

Phase Separation in Biology and Disease; Current Perspectives and Open Questions

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Abstract

In the past almost 15 years, we witnessed the birth of a new scientific field focused on the existence, formation, biological functions, and disease associations of membraneless bodies in cells, now referred to as biomolecular condensates. Pioneering studies from several laboratories [reviewed in[1–3]] supported a model wherein biomolecular condensates associated with diverse biological processes form through the process of phase separation. These and other findings that followed have revolutionized our understanding of how biomolecules are organized in space and time within cells to perform myriad biological functions, including cell fate determination, signal transduction, endocytosis, regulation of gene expression and protein translation, and regulation of RNA metabolism. Further, condensates formed through aberrant phase transitions have been associated with numerous human diseases, prominently including neurodegeneration and cancer. While in some cases, rigorous evidence supports links between formation of biomolecular condensates through phase separation and biological functions, in many others such links are less robustly supported, which has led to rightful scrutiny of the generality of the roles of phase separation in biology and disease.[4–7] During a week-long workshop in March 2022 at the Telluride Science Research Center (TSRC) in Telluride, Colorado, ∼25 scientists addressed key questions surrounding the biomolecular condensates field. Herein, we present insights gained through these discussions, addressing topics including, roles of condensates in diverse biological processes and systems, and normal and disease cell states, their applications to synthetic biology, and the potential for therapeutically targeting biomolecular condensates.

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Introduction

In the past almost 15 years, we witnessed the birth of a new scientific field focused on the existence, formation, biological functions, and disease associations of membraneless bodies in cells, now referred to as biomolecular condensates. Pioneering studies from several laboratories [reviewed in[1–3]] supported a model wherein biomolecular condensates associated with diverse biological processes form through the process of phase separation. These and other findings that followed have revolutionized our understanding of how biomolecules are organized in space and time within cells to perform myriad biological functions, including cell fate determination, signal transduction, endocytosis, regulation of gene expression and protein translation, and regulation of RNA metabolism. Further, condensates formed through aberrant phase transitions have been associated with numerous human diseases, prominently including neurodegeneration and cancer. While in some cases, rigorous evidence supports links between formation of biomolecular condensates through phase separation and biological functions, in many others such links are less robustly supported, which has led to rightful scrutiny of the generality of the roles of phase separation in biology and disease.[4–7] During a week-long workshop in March 2022 at the Telluride Science Research Center (TSRC) in Telluride, Colorado, ∼25 scientists addressed key questions surrounding the biomolecular condensates field. Herein, we present insights gained through these discussions, addressing topics including, roles of condensates in diverse biological processes and systems, and normal and disease cell states, their applications to synthetic biology, and the potential for therapeutically targeting biomolecular condensates.

Condensates in Biology: Evidence and challenges

Biomolecular condensates in the nucleus (*Ben Sabari, Shasha Chong, Serena Sanulli, Lucia Strader)

The cell nucleus contains diverse biomolecular condensates that selectively concentrate the machinery responsible for the regulation of chromatin structure, DNA replication, DNA repair, DNA recombination, RNA transcription, RNA processing, and pre-ribosome assembly (Figure 1).[8,9] Condensates are typically defined by a specific constituent investigated under the microscope or through reconstitution, yet as the diversity

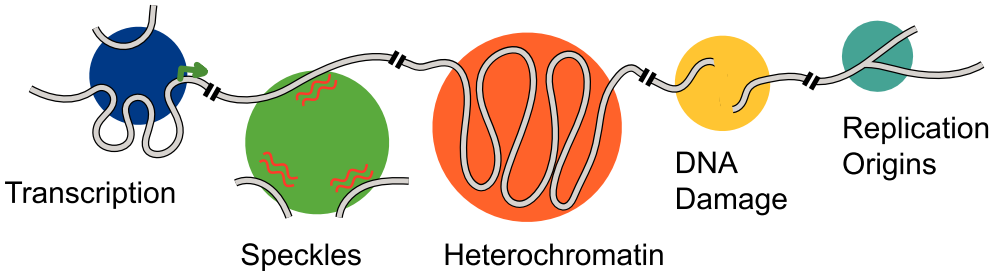


Figure 1. Diverse nuclear processes are compartmentalized within condensates with distinct compositions, locations, and dynamics. Five different nuclear condensates (colored circles) are illustrated to highlight how each engages with specific genomic loci and/or chromatin features (grey line). Investigating differential composition, relationship with genomic loci, and dynamics of formation are crucial to understanding the regulation and function of these high-order assemblies. Reprinted from[9] with permission from Elsevier.

of condensate-associated processes highlighted above suggests, following one component misses many important features of nuclear condensates. Here, we highlight how composition, location, and dynamics, three features for which we generally lack unbiased information, are particularly important to understanding the regulation and function of nuclear condensates.

A defining feature of any condensate is that it concentrates specific sets of biomolecules through a network of weak multivalent interactions.[1] This feature is distinct from high-affinity macromolecular complexes that rely on interactions with fixed stoichiometry. Due to the molecular complexity and dynamic nature of condensates, we lack effective tools to investigate their composition and structure. The small size of some nuclear condensates and their association with the genome makes isolation and characterization challenging. We often do not know much more than the fluorescence intensity of the labeled protein within a condensate, often giving the false impression that the condensate is composed of that factor alone. This lack of knowledge is particularly problematic for nuclear condensates, where a single protein exists in dozens or more discrete foci each with potentially distinct compositions. In addition to understanding condensate composition, it is important to also address how composition is regulated and how composition relates to function.

The location of a condensate within the nucleus, whether associated with a specific genomic locus or within the nucleoplasm will have significant consequences on the function and biophysical properties of the condensate. What determines the formation of condensates at specific genomic loci? The formation of the nucleolus occurs at nucleolar organizing regions (NORs), promoting transcription and processing of rRNA.[10,11] Other condensates sequester regulatory machinery away from the genome and inhibit transcription.[12] What determines whether a condensate will form on the genome? If on the genome, how are specific genomic regions included or excluded from the condensate? And how does the genome behave across the phase boundary? The distinction between condensates on or off the genome or at different genomic loci is critical yet understudied.

Many condensates are highly dynamic assemblies that can rapidly form and dissolve with a large distribution of lifetimes. This dynamic nature represents a challenge to track condensates with high temporal and spatial resolution, therefore limiting our ability to define the precise relationship between condensate formation and function. For example, transcription and the biomolecular interactions that drive condensate formation are both dynamic processes.[13,14] Does transcription of an endogenous gene start upon assembly of a condensate and stop upon its disassembly? Do longer lived condensates (*e.g.,* nucleoli or heterochromatin) enable constitutive “housekeeping” activities and more dynamic condensates enable tightly regulated discontinuous activities (*e.g.,* transcription of specific mRNAs)? The wide distribution of nuclear condensate lifetimes[15,16] suggests that there are likely to be unknown regulatory mechanisms that govern the dynamics of assembly and dissolution.

As we have highlighted here, many nuclear processes are compartmentalized by condensates, but how this higher-order organization regulates the compartmentalized process requires an understanding of the composition, location, and dynamics of the condensates. Whereas we have well-developed tools to investigate these features for macromolecular complexes, often relying on the stability of complexes in a wide range of dilutions and solvent conditions, comparable tools are limited for investigating condensates. Multiple orthogonal approaches and likely newly developed techniques will be required to address these important questions.

Biomolecular condensates in neurons in normal and disease contexts (*Dragomir Milovanovic, Steven Boeynaems, Bede Portz, James Shorter, Markus Zweckstetter)

Neurons are a prime example of non-dividing, highly polarized cells in which axons and dendrites can be several orders of magnitude longer than the diameter of a cell body. This architecture poses a major challenge for the trafficking of proteins and RNAs from the cell body to synapses, the contacts between neurons that allow for signal propagation. Interestingly, many RNA-binding proteins (RBPs) implicated in axonal trafficking are found aggregated in patients with ALS, FTD, AD, and other neurodegenerative diseases.[17,18] Several of these same RBPs are also involved in the formation of stress granules, which provide an invaluable paradigm for investigating the principles of phase separation involving RNAs[19,20] (Figure 2). However, several important considerations arise: (i) While some neuronal transport granules have been shown to regulate local translation,[21] how does phase separation relate to the function of other types of RNA granules? (ii) One type of RNA transport granule was reported to be translocated in cells by hitchhiking on lysosomes[22]; do other cellular structures play roles in granule transport (e.g., the neuronal cytoskeleton or membrane-bound organelles)? (iii) How are the organization and dynamics of transport granules changing in disease and aging? (iv) What quality-control mechanisms are in place to reverse aging/disease-associated alterations in these condensates, and can these mechanisms be leveraged or even reengineered for therapeutic purposes? Developing answers to these questions will shed light on the structural roles of RNAs and RBPs in the formation of neuronal granules and the regulation of translation.

Neuronal communication relies on the coordinated release of messenger molecules, neurotransmitters, at the specialized sites referred to as synapses. Synapses are composed of the presynaptic site, where synaptic vesicles (SVs) loaded with neurotransmitters are clustered; the postsynaptic site, where neurotransmitter receptors are located so that they can activate downstream signalling upon the binding of neurotransmitters for the cognate receptor; and the synaptic cleft where adhesion proteins keep the pre- and post-synapse together. Phase separation underlies the formation of subcompartments both at the pre- and post-synapse, such as SV clusters,[23,24] active zone proteins,[25,26] endocytic sites,[27,28] and postsynaptic densities in both excitatory and inhibitory post synapses[29,30] (Figure 2). These subcompartments are critical to ensure the fidelity of the SV cycle at the pre-synapse and the signaling amplification at the post-synapse, allowing for the coordinated neurotransmission. Within a pre-synapse, individual synaptic subcompartments are only a few hundred nm in size, with many of them tightly juxtaposed against each other. These properties raise several questions: (i) How is the specificity of synaptic condensates encoded? (ii) What are the roles of mem-

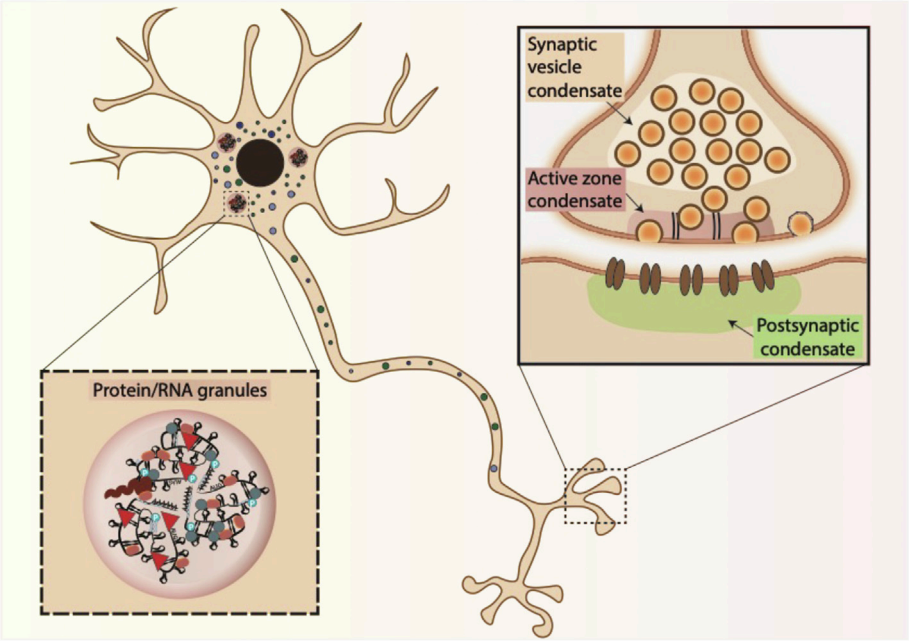


Figure 2. Different types of biomolecular condensates form in neurons. Left: Neuronal storage and transport granules composed of RNA-binding proteins and RNAs. Right: The synapse is as an example of a multiphase system. Scheme modified from Milicevic, et al.[118]

branes and membrane-bound organelles for phase separation at the synapse? (iii) How is phase separation regulated during repeated rounds of depolarization? Deficits in neurotransmission are associated with neurodegeneration; thus, understanding the emerging roles of phase separation in the organization and dynamics of the synapses promises to be critical for targeting dementia and related disorders.

Several methodological challenges need to be addressed by the community, such as the limitations of extrapolating data from cell lines to neurons; the lack of data measuring the functional outcome (*e.g.,* translational output and neurotransmitter release); the absence of tools that allow visualization, quantitation, and manipulation of RNAs within the condensates in neurons and glia. These challenges can be, at least in part, solved by validation of findings from cell lines in primary murine neurons. Patient-derived iPSC neurons promise to be a system of choice to address how the phase separation-related mutations in proteins affect specific neuronal populations (*e.g.,* motor neurons, dopaminergic neurons). Together, addressing these challenges will advance understanding of the roles of phase separation in neuronal physiology and how the dysfunctional condensates lead to disease onset.

Phase separation within the complex structure of the cell (*Emily Sontag, Liam Holt, Jeanne Stachowiak)

The interior of the cell is highly crowded with macromolecules and organelles. This complex environment strongly influences phase separation. Altering crowding in the cytoplasm by changing ribosome concentration has been shown to drive phase separation through depletion-attraction effects.[31] Conversely, mechanical confinement within chromatin can frustrate the growth of phase separated structures by preventing droplet fusion.[32] The cell is also "active matter", *i.e.* far from chemical equilibrium. The conversion of chemical potential (ATP) into motion, *e.g.* through molecular motors, is crucial for molecular movement. Depletion of ATP in *E. coli* leads to conversion of the cytosol to a glassy solid.[33] In *S. cerevisiae*, ATP depletion combined with cytoplasmic acidification, can cause large-scale material conversion of the cytoplasm from liquid to gel-like states.[34] Active processes are also likely to impact phase separation within subcellular compartments, as observed for the nucleolus,[35] but more work is needed in the future.

Membrane surfaces provide a mechanism to locally confine proteins.[36] As proteins move from the 3D cytosol to the 2D surfaces of membranes, they concentrate, potentially nucleating phase separation. Early studies on immunological receptors[37,38] and actin-interacting proteins[39] have demonstrated the ability of membranes to drive local LLPS. Several labs have demonstrated the role of phase separation during endocytosis.[28,40,41] Lipids also undergo phase separation on synthetic[42] and cellular membranes,[43] and recent work shows that lipid and protein phase separation on a membrane surface can be coupled.[44]

Beyond concentrating biomolecules, membrane-rich organelle contact sites help transfer materials (lipids, ions, proteins, etc) between organelles. Recent evidence in yeast suggests that organelle contact sites are formed through phase separation. The nucleus- vacuole junction is a particular hub for this activity as both intranuclear and juxtanuclear phase separation migrate to these junctions to facilitate clearance.[45] Additionally, phase separated bodies containing misfolded proteins can associate with mitochondria and accumulate at mitochondria- vacuole junctions.[46] The misfolded proteins are trafficked to mitochondria and mitochondria-vacuole junctions via COP-II vesicles that form at ER exit sites.[46] The ER makes contacts with all other organelles[47] and many of these ER contact sites (such as ER-mitochondria, ER-mitochondrial encounter structure, and ER-vacuole) are critical for condensate formation and transport.[48,49] Further, the ER membrane itself can form liquid-ordered microdomains at organelle contact sites.[50] Future studies will determine how this process functions in mammalian cells and neurons.

In conclusion, LLPS is critical for many cellular processes from environmental sensing to concentration and storage of macromolecules, generating organelles and subcellular structures, as well as protein clearance. Future work will further elucidate how the complexity of the cell is both defined by and influences phase separation.

Biomolecular condensates in bacteria and protists (*Stephanie C. Weber, Jörg Gsponer, Oliver Mueller-Cajar)

The list of biomolecular condensates identified in bacteria and protists is growing rapidly. For example, polar condensates containing PopZ in *Caulobacter crescentus* promote asymmetric cell division[51] while Rubisco condensates – carboxysomes in *Synechoccus elongatus* and pyrenoids in *Chlamydomonas reinhardtii* – enhance carbon fixation[52–54] (Figure 3). Phase separation has also been implicated in transcriptional regulation in *Escherichia coli*,[55] as well as virulence in *Mycobacterium tuberculosis*.[56]

The discovery of bacterial and protistal condensates (Figure 3) presents a number of exciting opportunities. First, carboxysomes have fewer components than condensates in multicellular organisms,[57] and are thus ideal for *in vitro* reconstitution. Indeed, the beta-carboxysome scaffolding protein CcmM recruits both Rubisco and carbonic anhydrase into conden-

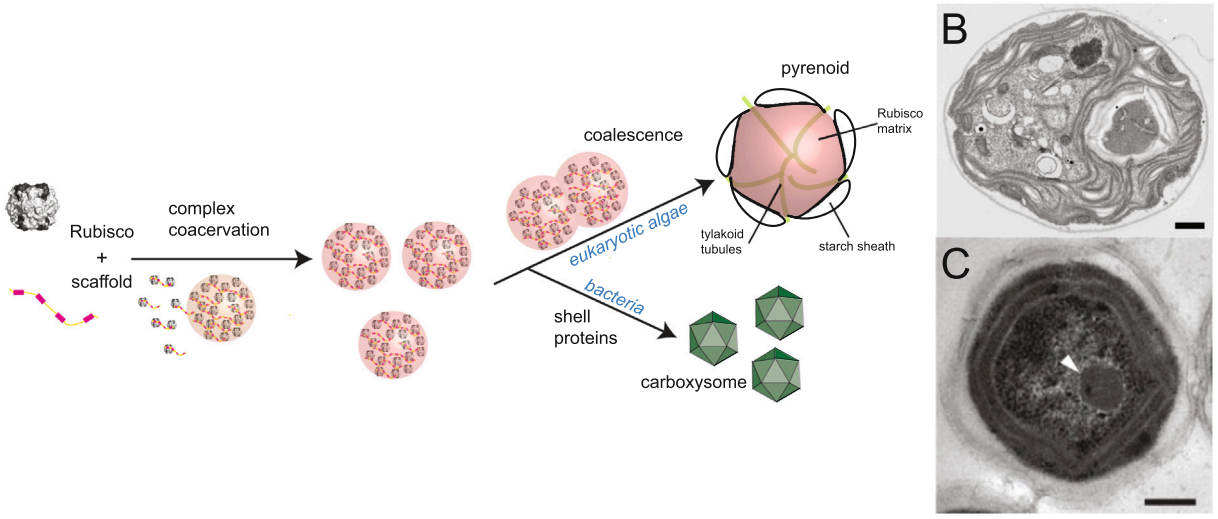


Figure 3. Examples of biomolecular condensates in bacteria and protists. (A) Rubisco and multivalent scaffold proteins phase separate through complex coacervation. Coacervates fuse to form the pyrenoid in eukaryotic algae. In bacteria, shell proteins may act to restrict coalescence of carboxysomes, controlling their size. Reprinted from[119] with permission from Elsevier. (B, C) Transmission electron micrographs of *Chlamydomonas reinhardtii* and *Synechococcus elongatus*, respectively. Arrowheads mark the pyrenoid (black) and carboxysomes (white). Reproduced from[53,71] with permission from Springer Nature.

sates that support carbon fixation activity *in vitro*.[58] Their relatively "simple" composition should also facilitate establishing direct links between phase separation and biological function. For example, loss-of-function mutations in *ccmM* result in a failure to assemble carboxysomes and an inability to grow photosynthetically.[59] Notably, sequence variants of PopZ that alter the material properties of polar condensates also impair cell fitness.[60]

Second, bacteria and protists span the tree of life and exhibit a wide range of metabolic and ecological strategies. This diversity allows for comparative analysis of condensate structures and functions. For example, clusters of RNA polymerase have been observed in fast-growing cells of both Gram-positive and Gram-negative bacteria,[61,62] across which the putative scaffold protein, NusA, is highly conserved.[55] In contrast, the scaffold proteins for alpha- and beta-carboxysomes and the pyrenoid - CsoS2, CcmM, and EPYC1, respectively - share no sequence homology, indicating that these condensates arose independently multiple times through convergent evolution (Figure 3).[63]

Finally, bacterial and protistal condensates offer powerful substrates for synthetic biology. The modularity of PopZ, which contains a trivalent oligomerization domain and an intrinsically disordered region, was leveraged to design synthetic organelles with tunable properties in human cells.[60] The alpha-carboxysome has been harnessed to engineer novel functions into *E. coli*, including carbon fixation[64] and hydrogen production.[65] However, these efforts used the proteinaceous shell rather than the condensed matrix. To that end, expression of the algal pyrenoid scaffold EPYC1 in *Arabidopsis* induced condensation of a plant-algal hybrid Rubisco within the chloroplast.[66] This result represents a critical step toward introducing a functional CO_2-concentrating mechanism into crop plants, with the ultimate goal of enhancing photosynthesis.

Despite this recent progress, challenges remain. Bacteria and protists are small, and their condensates are even smaller. Most of these systems fall below the diffraction limit, and thus require super-resolution, single-molecule techniques, or both for characterization.[55,60,67,68] Promisingly, cryogenic methods can now visualize condensates with unprecedented resolution, revealing both molecular architecture and cellular context.[53,69–71] In addition, genetic tools for protists are currently limited, so continued development of transformation protocols[72] will be necessary to take full advantage of the diversity of bacteria and protists, and the likely many condensates still to be discovered.

Links between biomolecular condensates and cancer (*Richard Kriwacki, *Huaiying Zhang)

Many cancer-associated proteins have been shown to localize within biomolecular condensates involved in diverse biological processes (reviewed in[73]). However, direct evidence of links between condensates and cancer phenotypes is available in relatively few cases. One example is SPOP, an E3 ubiquitin ligase which undergoes phase separation with substrates and mediates their polyubiquitation and degradation. Importantly, SPOP is recurrently mutated in prostate and endometrial

cancers. Prostate cancer-associated mutations reduce substrate binding and ubiquitination, and also increase the saturation concentration for phase separation of substrates with SPOP.[74] In contrast, mutations seen in endometrial cancer alter substrate specificity, enhancing interactions with some and weakening those with others,[74] although whether these alterations affect phase separation is currently unknown. Another example is YAP, a transcription factor upregulated in many cancers.[75] YAP forms enhancer-associated condensates that drive gene expression in response to cellular osmotic stress.[76] Further, mutant forms of ENL were recently shown to promote formation of aberrant transcriptional condensates that drive oncogenic gene expression.[77] Finally, PML bodies are mislocalized to telomeres in alternative lengthening of telomeres (ALT) cancer cells to promote telomere lengthening,[78] linking altered condensation behavior with a hallmark of cancer and replicative immortality[79] (Figure 4(A)).

Gene translocations, observed in ~15% of cancers, cause expression of fusion oncoproteins (FOs) that are oncogenic drivers in diverse

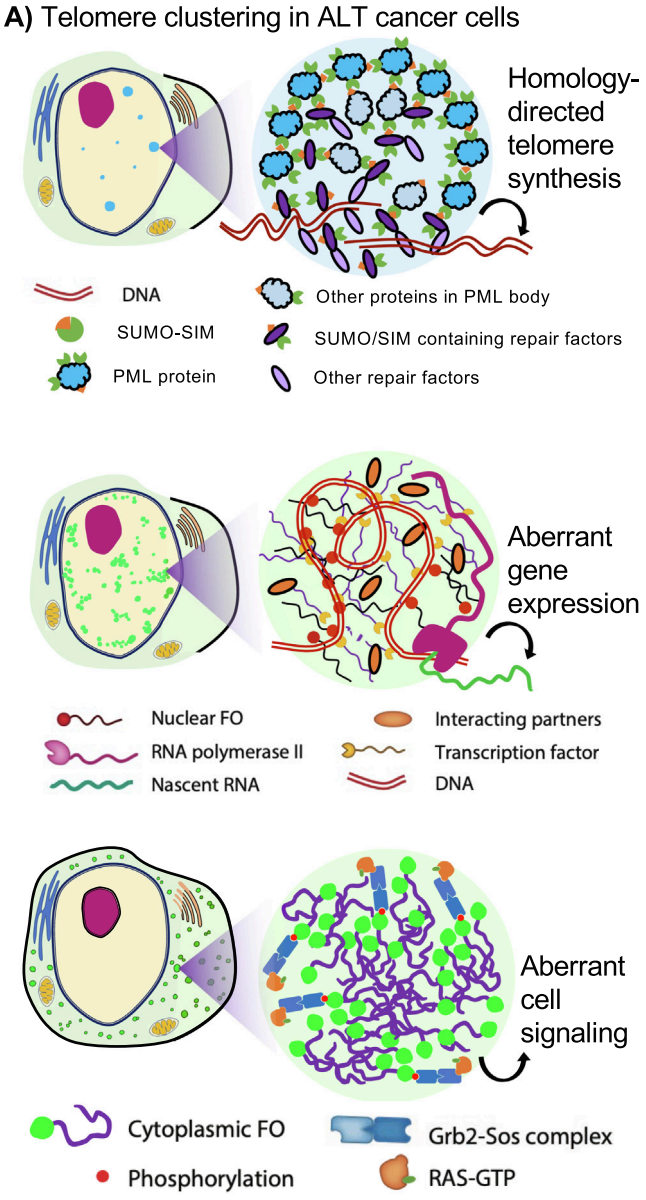


Figure 4. Aberrant biomolecular phase separation in cancer. (A) Mislocalized PML bodies cluster telomeres to promote homology-directed DNA synthesis for telomere maintenance in ALT cancer cells. (B, C) Several fusion oncoproteins that drive oncogenesis in diverse cancers have been shown to undergo phase separation to form aberrant (B) nuclear transcriptional condensates or (C) cytoplasmic signaling condensates. B and C reproduced from[81] with permission; copyright 2023 by the authors.

cancers.[80] Several FOs have been shown to function by forming aberrant condensates in cells[81] (Figure 4(B) and (C)). For example, the EML4-ALK and CCDC6-RET FOs, drivers in lung cancer, form cytoplasmic condensates that promote aberrant Ras signaling.[82] These FOs contain tyrosine kinase domains, enabling the resulting condensates to drive cell signaling independent of membrane-associated receptors.[82] In contrast, several other FOs, including the NUP98 FOs associated with acute myeloid leukemia (AML) in children and FET FOs associated with Ewing Sarcoma, form chromatin-associated nuclear condensates that cause aberrant gene expression.[81] NUP98 FOs undergo phase separation at specific genes, creating aberrant transcription centers that drive transformation of hematopoietic cells, causing AML.[83–85] Many other FOs are known to alter cell signaling or gene expression,[80] raising the possibility that aberrant condensate formation underlies the oncogenic activity of other FOs.

Many of the condensates discussed above form only in cancer cells, providing opportunities for onco-condensate targeted therapies in the future. It will be critical, however, to broaden our understanding of links between biomolecular phase separation and cancer mechanisms to realize this therapeutic promise. Does oncogenicity arise from gain of function through condensate formation, or through loss of normal physiological functions? What roles do the chemical, structural, and material properties of condensates play in oncogenesis? Many biochemical and cell culture studies are emerging to answer these questions. Studies into the molecular grammar of phase separation enable the design of mutants that alter phase separation or condensate properties, enabling interrogation of links with disease processes. Alternatively, chemical or optogenetic tools can be used to study condensate formation and assess functional consequences. Ultimately, *in vivo* studies are needed to link phase separation with tumorigenesis. In summary, multidisciplinary studies into how biomolecular condensates contribute to cancer mechanisms will create opportunities for novel approaches to anti-cancer therapies in the future.

Establishing rigorous relationships between phase separation and biology (*Liam Holt, Shasha Chong, Ben Sabari, Stephanie C. Weber)

There has been an explosion of publications on roles of phase separation in biology. However, we believe that some of the data supporting the function of condensates have not been fully considered, leading to rising skepticism regarding the physiological relevance of phase separation.[4,6,7] As the field matures, now is an excellent time to reflect on what evidence is needed to rigorously implicate the physical process of phase separation as a mechanism of biological organization and control. A key point that we highlight is that loss-of-function experiments are rarely sufficient. For example, condensates are often formed through phase separation based on weak interactions between disordered protein regions with low-complexity sequences (LCSs), also referred to as intrinsically disordered regions (IDRs). A truncation of an IDR can abrogate phase separation, and may lead to a phenotype. However, we argue that this experiment *is not sufficient to conclude that phase separation is important for the biological function*. This is because there is a second, competing hypothesis that the IDR was crucial for biological function through a mechanism(s) apart from its role in phase separation. How can we experimentally distinguish between these hypotheses?

We propose three rigorous tests of the importance of phase separation *per se.* First, extensive mutagenesis, or evolutionary diversity, can be leveraged to demonstrate that the physical properties of condensates strongly correlate with biological outcomes. For example, in seminal work, evolutionarily guided, systematic mutation of the yeast P-body protein, Pab1, altered phase separation *in vitro* and demixing *in vivo*. Strikingly, mutations that increased the temperature required for demixing led to loss of fitness upon heat-shock.[86] Similarly, several orthologues of the yeast stress-granule protein, Ded1p, were shown to have different critical temperatures both *in vitro* and *in vivo* and to differentially regulate translation.[87] In a third example, rational design of the bacterial protein PopZ revealed that condensate fluidity is optimally tuned for cellular fitness.[88] Increasing the length of PopZ's IDR or the valency of its oligomerization domain resulted in poorly-growing cells with condensates that were less viscous, or more solid-like, respectively, than those formed by the endogenous protein. Remarkably, a "Goldilocks" combination of these perturbations restored not only the material properties of the condensates, but also cell growth.

Second, rescue experiments can be used. For example, if the only function of the IDR is to provide general interactions that lead to condensation, orthogonal sequences (*e.g.,* those with low sequence similarity) that restore phase separation, should lead to rescue of the phenotype. If orthogonal sequences only give partial rescue this may indicate that the original IDR also conferred important specific interactions. This approach was used *in vitro* to illustrate the importance of phase separation for the microtubule nucleation activity of TPX2[89] where only certain chimeras fully recovered nucleation rates. Another study of stress-induced NELF condensates found that chimeras recovered condensate formation and function, but lost stress

inducibility.[90] Studies of the tumor suppressor UTX similarly found that swapping IDRs had varying effects on both condensate formation and cell growth assays.[91] The particular physical properties of the condensate may also be crucial for function, in which case finding an ideal orthogonal complementing sequence may be challenging, but also highly informative.

Third, synthetic biology can reveal general principles for how phase separation can organize and drive biological processes. For example, recruitment of kinases to synthetic condensates can accelerate reaction rates, expand kinase specificity and make the system responsive to changes in the biophysical properties of the cellular environment.[92] Optogenetic activation of condensates was shown to increase transcription by increasing effective transcription factor affinity, and also made transcriptional responses more switch-like.[93] On the other hand, a separate study of synthetic condensates concluded that phase separation was not the main determinant of TF activity.[94] Therefore, the details of the system are important, and phase separation will not always be a crucial determinant of biological activity.

In conclusion, we propose that the biological importance of phase separation is best demonstrated by orthogonal complementation, or fuctional modulation (*e.g.,* mutagenesis to modulate phase separation coupled with function) experiments. Deeply exploring how these chemical and material properties relate to biological function will give far deeper insights into the regulatory potential of phase separation.

Defining, Designing, & Targeting Condensates

Material properties of biomolecular condensates (*Shana Elbaum-Garfinkle)

Biomolecular condensates are inherently defined by their material properties, with the recently coined terminology[1] reflecting the diverse spectrum of *condensed* matter that these assemblies are now known to form. Ranging from liquid-like to solid-like features, the material properties of condensates, and their modulation as a function of aging and other cellular cues, are specifically implicated in disease.[95,96] Defining the material states of condensates and how material properties impact molecular behavior and functional outputs remain central open questions in the field.

Our understanding of how to best define and model material states of condensates is continuously evolving. For example, early work highlighted the 'liquid'-like properties of membraneless organelles and the role of liquid–liquid phase separation (LLPS) specifically in driving their formation.[2,97] However, there is growing appreciation for the viscoelasticity, i.e., the confluence of both viscous and elastic properties at unique time- and length-scales of apparent 'liquid-like' condensates.[98,99] This, together with increasingly complex condensate architectures[100] including the coexistence of multiple unique phases, justify the need for new models and definitions. Recent efforts to meet these needs include the incorporation of a LLPS-distinct, polymer–polymer phase separation (PPPS) process to describe the complexity of chromatin states,[101] and a phase separation coupled to percolation (PSPC)[5] model which offers a unifying framework for understanding diverse mechanisms of phase separation observed across many systems. Further refinement of the fundamental frameworks that define the assembly and properties of biomolecular condensates will continue to evolve alongside new discoveries in this rapidly growing field.

Defining condensate properties is an important first step in the advancement of a greater central question: how and when do material properties impact molecular behavior and ultimately function? From a mechanistic perspective, answering this question requires a more holistic interrogation of condensate properties across length-scales, from the molecular level to the mesoscale. For example, how do the unique material states along a nuanced viscoelastic spectrum specifically impact the behavior of individual molecules with respect to their diffusion, dynamics, stability and accessibility? And how might the size, chemistry, and interaction specificity of individual molecules dictate these behaviors? Importantly, addressing these questions will require an integration across system complexity, including computational approaches, in vitro reductionism, and in vivo model systems to yield insights that not only increase understanding of condensate biology, but crucially, can be leveraged for the development of novel therapeutic strategies to treat condensates-associated disease states (discussed below).

Biological applications of synthetic condensates (*Christopher D. Reinkemeier)

Synthetic biologists strive to reengineer cells for biotechnological or medical applications. To this end, directed evolution and *de novo* design[102,103] are powerful technologies, but more recently, spatial compartmentalization is becoming an increasingly exciting tool as well. Synthetic compartments can, for example, concentrate molecules or separate incompatible reactions, and thereby organize how biochemical processes proceed in cells. However, it is critical that such synthetic compartments can exchange components with the surrounding cellular milieu to access starting materials and co-factors, and subsequently release products to function in the cell. Biomolecular condensates are an especially attractive choice for encoding compartmentalization of cellular components (see[104–106]

for reviews), as they do not rely on a membrane boundary, and can thus operate without a dedicated transport machinery. The applications for this are vast; condensation principles have been successfully used to transiently sequester proteins,[107–109] to cluster enzymes for regulating product flux,[110] and even to establish orthogonal translation systems which allow site-specific incorporation of non-canonical amino acids into selected proteins.[111–113]

Synthetic condensates can be built either using naturally phase-separating proteins, or by constructing artificial multivalent networks (see[106] for a more detailed comparison). Although synthetic networks are orthogonal to the host, their behavior *in vivo* can be challenging to predict. Meanwhile, naturally phase-separating proteins are often derived from the host and can potentially interact with endogenous components, but their behavior has often been studied in cellular contexts and thus they typically can form condensates robustly *in vivo*.

Crucial to implementing the long-term usage of synthetic condensates *in vivo* will be developing condensates orthogonal to host components. Furthermore, it is of particular interest to develop clear, reproducible design guidelines that will allow customizing the properties of the condensates for specific processes. Together, these advancements will help incorporating ever more complex processes into synthetic condensates; such condensates will equip the host system with bespoke and useful functions, and thus come to the fore as the next generation of broadly applicable synthetic biology tools.

Drugging biomolecular condensates (*Diana M. Mitrea, John F. Reilly, Michael R. White)

Targeting biomolecular condensates has the potential to enable the development of novel and diverse therapeutic approaches. This is evinced by the new appreciation that most cellular proteins may participate in condensate formation at some point in their life cycle.[114] Condensates play key roles in the regulation of many cellular processes, and act as central nodes in multiple diseases.[2,96,115] Therefore, several companies are pursuing condensate-targeted therapeutics for diseases ranging from autoimmunity to virology, with many companies focused on neurodegeneration and oncology. However, the exploration of condensate-centric therapeutics is in its infancy; platform technologies and drug design strategies

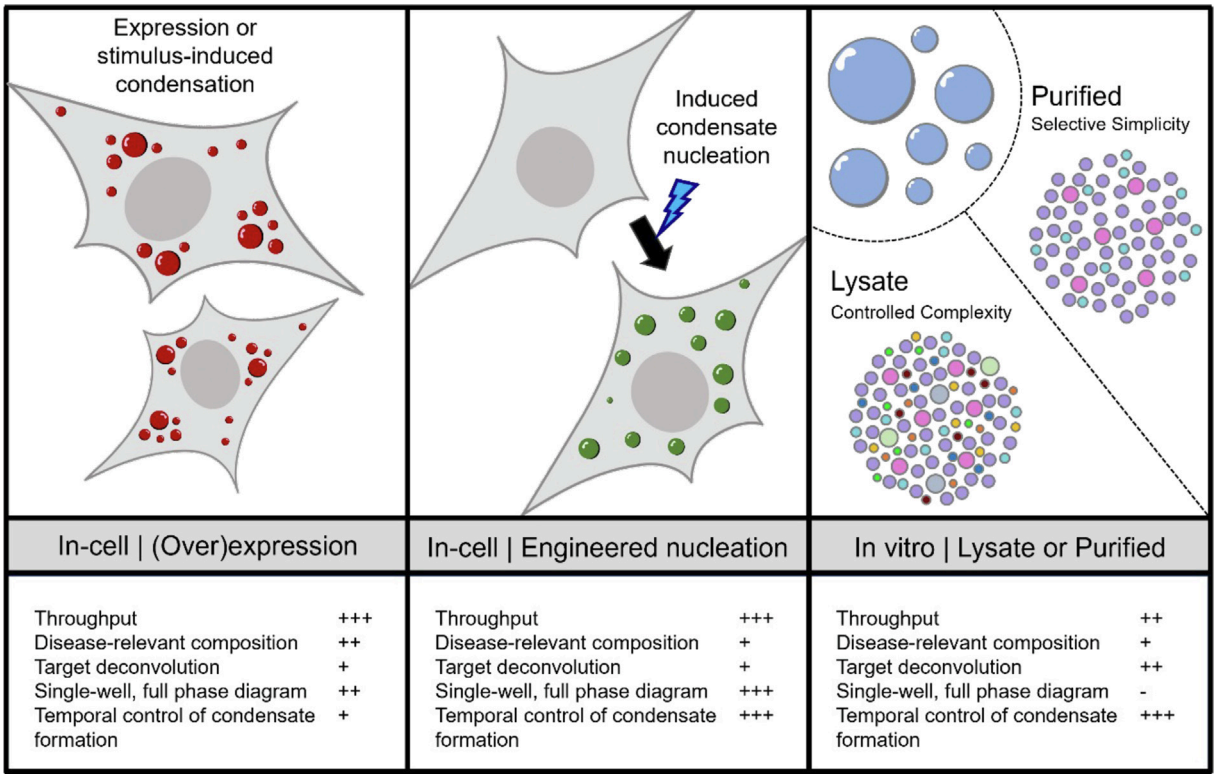


Figure 5. High throughput, plate-based screening approaches for identification of condensate modulators. (A) In-cell screen, with endogenous levels or overexpression of the marker protein; condensation can be induced by stimuli or by the level of expression of constituents; (B) In-cell screen with light-induced condensates; the marker protein is engineered to express a light-inducible oligomerization domain which nucleates condensate formation,[115] (C) In vitro screens; the condensates are reconstituted in buffer with a controlled number of recombinant components (selective simplicity), or by seeding cell lysates with the scaffold of choice (controlled complexity).

are being developed and/or adapted from established methods in parallel with the quest for new and better drugs.

Multiple approaches to condensate drug discovery were discussed during the workshop (Figure 5). As in traditional efforts, screens and validation assays can be performed *in vitro* or in-cells, each with their own advantages and disadvantages. *In vitro* reconstitution assays, for instance, examine the effects of small molecules on condensates, due to direct interaction with a specific component. This approach, being reductionist in nature with limited complexity compared to biological conditions, offers a more straightforward path to structure–activity relationship assessments. Any effects on condensates must be due to direct interactions with one of their constituents. Increased complexity, while maintaining a similar level of compositional control, can be achieved by reconstituting condensates using cell lysates. Alternatively, in-cell screens cast a wider net, identifying direct interactors and compounds that modulate the activity of upstream regulators of condensates, such as enzymes that introduce post-translational modifications. In-cell systems come closer to representing the biological complexity of the condensate composition and environment but complicate the target deconvolution process.

Performing condensate-targeted drug discovery enables a series of previously unexplored opportunities, including functional modulation of proteins previously considered "undruggable". Leveraging the emergent properties of a condensate[1] can help maximize efficacy and minimize off-target and toxic effects. We are just beginning to understand that some drugs currently used in the clinic interact with condensates,[116] and that their clinical outcomes could be positively or negatively impacted by interactions with condensates.[117] This realization begs multiple questions: How many drug candidates have failed due to disruption of off-target condensate function? What are the rules for rationally designing specificity and selectivity for a community of molecules, as found in a condensate? How do they differ from the well-established rules for individual biomolecule targets? Can targeting condensates that act as central nodes in polygenic diseases deliver drugs that benefit a larger patient population compared to those targeting a single protein? These questions are some of the many we hope to answer in the coming years.

Concluding remarks

There are growing numbers of biological processes that have been linked with biomolecular phase separation, although, as discussed above, the rigor with which these links have been established is variable and warrants strengthening in the future. Despite this limitation, it seems clear that the ability to undergo phase separation is an intrinsic property of many biomolecules. What is less clear is whether phase separation is a requirement for function in the various biological contexts for which it is reported.

In this Perspective, we have surveyed the involvement of biomolecular phase separation in diverse biological processes that span distinct cell regions, cell types, and branches of the evolutionary phylogenetic tree, as well as their implication in human diseases, including neurodegeneration and cancer. We further discussed the call for increased rigor in the field as well as current efforts to define, design and therapeutically target condensates. Results from these efforts have the potential to generate novel avenues of treatments and to expand the broader understanding of how condensates interface with, and potentially enhance biological processes.

As noted above in the Introduction, this Perspective is the outcome of a TSRC workshop on phase separation in biology and disease and is essentialy a series of snap-shots of the field at the time of the meeting and since. We look forward to tracking the development of biomolecular phase separation field as the ideas discussed here are rigorously tested and the questions raised answered in the future.

CRediT authorship contribution statement

Steven Boeynaems: Writing – original draft, Writing – review & editing. **Shasha Chong:** Writing – original draft, Writing – review & editing. **Jörg Gsponer:** Writing – original draft, Writing – review & editing. **Liam Holt:** Writing – original draft, Writing – review & editing. **Drago Milovanovic:** Writing – original draft, Writing – review & editing. **Diana M. Mitrea:** Writing – original draft, Writing – review & editing. **Oliver Mueller-Cajar:** Writing – original draft, Writing – review & editing. **Bede Portz:** Writing – original draft, Writing – review & editing. **John F. Reilly:** Writing – original draft, Writing – review & editing. **Christopher D. Reinkemeier:** Writing – original draft, Writing – review & editing. **Benjamin R. Sabari:** Writing – original draft, Writing – review & editing. **Serena Sanulli:** Writing – original draft, Writing – review & editing. **James Shorter:** Writing – original draft, Writing – review & editing. **Emily Sontag:** Writing – original draft, Writing – review & editing. **Lucia Strader:** Writing – original draft, Writing – review & editing. **Jeanne Stachowiak:** Writing – original draft, Writing – review & editing. **Stephanie C. Weber:** Writing – original draft, Writing – review & editing. **Michael White:** Writing – original draft, Writing – review & editing. **Huaiying Zhang:** Writing – original draft, Writing – review & editing. **Markus Zweckstetter:**

Writing – original draft, Writing – review & editing. **Shana Elbaum-Garfinkle:** Writing – original draft, Writing – review & editing, Project administration. **Richard Kriwacki:** Writing – original draft, Writing – review & editing, Project administration.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: D.M. M. is an employee and shareholder of Dewpoint Therapeutics; B.P. is an employee and shareholder of Dewpoint Therapeutics; J.F.R. is an an officer and shareholder of Nereid Therapeutics; J.S. is a consultant for Dewpoint Therapeutics, ADRx, and Neumora, and a shareholder and advisor for Confluence Therapeutics; L.C.S. is on the Prose Foods Scientific Advisory Board; M.W. was an employee and shareholder of Faze Medicines when this article was conceived and initially written, and currently is an employee of IDEXX Laboratories; R.K. reports personal fees from Dewpoint Therapeutics, GLG Consulting, and New Equilibrium Biosciences outside the submitted work.

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