its interactions with repressive granules in dendrites, which is associated with loss of translational repression of CPEB3 target mRNAs.[110] This shift in RBP–RBP interactions upon stimulation has also been demonstrated for other RBPs: for instance, TDP-43 shifts toward interacting with FMRP and Staufen 1 upon repeated stimulation with KCl in the dendrites of cultured hippocampal neurons.[111] In addition, condensate properties can regulate LPS via sequestration of the translational machinery rather than of mRNA: RNPs can contain a densely packed core of ribosomes that is released upon depolarization in dendrites.[112]

Functional restructuring of RNPs can occur not only through traditional signaling pathways and PTMs, but also through changes in cytoplasmic properties, which has again been reported in neurons specifically (**Figure 4**). For instance, it has been suggested that activity-associated changes in ion

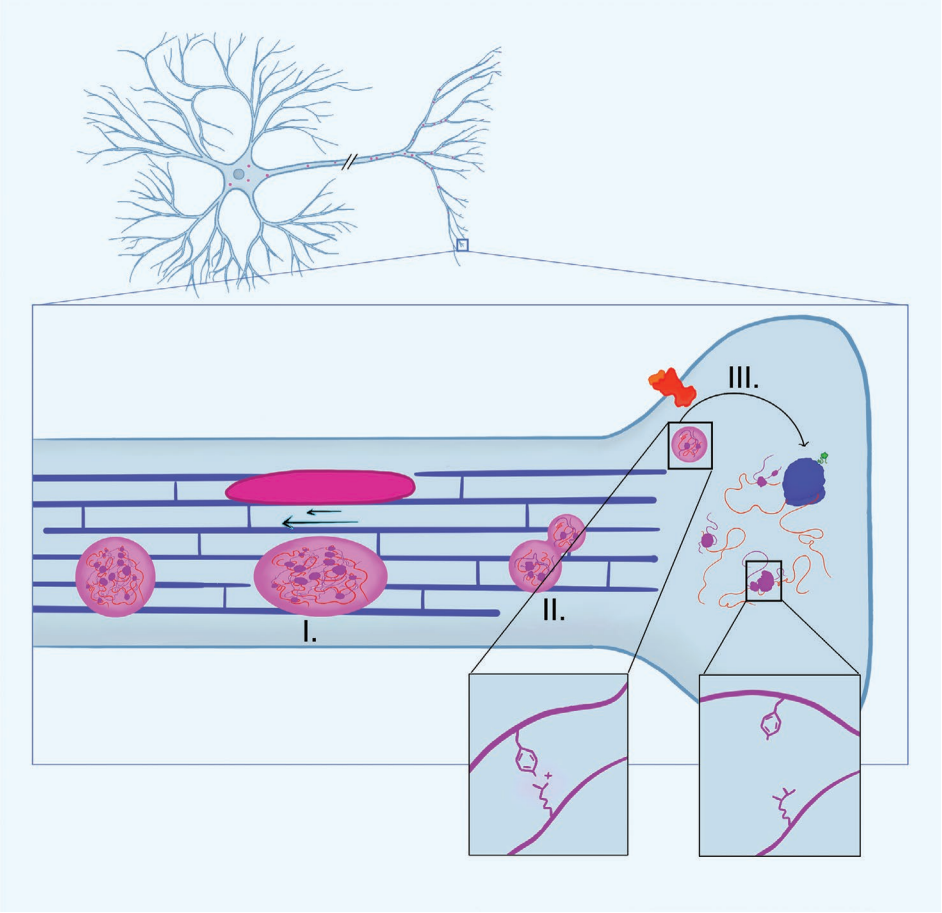

Figure 4. RNP granules (lilac) are sensitive to local context, as exemplified within axons. I) Droplets can deform under shear (for instance generated by active transport of large particles (pink)), which may affect their viscosity. II) Droplets can fission (or fuse) to generate smaller droplets with fewer mRNA molecules (red) and RNA-binding proteins (purple). III) Droplets may disassemble in response to local cues (activation of orange receptor by ligand), for instance via post-translational modification (arginine methylation depicted here). This may be associated with an increase in translational efficiency (ribosome in blue).

concentran could affect neuronal RNPs directly, for instance through Ca^{2+} influx in the case of granules containing the phase-separating RBP FMRP.[113] Furthermore, as liquid-like condensates, RNPs are affected by local force generation, and can adapt in scale to their local environment. RNPs have been observed to deform under shear stress associated with fast axonal transport,[114] and have also been observed to fuse and relax to a spherical shape within axons.[114,115] These granules may "mature" as they are transported through neurites:[116] smaller granules have been observed to form by fission (bud from larger granules) near dendritic spines, allowing entry into these structures.[117] This permits much more fine-tuned regulation of LPS in neurites, with mRNA species' regulation becoming more decoupled when needed: RNP granules being transported into neurites may contain only a single mRNA molecule, even where localizing mRNAs contain the same zipcodes.[118]

3.4. Outlook: Localized Changes in Cytoplasmic Properties in Synapses

So far, we have discussed how the properties of RNPs enable them to provide the functional autonomy that neuronal processes require, through locally regulated LPS. However, these processes' narrow protrusions represent a structural feature that may in fact affect the condensates that regulate LPS, but little is known about this. It is known that proteins behave differently in synaptic boutons: diffusion of the same proteins is generally much slower within synaptic boutons than in axons, with the diffusion coefficient of actin being halved in synapses.[119] Furthermore, the cytoplasm of synaptic boutons can be compositionally partially isolated from that of the rest of the neurite: the actin cytoskeleton restricts diffusion in a size-dependent manner in dendritic spines, with the extent of restriction being regulated by synaptic activity to modulate signal transduction.[120] Therefore, it would be interesting to consider whether bursts of translation in synapses significantly affect crowding locally, and whether this affects condensates, potentially to attenuate bursts in translation.

In this context, local synthesis of condensation-competent proteins like RBPs would be particularly interesting. This may act to modulate RNP dynamics, creating feedback loops that could be functionally important. For instance, the microtubule-associated protein Tau, which aggregates in several forms of neurodegenerative disease,[121] is locally synthesized in axons.[122] Tau is increasingly recognized to interact not

only with microtubules via phase separation,[123,124] but also with RNA and RBPs, and tauopathies are now thought to be associated with deregulated translation.[125] From these observations, it is possible that locally synthesized Tau could alter RNP behavior to deregulate translation. More generally, the local synthesis of condensation-competent proteins could alter the local compartmentation of the cytoplasm. This could occur through interaction with the cytoskeleton or with RNP granules. Such changes could in principle be self-amplifying, creating bistability, or self-repressing, aiding in the maintenance of local homeostasis. Such local synthesis of a particular RBP (or RBPs) could affect which subset of localized mRNAs is actively translated. Though not much is known about the extent of this process and its functional significance, RBPs emerge as prominent candidates in screens of neurite translatomes. For instance, the RBP Pum2 was recently found to be more postsynaptically than somatically translated in a screen using excitatory hippocampal neurons.[126]

4. Changes in Protein Behavior and Cytoplasmic Properties Occur in Stress Responses

While RNPs' dynamic properties make them suitable for local regulation of specific mRNA species, for instance via modulation of PTMs on specific RBPs, their shared sensitivity to changes in the cytoplasm's properties also allow them to act as stress sensors that can induce changes in global (as opposed to local) protein synthesis in response to stress. Furthermore, phase separation may have a more general cytoprotective role during stress: it has been proposed that phase separation is an evolutionarily conserved feature of proteins conferred by LC domains, which aids in stress survival by preventing protein aggregation.[127] This stress-mediated condensation can occur through specific signaling pathways, or downstream of changes in the cytoplasm's properties. These changes can also affect other aspects of protein behavior.

Here, we highlight three examples of stressors that trigger changes in cytoplasmic properties or organization, either directly or downstream of signaling pathways, with a focus on eukaryotic cells. We first discuss stress granules (SG) formation, in which signaling pathways facilitate nucleation of a specialized type of RNP. We then describe the entry into metabolic dormancy in yeast, in which glucose starvation triggers changes in cytoplasmic properties, which in turn affect protein behavior, including condensation. Finally, we describe the response to shock-induced loss of water, in which the cytoplasmic property change itself is the signal that automatically induces changes in protein behavior, with which cells must cope.

4.1. A Range of Stressors Trigger Specific Granule Assembly

Many different stressors induce the formation of SGs in eukaryotic cells,[128] which are distinct from for instance neuronal transport RNPs. Their exact composition depends on the stressor, as has been shown by comparing SGs formed upon arsenite treatment (oxidative stress), UV irradiation, and heat shock.[129] Generally, these sequester highly expressed mRNAs, as well as other cellular components such as translational machinery, and this is important for post-stress viability, as has been shown in yeast.[130]

SG assembly is modeled as a multi-stage process. Translational inhibition results in ribosomes dissociating from mRNAs, which are then free to form RNPs with specific proteins such as G3BP1 that nucleate SGs, and these nuclei then assemble into SGs.[131] It has also been suggested that SG formation is a direct physical consequence of ribosome disengagement.[77] In this model, the increase in exposure of RNA sequences and loss of ribosome helicase activity upon translation inhibition result in promiscuous RNA–RNA interactions that contribute to granule assembly. This was suggested to be an RNA analogue of protein aggregation, with "RNA chaperone" function being overwhelmed due to the number of released mRNAs (chaperones here being RBPs and RNA helicases, amongst others).[77]

During the assembly process, SGs form a biphasic structure with a more stable core and a more dynamic outer shell,[132] and the formation of this complex topology is under investigation. Two models have been proposed to account for core assembly: in one model, cores form first, concentrating IDR-containing proteins that facilitate shell assembly via phase separation; in the other model, phase separation results in formation of a droplet in which more stable cores nucleate.[133] Live imaging of SG assembly favors the core-first model.[134] However, RNPs can mature to fibril-like states in vitro,[60,61,135,136] and this process may occur in other contexts.

The different phase properties of the core and shell have consequences for the associated mRNAs. In U-2 OS cells, translationally active mRNAs interact with SGs much more dynamically, while silenced mRNAs are stably associated with the core of SGs and display limited movement.[137] Notably, up to 30% of SG-associated mRNAs may be in this translationally active category, based on imaging data acquired for HeLa cells.[138]

There is an interplay between SG formation and the considerable translation inhibition and reprogramming observed during stress responses,[139] but this is not a matter of SGs simply sequestering the majority of cellular mRNA within their cores. Based on RNA-seq of purified granules, only 10% of poly-adenylated mRNA molecules in the cytoplasm are recruited to SGs, with all different mRNAs species being found in granules, though longer and translationally inactive mRNAs are enriched.[140] This is consistent with the finding that loss of the SG-nucleating protein G3BP results in loss of SGs, but not stress-induced translational repression.[141] As SG formation is associated with improved viability in response to a range of stressors, SGs may act to protect and store a subset of mRNAs for future release when cellular conditions improve, delaying cellular decision making.[142] Consistent with this, it has recently been shown that SG localization does not affect an mRNA's translation following disassembly, at least in HeLa cells.[143]

There may also be cell type-specific roles of SGs that rely on their ability to sequester RNA and RBPs into large particles, for instance in asymmetric cell divisions. In neural progenitor lines, the intermediate filament protein vimentin has

been shown to form a localized mesh that locally promotes SG formation, facilitating asymmetric partitioning of SGs away from differentiating cells.[144] Loss of vimentin resulted in defects in neuronally differentiating cells, which have reduced ubiquitin-proteasome activity compared with progenitors, and so this SG (and protein aggregate) partition mechanism was proposed to enhance differentiating cell survival.[144]

4.2. Metabolic Dormancy Is Associated with Increased Condensation and Solidification of the Cytoplasm

In the context of more extreme stress, micro-organisms can respond with more dramatic changes in their cellular organization and become dormant. This dormancy is a reversible entry into a state of low metabolic activity that occurs in unfavorable environmental conditions, and has evolved in different ways across different taxa.[145] It is associated with considerable changes in cytoplasmic properties, including extensive compaction and filament formation in yeast.[146] These changes can be considered to be an integral part of the dormancy response: in the case of bacteria, such immobilization of larger particles has been suggested to provide a basis for the conservation of cellular architecture.[21] For eukaryotic cells, budding yeast has been a model system for entry into dormancy.

In yeast, dormancy is associated with strongly reduced mobility of organelles and increased mechanical stability[147,148] mediated by changes in protein organization. As yeast generally lives in acidic environments, energy depletion triggers acidification of the cytoplasm,[149] which directly affects protein behavior. For instance, the metabolic enzyme Gln1 forms filaments upon acidification, likely due to reductions in its overall charge reducing repulsive soft interactions, as its isoelectric point is around 6.[150] This filament formation results in Gln1's inactivation and storage, with Gln1 regaining catalytic activity upon filament disassembly.[150] As protein isoelectric point distributions are multimodal and largely not within the neutral range,[151] the charge of many proteins will be affected by acidification.[150] Therefore, this assembly formation is more widespread upon acidification to pH 5.5, which has been shown to result in solidification of the cytoplasm, likely by formation of a filamentous-colloidal network.[147] This filament formation could mediate cell shrinkage through release of bound water, which is observed in yeast's entry into dormancy.[147] However, when yeast is grown in glucose-depleted medium at neutral pH, reductions in cell volume can enhance macromolecular crowding without acidification, through unclear mechanisms, also resulting in macromolecular confinement.[148] Therefore, cell volume-regulation pathways likely contribute to cytoplasmic property changes during dormancy.[148]

Certain yeast proteins can specifically sense pH through their disordered domain's charge, and so their condensation can be directly affected by starvation-induced acidification, which can regulate their function. For instance, the translation termination factor Sup35 phase-separates upon acidification: with time, Sup35 condensates harden into cross-linked gels in vitro, but these are readily dissolved upon neutralization of pH.[152] This reversible behavior relies on a disordered pH-sensing domain that contains several charged residues, and Sup35 lacking this prion domain instead irreversibly aggregates upon acidification.[152] Therefore, gel formation protects Sup35 from irreversible aggregation during stress, allowing translation to resume during stress recovery.[152] However, disordered domains can also act as pH sensors that positively regulate activity upon acidification. The SWI/SNF chromatin remodeling complex is essential for yeast's carbon starvation response, and was recently reported to sense pH change via its disordered domain, which allows it to interact with a different set of transcription factors to mediate transcriptional reprogramming.[153] Modeling studies indicate histidine protonation results in its conformational expansion, which may facilitate the observed changes in protein–protein interactions.[153]

It should be noted that not all metabolic stressors result in the same induction of dormancy: in contrast to glucose starvation, amino acid depletion may decrease cytoplasmic crowding in budding yeast, due to a reduction in ribosome concentration.[154] Recently, it was shown that mTORC1, which acts as an amino acid sensor that controls protein synthesis rates,[155] mediates changes in budding yeast cytoplasmic viscosity.[154] In this system, reduced mTORC1 signaling upon amino acid depletion increased effective diffusion of particles at the 40-nm, but not 5-nm length scale. This effect was independent of translational inhibition or cytoskeletal modulation, but rather was mediated via a reduction in ribosome concentration. In mammalian HEK cells, the same effect of reduced ribosome concentration following mTORC inhibition was seen.[154] In both model systems, this reduction in ribosome concentration resulted in changes in crowding and therefore condensation with time: in budding yeast, 2 h of inhibition of mTORC resulted in at least a halving of total droplet area of a synthetic intracellular reporter protein that phase-separates.[154]

4.3. Shock-Induced Loss of Water Alters Crowding and Condensation

Changes in crowding and associated protein behavior can also be induced on very short timescales, by rapid changes in cell volume due to changes in water content. Such changes can occur in a range of circumstances. For example, mechanical pressure can cause volume changes: while cells maintain their volume upon slow deformation via a mechano-osmotic feedback loop, rapid deformation is associated with changes in cell volume.[156] This can affect cytoplasmic properties: mechanical compression of *E. coli* cells results in significant slowing-down of cytoplasmic diffusion of proteins, which has been speculated to be due to loss of water and associated increased viscosity.[157] Osmotic shock also alters cellular water content, and this has been a model system for volume change-associated alteration of cytoplasmic organization.

Hyperosmotic shock occurs when cells are exposed to a high-osmolarity extracellular medium, resulting in a loss of water to the extracellular medium that alters protein behavior. In yeast, this results in cellular shrinkage within tens of seconds.[158] As a consequence, there is a reduction in speed of diffusion of proteins involved in shock-related signaling, in a manner that is proportional to the severity of the shock, and therefore presumably to the increase in

macromolecular crowding.[159] This increase in crowding will enhance attractive soft-interactions and may therefore also destabilize protein folds, as has been shown for a model protein in osmotically stressed *E. coli*.[160]

Hyperosmotic shock also triggers SG assembly, in a manner that is independent of the SG-nucleating proteins G3BP1 and G3BP2.[141] Instead, SGs are proposed to form as a direct consequence of increased macromolecular crowding upon cell shrinkage.[161] In particular, the RBP and SG component IGF2BP1 forms small cytoplasmic clusters together with mRNA within seconds of hyperosmotic shock, which coalesce within minutes to form nuclei for recruitment of other SG proteins and mRNA.[162]

Similar rapid assembly of condensates upon hyperosmotic shock has also been described for non-SG condensates. In mammalian cells, multivalent proteins condense within seconds upon hyperosmotic shock, unlike classic SG components like G3BP1, indicating this process is distinct from typical SG assembly and a direct consequence of altered crowding and hydration.[163] This process that has been described as being akin to formation of clouds, with proteins in the cell being "poised on the phase boundary between a 'vapor'-like dispersed state and a more condensed phase."[164] Cells use these changes in condensation to sense osmotic stress: apoptosis signal-regulating kinase 3 condenses upon hyperosmotic shock, which regulates signaling to mediate cell volume recovery.[165] Formation of many dense foci within seconds of hyperosmotic shock has also been observed in yeast, in which proteins formed an interconnected network of elongated structures, which could also be a form of condensation.[166] However, the authors suggest that formation of these foci could also result from pre-existing heterogeneity within the cytoplasm, with some regions losing water more readily and therefore becoming the site of focus formation. In this model, foci represent pockets of liquid phases that have become more concentrated, while focus-free areas represent a solid-like phase, which for instance may be enriched in ribosomes.

4.4. Outlook: Cytoplasmic Properties from Stress to Disease

Outside of the above-described physiological contexts, loss of cellular homeostasis due to injury or disease can similarly cause changes to cytoplasmic properties and/or protein behavior. This has been described for acute stresses: for instance, acute cell swelling commonly occurs upon injury in a range of cell types, from epithelia to neurons,[167] and ion levels can change in neurons and glia upon energy starvation, also resulting in intracellular acidification.[168] However, it has also become apparent that changes in cytoplasmic properties or associated protein behavior occur outside of acute disease contexts, in age-associated diseases where loss of cellular homeostasis occurs over time. This is an area of current research.

Cancer is associated with loss or deregulation of the homeostatic mechanisms and stress responses that maintain cell phenotype, and these can affect the organization of the cytoplasm. Such links have been established most strongly for condensates within the nucleus and at the plasma membrane, but are also suggested to exist for SG condensation.[169–171] However, changes in water content and/or cytoplasmic mixing also occur in some tumor cells,[172,173] as do changes in pH.[174] This could indicate that some invasive cells will display broader compromised cytoplasmic condensate formation and associated deregulated protein synthesis. This link between altered RNP behavior and deregulated protein synthesis is well-established in the context of neurological disorders: within neurons, RBP-induced changes in LPS are thought to occur in a range of neurodevelopmental and neurodegenerative conditions.[100]

The tendency of multivalent proteins to locally concentrate may also carry an inherent risk of loss of homeostasis through protein aggregation. In particular, SG formation is linked to neurodegenerative disease, and it has been suggested that aging-associated chronic stress can lead to pathological RBP aggregation via SG persistence.[175] This can have consequences for condensate function: repression of (local) protein synthesis has been linked to changes in RBP phase separation in axonal growth cones.[176] However, to what extent changes in RBP phase state in neurodegenerative diseases exert their pathological effects due to perturbation of RNA translation, rather than direct toxicity of aggregation, remains a partially open question.[100]

5. Adaptation to Extreme Niches Requires Protein and Organizational Changes: The Case of Extreme Temperatures

Some organisms live in "extreme" ecological niches that would trigger stress responses in others, which requires specific adaptations to cytoplasmic and protein properties. Extreme temperatures are a key example that is also relevant to animal biology. Cells or organisms generally are viable over a specific range of temperatures, with the organization of their cytoplasm being adapted accordingly. Many organisms are "mesophiles," which for micro-organisms means they thrive at temperatures between 20 and 45 °C.[177] Where organisms have adapted to habitats characterized by temperatures significantly above or below this range, they are referred to as thermophiles and psychrophiles respectively. For these organisms, adaptations are required in all the aspects of protein behavior and cytoplasmic organization that have been previously discussed, including protein folding, diffusion, and condensation. However, for psychrophilic and thermophilic micro-organisms, characterization of these adaptations appears to have largely focused on protein folding and activity, with some discussion of maintenance of osmolality and prevention of ice nucleation for psychrophiles,[178,179] leaving many open questions. For psychrophilic vertebrates, cell type-specific adaptations remain largely uncharacterized.

5.1. Folding and Protein Assemblies

Thermophile adaptations to increase protein fold stability fall into two broad categories, depending on whether the organism descends from a thermophilic or mesophilic ancestor. Where the ancestor is thermophilic, proteins are generally more compacted and hydrophobic,[180] which represents adaptation

of the structure at many points along the protein sequence. Where the ancestor is mesophilic, however, a small number of strong interactions make the protein more stable compared with homologues from mesophiles,[180] which is a more feasible form of adaptation at shorter evolutionary timescales. This mesophile adaptation to higher temperatures is only possible because the free energy change associated with protein folding is not very large, allowing temperature optima to be shifted by a few altered intramolecular interactions.[181] For instance, salt bridges can rigidify thermophilic enzymes,[182] and they often have a greater number of hydrophobic core residues and surface charged residues.[183] Protein complexes may similarly be stabilized by a small number of adaptations, such as by formation of disulfide bonds that may even topologically interlink polypeptide chains.[184]

Less intuitively, low temperatures are also associated with reduced stability of protein folds, and psychrophilic proteins must be adapted to this.[185] This cold denaturation has been explained by solvent effects. As protein folding is associated with a decrease in solvent-accessible surface area, it increases conformational entropy of solvent water molecules; however, this effect becomes smaller as water density decreases below 4 °C,[186] and disappears as water molecules become favorably ordered in ice-like states.[187] The formation of water "cages" around unfolded chains is associated with an entropic cost, but with a small favorable enthalpy, as more hydrogen bonds are saturated, and the enthalpic penalty increases faster with cooling than the entropic stabilization.[188,189] As a consequence, protein folds are destabilized at low temperatures, and specific adaptations must increase their stability in psychrophiles.

Psychrophile adaptations to stabilize protein folds are diverse. These include increased expression of protein chaperones[190,191] as well as adaptations within protein sequences.[178] For prokaryotic proteins, the latter include reduction of proline content, since prolyl isomerization is a rate-limiting step in folding and is temperature-dependent,[192] and changes in amino acid composition, with amino acids with aliphatic, basic, aromatic, and hydrophilic side chains, being underrepresented in the helical regions, for instance.[193] In general, for psychrophilic micro-organisms, it has been argued based on genomic and proteomic data that cold adaptations are superimposed on pre-existing cell organization, and are therefore species- and ecological niche-specific to an extent.[190,194] This argument can be extended to eukaryotic and particularly animal psychrophiles. For instance, Antarctic fish species do not show shifts in amino acid composition compared with more mesophilic relatives, other than increased incorporation of methionine, which may act as a redox sensor.[195] However, as might be expected from species with mesophilic ancestors, they do display adaptation of a small number of key residues in proteins that have been investigated, which do include replacement of charged or large hydrophobic residues with non-polar residues in regions that are required to be flexible.[195]

Further changes to protein structure could be required to allow continued function in psychrophiles (as opposed to fold stability alone). Catalytic proteins in polar fish display adaptations to favor entropic rather than enthalpic contributions to catalysis, such as for myofibrillar ATPase.[196] Cold-adapted enzymes generally have a range of structural changes that facilitate continued required flexibility at the active site, but at the expense of stability at higher temperatures, rendering them thermolabile.[197] The interactions underpinning protein interactions in assemblies must also be adapted. For instance, microtubuli from mesophilic eukaryotes disassemble completely at 4 °C.[198] However, this is not the case for polar fish microtubuli, which appears to be due to a small number of amino acid substitutions that stabilize monomers in conformations favorable to polymerization and strengthen inter-filament interactions.[199] In addition, it has recently been reported that increased cytoplasmic viscosity decreases the rates of microtubule polymerization and depolymerization.[200] As viscosity may be higher in psychrophiles' cytoplasm, if adaptation to low temperatures is imperfect, microtubule dynamics may therefore be altered, or will require further adaptation.

5.2. Outlook: Diffusion and Extreme Temperatures

Properties of water such as viscosity change substantially at low or sub-zero temperatures,[201] and so changes in a cell's temperature would be expected to change the efficiency of diffusion-limited processes. Some cells are able to compensate for this within a given temperature range: in budding yeast, a change of ambient temperature triggers compensatory changes in synthesis of glycogen and trehalose, which alters cytoplasmic viscosity and so makes diffusion rates temperature-independent, at least between 22 and 40 °C.[202] Upregulated trehalose synthesis has been described in cold-shock for micro-organisms, in which it can prevent protein unfolding aggregation and ice nucleation,[203] and in cold-adapted fungi.[204] However, no vertebrate capable of synthesizing trehalose has been identified, though it can confer desiccation tolerance to human cells when introduced experimentally.[205] In thermophiles and psychrophilic animals, more pronounced adaptations to maintain cytoplasmic properties would likely be required. However, to our knowledge, these adaptations have not been characterized extensively.

In general, increased temperature would be expected to enhance macromolecular diffusion within thermophilic cells. However, within the crowded cytoplasm, the increased charge density on the surface of some thermophilic proteins would be expected to reduce diffusion. This effect would be more pronounced at higher temperatures: the lowered dielectric constant of water at higher temperatures stabilizes salt bridges, and so proteins would be expected to form more non-specific transient complexes, slowing their diffusion.[206] This could partially compensate for thermal effects.

In psychrophiles, conversely, diffusion would be expected to be reduced. Not only would Brownian motion be slowed down, but the loss of thermal energy also means that non-specific intermolecular interactions are less easily broken. Furthermore, lower levels of metabolic activity in organisms such as Antarctic fish[207] might mean cytoplasmic mixing is decreased. Thermal effects on density may also affect crowding. However, other factors may partially compensate for this again. The increased relative permittivity of water would reduce the degree of attraction between oppositely charged ions,[204] partially compensating for the effect of electrostatic patch–patch interactions. Altered

protein self-organization might also ameliorate some of these effects: enhanced formation of enzyme complexes for substrate channeling[208] could enhance reaction rates, if these become diffusion-limited at low temperatures. Furthermore, condensate formation could locally concentrate species.

5.3. Outlook: Condensation and Extreme Temperatures

At the level of condensation, thermophiles and psychrophiles must also adapt, given that phase transitions are sensitive to temperature. In the context of RNPs, adaptations could occur in RBP concentration, sequence composition, and PTMs, as well as in RNA concentrations or secondary structures. In addition, changes in crowding would affect condensation.

In thermophiles, little is known as to whether adaptation of condensate formation may be possible. Interestingly, thermophily is much less common in eukaryotes than prokaryotes, indicating eukaryotic cells have features that are not thermoadaptable.[209] Concretely, there are almost no eukaryotes that can survive above 45 °C, with the exception of a group of thermophilic fungi that can survive temperatures up to 62 °C, which have a range of adaptations including enhanced protein turnover and chaperone expression.[210] In contrast, the most heat-adapted prokaryotes have their upper growth boundary at 113 °C.[211] As principles of protein folding would be expected to be broadly similar, this could correspond to an inability to adapt eukaryotic subcellular organization to extreme temperatures. This would include the stability of organelles, as has been suggested,[209] though organelle-like features have now been described for some archaea,[212] and potentially the stability of some self-organized protein structures. Little literature is available on condensation in these cells. For these to continue to form, RBP valency may need to increase.

In psychrophiles, adaptations to RNP condensation would be of particular interest, given that protein synthesis is thought to be a major constraint in cold adaptation.[213] As protein folding becomes more inefficient, specific adaptations are required to increase protein turnover.[213] These include increased RNA levels: developing Antarctic sea urchin embryos have tenfold higher poly-adenylated mRNA levels normalized to mass than temperate relatives, allowing comparable rates of protein turnover, albeit at lower metabolic cost.[214] Furthermore, at least in some psychrophilic microorganisms, RNA chaperones are constitutively upregulated, including RBPs and RNA helicases.[215] Depending on the concentration of RNA molecules relative to that of RBPs, as well as their secondary structures, this could make re-entrant phase transitions more likely. In addition, characterized RBPs can form hydrogels rather than liquid droplets at 4 °C in vitro,[60] and so the composition of IDRs may need to be adapted to prevent aggregation of IDR-containing proteins.

5.4. Outlook: The Biology of Extremes in Animals

The "biology of extremes" is comparatively understudied beyond its effect on protein folding, including in the context of multicellular eukaryotes that have adapted to these challenges. We discussed temperature as an example of this: little is known as to how cytoplasmic properties and cellular organization at the mesoscale are adapted. For instance, it is unclear to what extent biomolecular condensation occurs in hyperthermophiles, and if yes, what adaptations facilitate this. Similarly, it is completely unknown how RNP granules behave in psychrophiles such as cold-adapted fish, and given that cytoplasmic properties are not characterized in these organisms, this remains an intriguing open question.

Other types of extreme environments arise on Earth, some of which are inhabited also by animals, and adaptations to cell organization could also be further investigated in these contexts. For instance, though the effects of temperature, osmolyte concentration, and protein concentration (activity) as a thermodynamic parameter on biomolecular condensation have been considered extensively, the effects of pressure are less explored.[216] However, it has been shown that high hydrostatic pressure affects phase separation at pressures up to an order of magnitude smaller than those leading to protein unfolding,[217] indicating high pressure poses a major challenge to cells. Adaptations in osmolyte levels may be an adaptation strategy to maintain both folding and condensation. Deep-sea fish upregulate the osmolyte trimethylamine-*N*-oxide (TMNO), around which structured water layers form, which may repel the unfolded protein backbone.[218] Recently, it has been shown that TMNO also stabilizes condensates of γ-crystallin under pressure in vitro,[217] and so it may further serve to preserve cellular organization in these organisms.

6. Conclusion

In conclusion, the behavior of proteins is dependent on their local cytoplasmic environment, and this allows that environment to become dynamically organized in a manner that is adaptable to cells' functional context (**Figure 5**). The cytoplasm is an aqueous but crowded solution, in which energy is continually expended to drive non-spontaneous processes. This influences protein folding, conformation, motion, and interactions. As a consequence, cytoplasmic compartmentation can arise. For instance, partially disordered RBPs may form condensates that can spatiotemporally localize protein synthesis, which is required for the maintenance and function of subcellular domains. More global changes in protein behavior occur in response to stressors, which can directly alter cytoplasmic properties or signal to change condensation. Some organisms have adapted to "stressors" that affect the cytoplasm in order to inhabit certain ecological niches, such as those characterized by extreme temperatures, which may require changes in both their proteins' and their cytoplasm's properties.

Many open questions remain in these interconnected fields. As models predict an influence of packing fraction on the effect of crowding, this should be investigated further in vivo. This would be particularly interesting in the context of subcellular compartments such as synapses, in which the effects of properties like crowding are comparatively unexplored. In this context, effects on biomolecular condensation are particularly

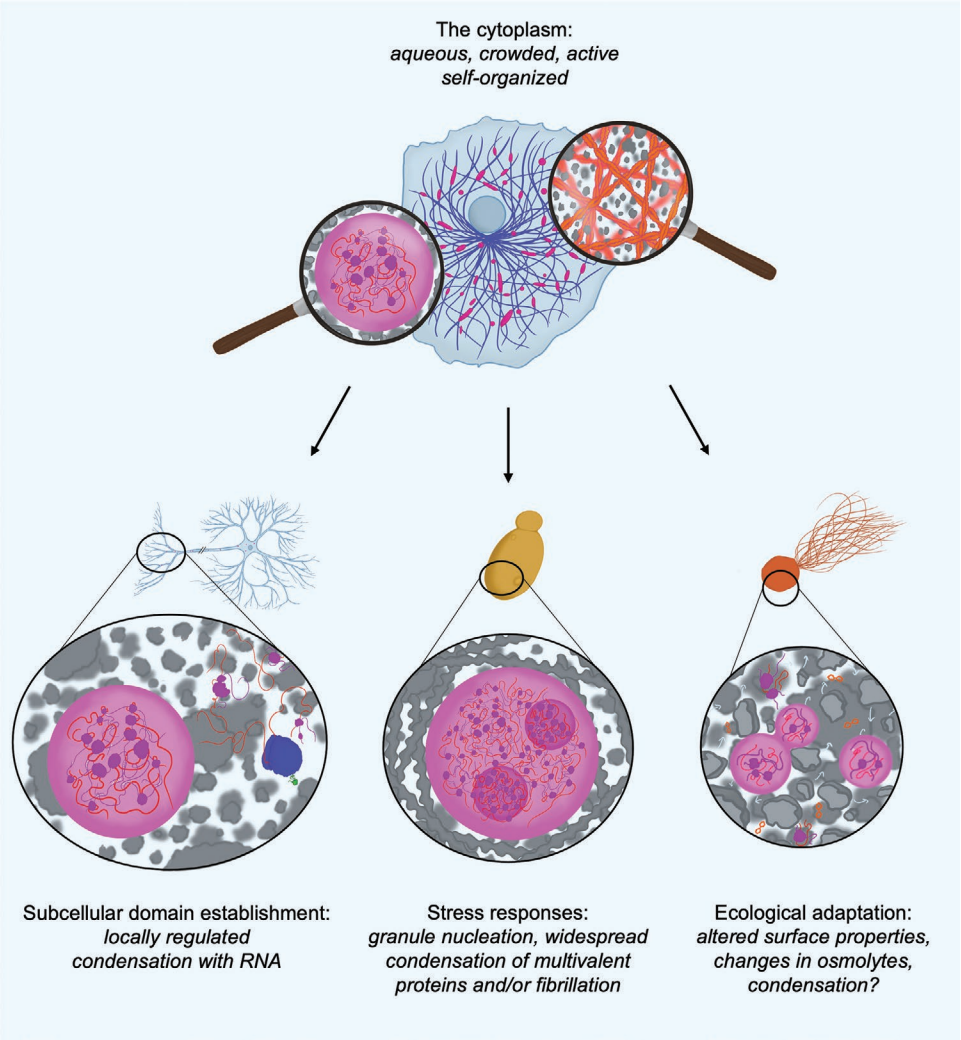

Figure 5. Context-specific modulation of cytoplasmic properties and protein behavior supports specialized phenotypes.

relevant. A more detailed picture of the local phase state, size, and composition of RNPs would also help to further understand regulation of LPS by condensate dynamics. More generally, the similarities and differences between different types of condensates are of interest, including in the context of stress responses. In such stress responses, the establishment of cause and effect between stress signaling, changes in cytoplasmic properties, and changes in protein behavior are also topics of interest for further study. In the context of extremophile adaptation, the interplay between these is comparatively unexplored, and so this is an exciting area for future study. Finally, there are relevant questions in related fields beyond the scope of this review, such as the influence of the tissue context and cell–cell interactions within multicellular organisms.

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Conflict of Interest

The authors declare no conflict of interest.

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