Bridging the Gap Between Primitive and Modern Phase Separation

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Abstract

Liquid-liquid phase separation (LLPS) is a process that often occurs due to binding between oppositely charged biopolymers, and has gained increasing attention recently due to their ubiquity in biological systems and ability to direct essential cellular processes. For example, aberrant biological LLPS can lead to the emergence and progression of various diseases and disorders, and many significant advances have been made in the biological phase separation field. However, while these discoveries in biology are recent, the field of origins of life has been investigating LLPS for nearly 100 years, ever since the first suggestions by Oparin and Haldane that primitive LLPS could have been precursors to the first cells on Earth. Since then, a significant amount of work has been done to elucidate different primitive LLPS systems that could have been relevant as protocellular models. Given the structural similarities between primitive LLPS and modern membraneless organelles, there may even be an evolutionary link between the two, although this remains a question to be answered. Nevertheless, in order to answer this, a source that compares aspects of modern and primitive LLPS is necessary. Here, we first focus on the assembly of membraneless organelles from intrinsically disordered proteins (IDPs) and nucleic acids, and discuss an example by which aberrant LLPS can result in progression of a disease (tumorigenesis). Then, as a parallel, we explore assembly of primitive membraneless compartments from simple biopolymers such as short peptides and nucleic acids. This is followed by a discussion of how the first biomolecules on Earth may have originated, analyzing the environmental and chemical conditions that could have favored primitive LLPS processes. Finally, we directly compare various aspects of LLPS assembly from both a primitive and a modern perspective, further discussing the potential of primitive IDPs on early Earth, but also the evolution from membraneless to membrane-bound cells. This review aims to provide a comparison of modern and primitive phase separation, including assembly and function, in order to help researchers in both fields understand the current state of knowledge, how this knowledge evolved, and the current gaps that need to be further addressed.

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Introduction

A cell is the basic building block of life, controlling and localizing complex chemical reactions within subcellular compartments, each a distinct chemical environment.[1] Compartmentalization, not just by organelles, thus defines the boundaries of biological systems by separating internal volume from external environment, which is essential for cellular activity.[2] Compartments within cells are mostly labeled as cell organelles, many of which are separated by membranes, such as the mitochondria, which generates energy in the form of ATP, or lysosomes, which digest cellular waste/byproducts.[3] However, membranes are not present in all organelles, such as stress granules, Cajal bodies, or nucleoli,[1] which are assembled *via* liquid-liquid phase separation (LLPS). Despite major recent advances in understanding biological phase separation,[1,4,5] how cells and organelles, especially membraneless organelles, emerged and evolved is still unknown. One way to tackle this question is to investigate the mechanism of assembly and evolution of primitive membraneless compartments.

Protocells are the simplest compartments that likely existed on early Earth and formed abiotically; it has been proposed that modern cells evolved from protocells.[6,7] There are several different protocell models that perform various prebiotically relevant functions, including lipid or polymer vesicles,[8] polyelectrolyte microcapsules,[9] colloidosomes,[10] proteinosomes,[11,12] supercritical carbon dioxide,[13] and coacervates.[14–16] Among these, coacervates are generated from LLPS where a macromolecule-rich condensed liquid phase separates from the aqueous solution.[2] Coacervates can be composed of synthetic polyelectrolytes, polysaccharides, nucleic acids, peptides, proteins, or (poly)nucleotides,[15,16] some of which were certainly available on early Earth. For this reason, and because coacervates can exhibit "life-like properties” similar to those of living cells (**Fig. 1**),[17,18] such as movement, growth, and division,[14,19,20] analyte uptake and concentration,[21] information processing,[22] adaptation,[23] and proliferation,[24] coacervates have been proposed as protocell models as early as 100 years ago.[25,26] However, coacervates cannot mimic all characteristics of a cell as they lack a membrane, which may lead to rapid coalescence,[27] instability,[28] or inability to selectively uptake nutrients and remove waste while retaining useful products.[2,29] Nevertheless, LLPS still allows regulation of internal biochemical reactions by concentrating specific prebiotic reactants,[21] while also being the foundation for emergence of complex functions, such as specific double-stranded DNA binding[30] or scaffolding of higher order structures such as liquid crystals.[16] This suggests the potential for membraneless LLPS compartments to be promising protocell

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candidates, perhaps before the advent of membrane-bound systems, and eventually leading to the emergence of cells.[31]

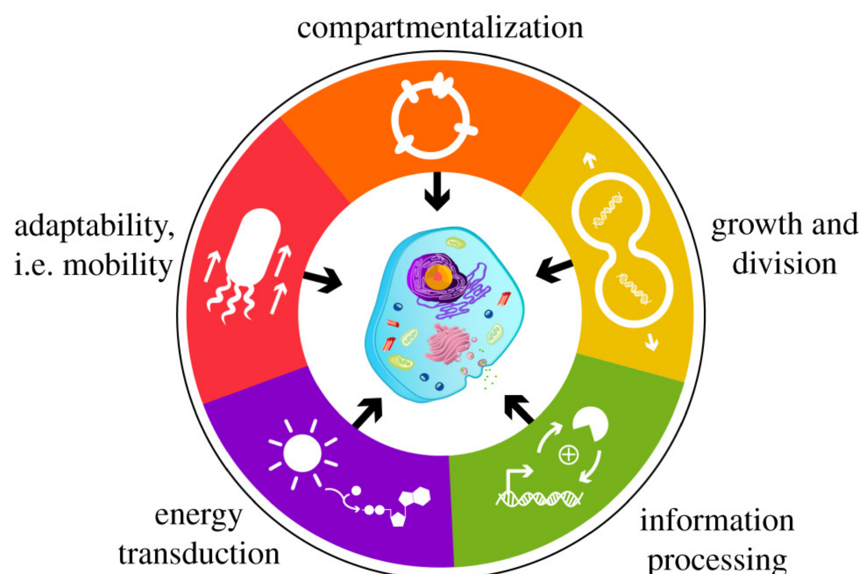

Figure 1. Cellular life-like properties. Reproduced with permission from[18] under a Creative Commons License.

The phase separation process itself is driven by different non-covalent intermolecular interactions such as electrostatic, pi-pi, cation-pi, hydrogen bonding, dipole-dipole interactions, van der Waals, etc., whether specific or non-specific,[2,32–34] resulting in different types of LLPS systems (**Fig. 2**):

1) Segregative: Two neutral polymers or a polymer and a salt, despite having a favorable mixing entropy (*i.e.*, the increase in total entropy or degree of randomness that occurs when multiple separate systems of different compositions are mixed; usually, favorable mixing entropy results in interaction), do not interact because of repulsive interactions between them.[35] They are consequently divided into two phases, each of which is enriched in one solutes.[2]
Examples: Polyethylene Glycol/Dextran.[29,36,37]

2) Associative: Formation of a dense polymer-rich phase and a dilute (polymer-poor) supernatant phase upon mixing of oppositely charged polyelectrolytes, resulting in associative phase separation due to ion-pairing interactions.[35] Due to attractive interactions (mostly charge-charge, but also other non-covalent

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interactions) between them, two or more polymers or polyions eventually reside in the same phase.[2,38] A specific case is the "complex" coacervate, where there are only two mixed species.

Example: oppositely charged polymers like gelatin (polycation) and gum arabic (polyanion) or cationic peptides and RNA with an anionic phosphate backbone.[39,40]

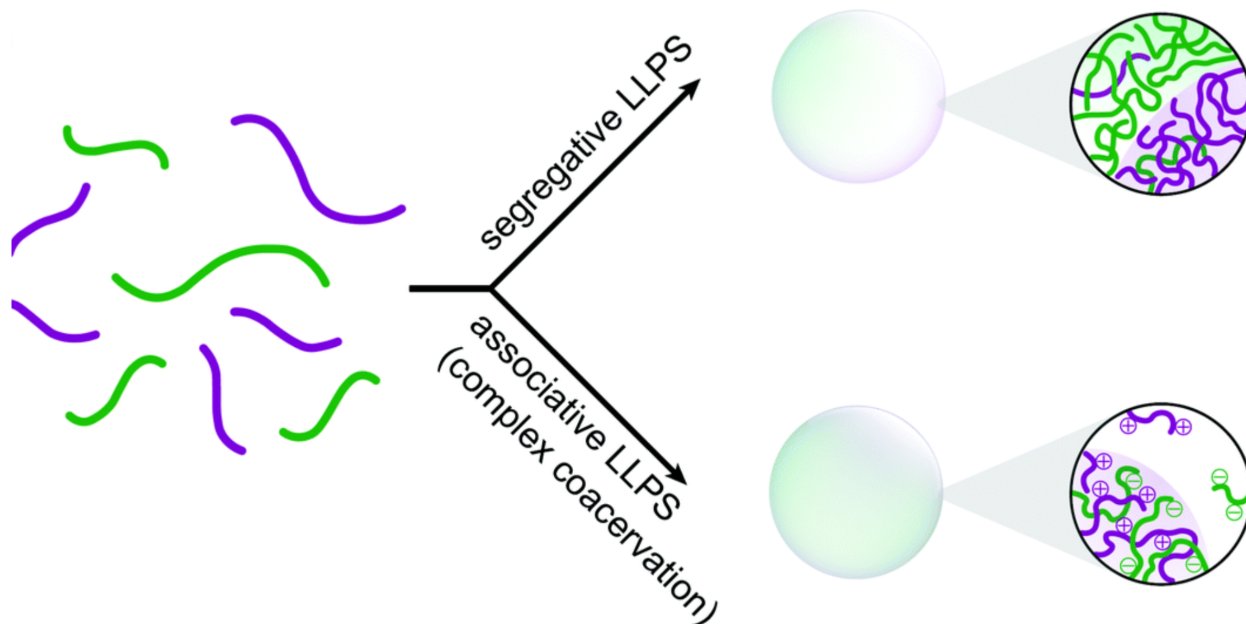

Figure 2. Segregative and associative LLPS. Reproduced from[2] under a Creative Commons License.

Within each category is a large range of possible structures,[4,41,42] and also systems that can transition between segregative and associative LLPS![43] Thus, given the breadth of systems that can phase separate, it is believed that membraneless droplets driven by LLPS could have formed from a variety of different chemistries and environments, an important consideration on early Earth, where chemical concentration, stability, and variety and environmental consistency may not be consistent.[44,45] The ubiquity of phase separated membraneless organelles in modern biology also suggests their importance universally in life; thus, is there some way to connect primitive phase separation with modern phase separation, whether through function, structure, or assembly? Here, as part of the Blue Marble Space Institute of Science Young Scientist Program in 2022, we sought to review and compare/contrast various aspects of modern and primitive LLPS. In particular, we review unique aspects of both modern membraneless organelles and primitive membraneless compartments/protocells. Finally, we attempt to directly compare/contrast aspects of these systems in hopes of finding direct connections between them.

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Aspects of Modern LLPS

Valency effects and intrinsically disordered proteins in cellular LLPS

Membraneless organelles formed through LLPS[46,47] come in different types highly present in both the nucleus and the cytoplasm such as nucleoli, stress granules, P-bodies, paraspeckles, Cajal bodies, etc. These organize and regulate intracellular biomolecules spatiotemporally through transient and dynamic microcompartments[48] as well as providing ribosome synthesis, signal transduction, stress regulation,[49] transcriptional noise buffering,[50] or nuclear heterochromatin area production functions.[51] One important component of membraneless organelles are intrinsically disordered proteins (IDPs), which do not have a fixed, ordered 3D structure, unlike typically "ordered" proteins, likely due to repulsion of like-charged residues.[49,52] Due to a lack of 3D structure, IDPs have high plasticity, *i.e.*, they fluctuate between multiple conformations instead of folding into a single well-defined 3D structure. However, IDPs can attain a fixed conformation when they bind with each other or with RNA/DNA, potentially forming stable membraneless organelles.[53] IDPs all contain low complexity (LC) domains, and it is believed that LC domains are the main drivers in biological phase separation, as they are frequently concentrated in membraneless organelles. LC domains uniquely contain only a few amino acids (AAs) among the 20 canonical AAs; in contrast, other domains/proteins contain most or all of the 20 AAs. Thus, to understand the biochemistry of membraneless organelles it is important to investigate the sequence to phase behavior relationship of IDPs. Here, we investigate the significance of how valency/AA sequence impacts/initiates phase behavior of organelles in the context of a "sticker and spacer" model.

It is known that composition and concentration of the participating macromolecules, the flexibility of these macromolecules, and environmental factors such as pH, temperature, and ion concentration affect phase separation.[54] In particular, multivalent interactions between macromolecules,[55] such as electrostatic interactions between residues in IDPs, appears to be essential for phase separation; at the molecular level, temporary electrostatic interactions between oppositely charged polyelectrolytes driven by valency can also result in phase separation.[56,57] For example, phase separation of IDRs in Ddx4, an RNA-binding protein found in germ granules, has been attributed to electrostatic interactions between clusters of opposing charges and cation-pi interactions between FG and RG motifs.[58] Additionally, the mitotic spindle protein BugZ and the RNA-binding proteins FUS, hnRNPA1, and hnRNPA2 phase separate due to Tyr-Arg interactions.[59–61]

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When considering valency effects on phase separation caused by binding of two macromolecules (considering the most simplistic case), the following factors must be considered: a) structural valency, *i.e.*, the number of binding sites available per molecule,[56] b) effective valency, *i.e.*, the number of sites that are sterically, simultaneously accessible for intermolecular contacts,[55] and c) interaction energy, *i.e.*, the affinity between binding sites on each molecule.[62] For a two-component system made up of associative polymers, a "sticker and spacer" model, which considers all of these factors, can be used to understand how multivalent interactions drive phase separation.[63] In this model, a polymer contains "sticker" groups along the chain that can bind reversibly with other sticker groups (on the same or a different polymer) through weak chemical or physical interactions such as H bonds, electrostatic interactions, hydrophobic interactions, etc.[47] Spacers are the regions between stickers, and enable cross-links by physically bringing stickers together[64] (**Fig. 3a**). With the help of spacers, the physical crosslinks between stickers on two interacting associative polymers can result in a density transition (also known as phase separation), whereby when both polymers are above a threshold concentration (c_{sat}), a polymer-dilute phase (comprising minimal inter-sticker crosslinks) and a polymer-dense phase (containing crosslinked stickers) will form.[63] This model requires two specific assumptions. First, that interactions between spacers, such as those between stickers and spacers, spacers and other spacers, and spacers and solvent, balance one another out and cause the spacer areas to operate as an ideal chain.[47] Second, that after phase separation, the polymers themselves have similar structural preferences regardless of which phase they are in, ruling out the idea that conformational changes due the physical/chemical properties of different phases could affect the valence of stickers (thus keeping all valence-related variables constant).[64]

To illustrate the usefulness of the sticker and spacer model, let us examine one case study in which the sticker and spacer model was used to demonstrate the mechanism of phase separation. The CT-10 regulator of kinase II (CrkII), a signaling adaptor protein, identifies proline-rich motifs (PRMs) in binding partners like cAbl kinase through its N-terminal Src homology 3 (nSH3) domain. Synthetic SH3/PRM system repetitions have thus been used to clarify the role of these interacting domains in promoting phase separation and are particularly of interest because of their role in cancer metastasis, cell spreading, and microbial pathogenesis.[20] Within this model, stickers on SH3 thus interact with stickers on PRM (**Fig. 3b**). These cross-links have a finite time span (making and breaking of these bonds/interactions) based on interaction strength, range, and directionality between these domains, and the timescale of the making and breaking these cross-links and the valency of the participating polymers determines the physical properties and dynamics of the system itself.[63] For example, SH3 and PRM constructs with higher valency (higher number of binding sites) form networks/cross-link faster, resulting in more rapid phase separation.[65–67] If

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SH3 and PRM are mixed at equimolar concentration, gradually increasing their concentrations at an identical rate of time results in more sticker-sticker interactions between SH3 and PRM.[67] When the concentrations of both SH3 and PRM exceed c_{sat}, phase separation occurs, resulting in separation of the polymers into a dense phase and a dilute phase. Within the dense phase, we observe a networked system of PRM and SH3, bound together by sticker-sticker interactions and highly concentrated in both PRM and SH3 (c_{dense}). However, in the dilute phase, the absence of sticker-sticker interactions results in an un-networked system and a lower concentration of PRM and SH3 (c_{dilute}) (**Fig. 3c**).

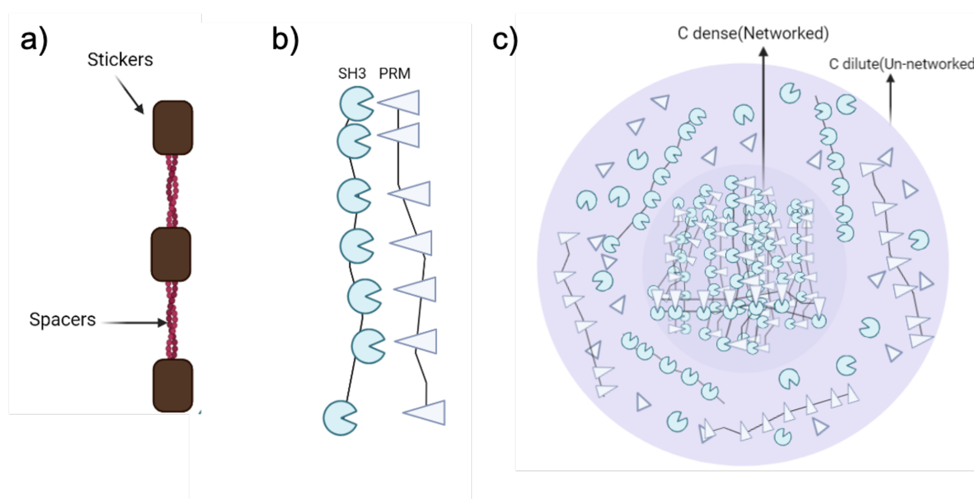

Figure 3. a) Stickers represent hotspots/domains that bind strongly to one another. Spacers are disordered regions between these stickers, acting to "space" stickers. b) SH3 and PRM domains represented in the sticker (pac-man and triangle) and spacer (lines) format. c) Graphical representation of the internal structure of an example phase-separated system composed of SH3 and PRM based on the sticker and spacer model, with c_{dilute} (un-networked) and c_{dense} (networked) phases.

RNPs and the importance of RNA in membraneless organelles

Although much previous research has focused on the role of IDP LC domains on intracellular condensation, RNA may be just as important[68] due to its anionic backbone, which could affect valency-dependent interactions with peptides or proteins. Indeed, a number of RNA-binding proteins (RBPs) phase separate in the cell upon binding with RNA, leading to membraneless organelles called ribonucleoprotein (RNP) condensates formed through a combination of hydrophobic interactions, hydrogen bonds, and electrostatic interactions.[47,57,69–71] The RNA-to-protein ratio is the key parameter for RNP assembly/disassembly, as low and high ratios tune up and down phase separation propensity, respectively.[72,73] In fact, RNA drives

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the phase separation process of certain reentrant transitions,[68] while RNA and RBPs are generally enriched in most membraneless nuclear bodies, suggesting that RNA and RBPs likely play a role in gene-regulatory functions of membraneless organelles.[74]

Numerous studies have revealed that inherent RNA characteristics are important in RNA-dependent condensates.[75,76] For example, generation of stress granules is preferentially influenced by relatively lengthy transcripts with low levels of or no translation.[77] In addition, RNA with complex secondary (or higher) structure can modulate protein aggregates to interact with proteins more frequently.[75] N6-methyladenosine modifications on RNA were even found to serve as a multivalent scaffold for the binding of YTHDF proteins, leading to phase separation.[78] Nuclear RNA concentration itself even governs RBP solubility by acting as a buffer![69] These above-mentioned parameters of RNA collectively impart unique biophysical qualities in condensates, which are necessary for condensate functions in homeostasis and are inherently encoded in RNA.

Variation of all possible interactions among RNA-protein condensate constituents (RNA-RNA, RNA-protein, protein-protein) will also result in changes in physical and structural condensate properties. For example, phase separation may be prevented by exclusion of multivalency in RNA structures, which results in weaker protein-RNA interactions, resulting in RNP droplets becoming more fluid.[79] On the other hand, abnormal accumulation of RNAs in the nucleus or cytoplasm as a result of aberrant LLPS might result in plaque formation and eventually lead to neurodegenerative diseases.[79] Timely disaggregation of these proteins and associated RNAs is thus essential to prevent and/or treat such diseases. There are some natural intracellular methods present to avoid these situations and prevent aberrant cellular RNA accumulation, such as nuclear import receptors, which chaperone RBPs, preventing phase separation.[80] Parallel therapeutic approaches to prevent aberrant cellular RNA accumulation includes addition of high concentrations of RNA, which prevents aberrant RBP LLPS by promoting strictly bimolecular RNA-RBP interactions (which inhibits phase separation) while inhibiting multivalent RNA-RBP interaction networks stretching across multiple components (which generally promotes phase separation).[81]

Although RNA participation in cellular LLPS has been well investigated, an integrative repository of these RNAs is currently lacking. For example, each disordered RBP has its own compendium of RNA binding specificities, but it is unknown both how RNA folding affects phase separation of RBPs and how multiple areas of structural complexity may combinatorially affect RNA structure and subsequent condensation.[79]

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The role of subcompartmentalization or biphasic RNA droplets,[82,83] which has been shown *in vitro*, also remains unknown. To fill these gaps, one tool that could be used are RNA databases, such as RPS, which reports RNA related to cellular phase separation.[84] However, as RPS only focuses on 20 specific condensates, further expansion of such databases is necessary.

Tumorigenesis induced by aberrant LLPS

While the typical function of biological condensates results in regulation of cellular processes, aberrant function may lead to development of various diseases or disorders such as neurodegenerative[85,86] or genetic diseases[87], including repeat expansion disorders.[88] However, one disease that is less well-known to be LLPS-mediated is cancer.[89] In particular, genetic mutations that impact DNA damage response and repair, chromatin organization, genomic stability, or transcription, influence the transformation of cells from benignancy to malignancy.[90] Specifically, malignant cells result in aberrant cellular activity such as uncontrollable proliferation, signal pathway disruption, metastasis, angiogenesis, and cancer cell evasion from growth suppressors.[90] A number of these oncogenic activities are, one way or another, driven by the dysregulation of LLPS and are critical factors in the development of cancer cells or tumors, *i.e,* tumorigenesis.[91] As a result, understanding the mechanisms of LLPS that result in these tumorigenic processes will help to demystify the complex role of LLPS in tumorigenesis, which could lead to LLPS-targeted cancer intervention treatments in the future.

It is known that aberrant LLPS disrupts signaling pathways that curb tumor growth. For example, the FERM domain protein Merlin, which is encoded by the NF2 gene, is a known tumor suppressor protein whose main function is to regulate intracellular signaling pathways and to facilitate innate immunity against cancer by minimizing oncogene expression.[92] However, when Merlin is inactivated due to promoter methylation in schwannomas, it becomes mutated in the NF2 gene and can phase separate upon binding with the interferon regulatory factor 3 protein. The resulting condensates then suppress the cGAS-STING signaling pathway (which typically inhibits tumor formation), resulting in potential tumorigenesis.[93] Cancer proliferation can also be regulated through the non receptor protein tyrosine phosphatase encoded by PTPN11, *i.e.*, SHP2, which is instrumental in regulating the RAS-MAPK signal transduction process (which itself regulates oncogenesis) and organism development in general.[94] SHP2 mutations can result in condensation *via* LLPS, leading to significant accumulation of wild-type SHP2 within the condensate itself and depleting the cell of SHP2 capable of regulating RAS-MARK, thus resulting in potential tumorigenesis.[95] Finally, while biological LLPS is typically limited to nucleic acids and proteins/peptides, other types of cellular biomolecules can phase separate, such as glycogen.[90] Glycogen typically

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accumulates in liver cells, but glucose-6-phosphatase (G6PC) hydrolyzes excess glycogen. However, in some pre-malignant cells, G6PC function is downregulated, leading to glycogen accumulation. This accumulated glycogen can phase separate, resulting in sequestration of a known tumor suppressor Mst1/2 (which inactivates the oncogene Yap in typical cellular conditions) within these condensates, thus leading to decreased suppression of cellular Yap and resulting in liver tumorigenesis (**Fig. 4**).[96]

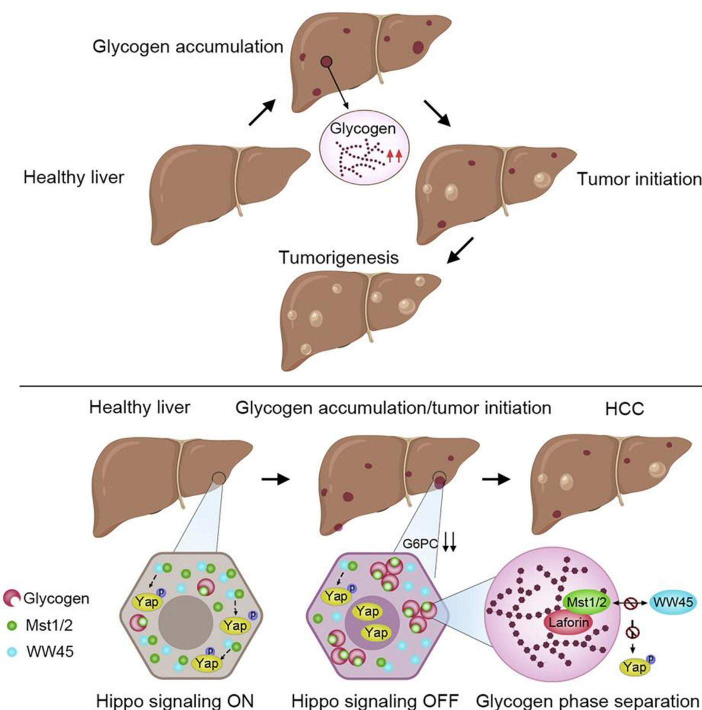

Figure 4. Glycogen accumulation and phase separation initiate liver tumorigenesis. Reprinted with permission from[96] with copyright of Cell Press.

Here, we choose to discuss only a few critical instances by which aberrant LLPS can inhibit antitumor signaling pathways, leading to tumorigenesis. However, this is just the tip of the iceberg, and a number of other mechanisms and systems likely exist. Nevertheless, there are currently some promising therapeutic strategies that can be applied to inhibit tumorigenic biomolecular condensate formation, such as normalizing the phase-separating proteins' concentrations through drug targets *via* protein post-translational modification enzymes or partitioning of small molecules which inhibit phase separation directly into condensates.[97] More studies on animal models are required to explore and expand our scope of potential anticancer drugs and medications that can moderate and control aberrant cancer-related LLPS, potentially preventing cancers early on before malignancy.

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Aspects of Primitive LLPS

Primitive biopolymer-based coacervates

The origin of life on Earth and the conditions under which it may have arisen remain a mystery, due in part to the lack of knowledge about the Earth's environment 4.2 billion years ago and the available chemicals during that time.[98,99] It is also difficult to imagine the setting of the origin of life to be in a single type of environment or location as a variety of reactions/chemical processes, such as spark discharge,[100] UV irradiation,[101] mineral catalysis,[102] or dehydration-rehydration cycles,[103] likely contributed to the emergence of early life, each of which may have been more favorable in different environmental settings.[44] Thus, the origins of life required a variety of settings:[98,99] at the bottom of the ocean floor,[104] near hydrothermal vents,[105] or in hot springs,[106] just to name a few. These chemical processes ultimately resulted in the abiotic synthesis of life's building blocks, *i.e.*, amino acids, peptides, ribose, nucleobases, fatty acids, nucleotides, and oligonucleotides,[98] as well as so-called "non-biomolecules" which are not as present in modern biology.[107] The assembly of these components into larger bio-macromolecules and supramolecular structures could have then led to emergence of key biological properties such as replication[7] or homeostasis.[108]

One key property of primitive systems that necessarily must have emerged is compartmentalization, which could contribute a variety of functions helpful for a growing/evolving primitive chemical system and could have been achieved by primitive compartments or protocells.[109,110] Ghosh and colleagues summarize the latest experiments on the characterization and utilization of membraneless coacervates as protocell models in origins of life (OOL) studies.[31] This includes the possibility that coacervates facilitated the transition from membrane-free to membrane-bound compartments, brought together RNA and peptides for co-evolution, localized prebiotic metabolites to initiate primitive metabolism, and evolved through growth and division while maintaining an out-of-equilibrium state, each of which could have been important steps in the origin of life. While there are other types of primitive membraneless protocell models, such as aqueous two-phase systems,[36] supercritical carbon dioxide,[13] and polyester microdroplets,[111–113] we focus on coacervates as membraneless protocell models.

Given that proteins are present in modern condensates, and chemically, peptides (simplistically, minimalized proteins) could have also led to primitive coacervate assembly. Peptides could have potentially been abundant on early Earth, as they could have polymerized (such as through volcanic gas,[114] mineral

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surface catalysis,[115] or dehydration synthesis[116]) from amino acid monomers that could have themselves arisen from hydrothermal synthesis,[117] spark discharge,[118] UV irradiation,[119] or meteoritic delivery[120] on(to) early Earth. The key feature of peptides used as building blocks for coacervates lies in the functional and chemical diversity of the amino acid residues available in the peptides (of course, constricted by availability on early Earth). For example, peptide formation in the cell generally results when the proteinogenic amino acids are biosynthetically incorporated into a growing chain through the translation system, but there non-proteinogenic amino acids (*e.g.*, ornithine, citrulline, gamma-aminobutyric acid, etc) also exist; these are either not incorporated into proteins or not produced directly and in isolation by standard cellular machinery.[121] However, while early peptides like contained some amount of proteinogenic amino acids, there's also the possibility (or, some would argue the likeliness, given the diversity of amino acids on early Earth[122]) of early peptide-based coacervates being composed of peptides with non-canonical amino acids formed by spontaneous abiotic synthesis. In particular, ornithine (a non-proteinogenic basic amino acid) is proposed to have been in early proteins, yet were "removed" over chemical evolution in modern proteins,[30] possibly due to the selective incorporation of proteinaceous amino acids into growing primitive peptide chains.[123] Poly-ornithine in particular has even shown the plausibility to phase separate upon binding with a synthetic anionic polymer,[124] suggesting that although many studies on peptide-based coacervates focus on those with canonical residues, that peptides with non-canonical residues may also have significant phase separation propensity and novel functions yet to be discovered.

However, a single peptide alone can not easily phase separate, and thus would need to bind to some type of counter-ion with the opposite charge in order for the phase separation process to initiate and progress. Peptides can in fact form coacervates upon binding with other peptides, as long as there are appropriate electrostatic interactions between a minimum of two peptides with differing/opposite charges.[125] On early Earth, a number of other non-peptide-based plausible types of counter-ion could have also existed, one of which may have been in the form of nucleotides or nucleic acids.[126] There has been much speculation about the existence of nucleotides on early Earth, especially through investigation of the RNA World theory of the origin of life (also plausible would be a pre-RNA[127] or an RNA-DNA[128] World), and researchers have uncovered many ways by which such nucleotides could have been synthesized, including by spark discharge,[129] on meteorites,[130] or as a result of syntheses in cyanosulfidic environments.[131] AMP, ADP, and ATP have all been observed to form in mineral pores,[132] suggesting that nucleotide mono-, di-, and tri-phosphates could have plausibly existed. Once formed, nucleotide monomers, in particular RNA

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monomers, could then potentially have polymerized through a variety of prebiotic mechanisms such as through dehydration synthesis,[133] clay mineral catalysis,[134] non-enzymatic template-directed polymerization *via* activated nucleotide monomers,[135] or even lipid-assisted polymerization.[136] It is possible that the early RNA molecules eventually could self-replicate,[137] similar to modern-day viroids, which are small, circular RNA molecules that infect plants and replicate autonomously, not using their own proteins but rather the proteins of the host for replication.[138,139] Similarly, the discovery of self-replicating RNA molecules, or replicators, that can evolve in the absence of proteins further lends to the possibility that RNA-based replicators could have given rise to the first viruses.[140]

Virus-like RNA could have facilitated the transfer of genetic material between RNA, helping to select for more efficient replicators, contributing to the continued evolution of organisms, and culminating in the DNA world.[141] For example, viral-like particles consisting of RNA molecules surrounded by a protein coat, similar to modern viruses, could have arisen spontaneously in the prebiotic environment and could have acted as intermediates in the evolution of more complex life forms; some systems using *in vitro* peptides and proteins (although not prebiotically plausible) have been demonstrated.[142] The ability of viral-like particles to transfer genetic material between RNA molecules or compartments encapsulating RNA could have also facilitated the emergence of more complex replicators and helped to select for more efficient self-replicating RNA molecules.[138] However, the role of viruses in the evolution of life is still not universally accepted. For example, it is unknown how viruses could have emerged in the absence of host cells (which are required for viral replication), which could not have existed on early Earth, although protocells could have existed and acted as primitive hosts. Additionally, it is unknown how viruses could have evolved into more complex structures, for example eventually giving rise to cells. The evolution of cells requires the development of complex cellular machinery, such as membrane-bound organelles and the cytoskeleton, which is simply not observed in viruses.[143] However, while the exact nature of their involvement in the origin of life is not yet clear, there is evidence to suggest that viruses may have played a role in the evolution of primitive RNA and the development of complex genomes.[141,144] Nevertheless, while there are still many unanswered questions regarding the plausibility of nucleic acids on early Earth, such as degradation[145] or sustained replication,[146] it is still very likely that nucleic acids emerged and were abundant at some point during the progression of early life.

Thus, with the possible existence of nucleotides, nucleic acids, peptides, and amino acids on early Earth, let us thus examine some examples of coacervates that can form from these biomolecules. An example of

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a peptide-peptide coacervate is composed of poly-lysine and poly-glutamate.[147,148] Peptide-polynucleotides and mononucleotides can also form coacervates due partially to the interaction between cationic peptide residues and the anionic nucleic acid backbone or phosphate moiety. For example, poly-L-lysine can coacervate with nucleotide monomers such as ATP[15,29] or with nucleic acid oligomers such as RNA.[149] Monomers with fewer phosphate groups/negative charges, such as AMP, have even been shown to coacervate, in this case upon binding with oligoarginine.[150] As this demonstration shows, peptide-nucleic acid coacervates are not limited to poly-L-lysine, as other peptides including oligoarginine have shown coacervation ability upon binding with nucleic acids as well.[72,151] In addition, droplets with increased structural complexity, such as multiphase droplets, may also form as a result of coacervation between two or more components such as certain cationic polypeptides and nucleotides or nucleic acids (both RNA or single-stranded DNA).[82,152] Higher order structures, such as liquid crystals, may also co-assemble with coacervates upon reaching the proper conditions, *i.e.*, binding of peptides with high concentrations of liquid crystal-forming DNA duplexes.[16,54,153]

Additionally, other than peptides, other types of primitive positively charged polymers or molecules could have also formed coacervates upon binding with nucleic acids or nucleotides. For example, one class of polycation that is of interest are polyamines, which are polymers containing multiple amino groups that have the capacity to be positively charged, and have been shown to condense certain nucleic acids in biology.[154] In particular, putrescine, a simple polyamine with four carbon atoms and two terminal amines (two potential positive charges) has been synthesized under prebiotically plausible conditions.[155] Putrescine is a biochemical precursor of more complex polyamines, such as spermidine (three amines) and spermine (four amines),[156] but to date, no prebiotically plausible syntheses of either has been demonstrated, although it is proposed that any prebiotic syntheses of spermidine or spermine would likely require putrescine as a reactant or intermediate.[155] As short polyamine-nucleic acid coacervation has been demonstrated, such as spermine/spermidine-RNA[157,158] coacervates, assuming spermine or spermidine could have been synthesized at some point during the origins of life, such coacervates could have existed on early Earth. Furthermore, at some point if longer polyamines could also be synthesized, longer polyamine-based coacervates could have provided even further function, such as up-concentration of analytes in polyamine-nucleotide coacervates[21] or increasing catalytic ribozyme reaction rates within polyamine-anionic peptide coacervates,[159] to a primitive system.

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How the early Earth environment might have affected compartmentalization and LLPS

To understand how coacervates and other phase separated compartments could have formed and evolved on primitive Earth, one must take into consideration the environmental parameters and how such parameters could have influenced the chemicals on primitive Earth to initiate reactions that would have lead to primitive phase separation (and those that reacted within those compartments).There are many different factors relevant in early Earth environments that could affect the formation of coacervates, such as variations in temperature, pH, solvent, or salinity, just to name a few.[44] At the molecular level, for example, peptides can become insoluble or inactive when the pH,[160] temperature,[161] solvent environment,[162] or salinity[163] changes, sometimes leading to the formation of an amorphous solid precipitate that becomes unable to interact with counterions to form coacervates. Similarly, RNA or other nucleic acids are also very sensitive to environmental conditions, often denaturing or degrading at high temperatures,[164] divalent cation concentrations,[145] or pH.[165]

At the compartment level, temperature could affect coacervation, as certain systems show coacervation ability only above a specific temperature, while others only coacervate below a specific temperature. For example, in systems composed of lysine-rich elastin-like polypeptide (ELP) and hyaluronic acid, coacervation occurs only when the temperature rises above 25–30 degrees C, *i.e.*, the lower critical solution temperature (LCST).[166] However, when protamine, an arginine-rich polypeptide, is mixed with either citrate or triphosphate, coacervation can only occur below 40–60 degrees C, *i.e.*, the upper critical solution temperature (UCST).[167] Additionally, coacervates are stable at a specific pH range, as changes in pH affects the charges of the polymers comprising the coacervates, resulting in inhibition of the electrostatic forces needed to maintain phase separation.[15] In one case, when a coacervate itself is dissolved in water, only the shorter peptides selectively self-coacervate into stable liquid droplets when either the temperature or the pH rises above the optimum value, leading to changes in the droplet structure.[168] While this environmentally induced structural change could result in emergent structures or function, it may also go the opposite way and result in inactivation of any coacervate functions.

Nevertheless, although it seems daunting to find real suitable primitive environments that could have sustained coacervate assembly and function, there are studies which have indeed found suitable candidates. For example, researchers discovered that gas bubbles within heated rock pores have the potential to initiate reactions and form membraneless coacervate microdroplets, offering a direct plausible scenario for the emergence of such membraneless protocells on Early Earth.[169] The study showed that the accumulation of

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components at the gas-water interface of a gas bubble leads to growth and fusion of the coacervate microdroplets, which were also observed to divide and fragment, representing a mechanism for growth and division. The thermal gradient in the environment also played a role in driving the evolution of protocells, as it resulted in the formation of different types of protocells with various chemical compositions, sizes and physical properties.[169] However, this is but one study, and more studies should take inspiration and explore more "habitats" and conditions that may have supported the emergence of membraneless protocells on early Earth. Additionally, while there are a number of studies which examine primitive coacervate function,[31,170] more work must be done in understanding how coacervates could transition from passive to active compartments, and later to active cells. For example, can coacervates convert external energy to force themselves out of equilibrium, resulting in further emergent functions similar to how electric fields drive coacervates to display some activity/function including life-like behaviors such as active growth, motion, and replication?[171] Similarly, is there a way that active droplets could divide through modulation of surface tension[19] like that observed in recent work on chemical fuels regulating different aspects of primitive coacervation?[172]

Comparing Primitive LLPS with Modern LLPS

Proteinoids, short peptides formed by primitive Earth-relevant conditions, can also form coacervate-like microspheres.[173,174] Such proteinoids are both of low complexity, mostly containing charged amino acid (AA) residues, and also hydrophilic, similar to modern IDPs. Therefore, it has been hypothesized that proteinoids may have been precursors of IDPs, and primordial IDPs could have also formed microsphere-like structures.[175] We thus explore the possibility of primitive disordered proteins or peptides forming coacervates at the origin of life by comparing membraneless organelle forming modern IDPs to primitive peptides on the basis of AA composition and length.

Comparison of AA composition

Not all canonical AAs originated and evolved at the same time. AAs with complex chemical structures, such as larger ones or those with ring-containing chains like tryptophan and other aromatic AAs, are postulated to have evolved later.[176,177] On the other hand, bioinformatics analysis suggests that aromatic AAs promote ordered structure formation and folding in proteins,[178] while aromatic AAs are present in limited proportions in IDPs.[179] IDPs and LC domains instead contain a large fraction of charged AAs[52] and internal repulsion from AA residues of like charges contained within the same chain makes them disordered.[49] Therefore, the late evolution of aromatic AAs on early Earth that established an ordered

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structural configuration in proteins indicates that IDPs might have originated first, before folded proteins.

As LC domains contain a large number of charged residues, phase separation is necessarily induced by charged residues or charged regions (as discussed previously). For example, LC domains and disordered positively charged RGG (Arginine-Glycine-Glycine) repeats are present in many RBPs.[180] P granules in *C. elegans* are formed by the LAF-1 RBP *in vivo.* RGG repeat regions present in LAF-1 that can be separately isolated from the entire protein can phase separate *in vitro*, suggesting that the entire LAF-1 protein is not an *ad hoc* requirement for phase separation.[181] Thus, RGG domains from LAF-1 have been modified to engineer switchable phase-separated reversible compartmentalization systems *in vitro* that can assemble/disassemble and recruit/release biomolecules with the help of specific biochemical reactions.[182] Similarly, CIRBP (an RBP) recombinants containing only the RGG domain can also phase separate upon RNA binding without the necessity of the entire protein *in vitro*.[183] These RGG repeats are also present in FUS family proteins and are critical for the formation of stress granules.[184] Hybrids containing LC domains of FUS (containing the RGG domains) as stickers and dextran polymers as spacers (replicating the sticker-spacer arrangement of modern IDPs) can also phase separate *in vitro*,[185] suggesting the importance of the RGG motif in phase separation. In fact, RGG domains are highly conserved,[186] and have been hypothesized to have played roles in primitive RNA metabolism and folding.[187] Specific AA sequences are also required for proper folding and structure in structured proteins. Abiotic, potentially error-prone synthesis of peptides during the OOL could have led to the random, non-specific addition of AAs in primitive peptides, potentially lead to the formation of unfolded disordered peptides on early Earth containing domains like RGG.[188] As RGG domains themselves can potentially phase separate *in vitro*, the formation of primitive coacervates composed of primordial short-disordered RGG and/or RGG-containing peptides bound to other biomolecules (like primitive nucleic acids) is certainly plausible.

Like RGG domains in FUS proteins and LAF-1, repetitively charged homopeptides like polylysine (poly-K), poly-glutamate (poly-E), and poly-arginine (poly-R) are all unstructured,[189] have low complexity, and contribute IDP phase separation.[180,190] Some of these homopeptides can form phase-separated systems with RNA or with each other *in vitro* and therefore, have been associated with the formation of coacervates on primitive Earth. For example, poly-K-ATP coacervates can encapsulate primitive biochemical reactions and biomolecules[15] while reversibly assembling and disassembling.[191] pH[192] and temperature-mediated[193] reversible compartmentalization of poly-K-ATP coacervates inside of liposomes represents a membrane-bound protocell model containing functional coacervates, while poly-K and DNA liquid crystal

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coacervates have been hypothesized to aid in the evolution of prebiotic peptides and nucleic acids through multiple structural and functional complexity increasing prebiotic pathways.[16] Poly-R has also been shown to form coacervates upon binding with nucleotides *in vitro*. poly-R-containing coacervates are more viscous than poly-K-containing coacervates, leading to the formation of a multiphase coacervate with a polyR core and a polyK shell.[194] Formation of such multi-phase systems[82,83,152] using simple homopeptides hint towards a possibility that such peptides could have paved the way towards the formation of coacervates of increasing complexity, thus bridging the gap between primitive and modern phase separation. Moreover, arginine is hypothesized to have formed even before lysine and could be considered to be the first canonical basic AA that originated on early Earth.[131,195,196] Other than poly-K homopeptides, anionic homopeptides such as poly-aspartate (poly-D) and poly-E have also been used to produce prebiotically relevant coacervates *in vitro*.[197] Thus, the ability of charged disordered homopeptides to form *in vitro* coacervates strengthens the hypothesis that primitive disordered peptides could have given rise to primitive coacervates on prebiotic Earth. However, rather than K, E, and R, G and alanine (A) homopeptides have been assumed to be the most abundant in the earliest peptides that formed on early earth.[198] Thus, rather than homopeptides, coacervation of simple heteropeptides containing charged residues as well as G and A should also be considered as a mechanism of primitive phase separation, and the order/disorder of such peptides should also be investigated.

Non-canonical AAs could also have been present on early Earth and contributed to primitive phase separation as well. For example, ornithine (O), a precursor of arginine,[30] is a non-canonical abiotically produced AA that participates in the urea cycle,[199] but is generally absent in modern proteins;[30] it is postulated that the sequence of emergence of the three basic AAs on early Earth is as follows: Ornithine->Arginine->Lysine. In one study, a DNA binding protein motif, Helix-hairpin-helix (HhH) was ancestrally reconstructed to two primordial protein forms by replacing all lysine residues with O and R, respectively. This reconstructed primordial ornithine-containing motif (pre-O) formed coacervates with poly-uridylic acid (polyU), albeit not as effectively as the reconstructed primitive arginine-containing motif (pre-R). Pre-O was found to be extremely unstructured and disordered, compared to pre-R. Progressive chemical conversion of ornithine residues to arginine led to an increase in DNA binding and folding of the resultant protein each time after conversion, suggesting an increase in structural conformation.[30] This study is one of the only examples that primitive proteins, such as those containing O, could have been more disordered,[188] while structural, globular,[200,201] and folded[202] proteins, such as those containing arginine, evolved from O-containing proteins. Therefore, the formation of primitive membraneless protocells with

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primordial disordered peptides composed of non-canonical AAs like ornithine might have been possible (or even likely) before the emergence of canonical AAs like arginine or lysine, although eventually lysine and arginine would have replaced ornithine in biology.[123]

Finally, while aromatic AAs are limited in number within IDPs,[52] their role in the functionality and phase separation of modern IDPs cannot be ignored. For example, weak non-covalent interactions (cation–π and sp2/π) between tyrosine and arginine residues within an IDP can result in phase separation; induced mutations of tyrosine residues within the IDPs have been shown to affect this ability.[203,204] Such weak interactions are also required for a synthetic mimic of FUS protein[185] and RGG domains of LAF-1 to phase separate *in vitro*.[205] Tyrosine and histidine interactions have also been shown to be important for phase separation, while hydrophobic interactions of phenylalanine can strengthen charge-charge intermolecular interactions that result in phase separation as well. In particular, phenylalanine present in FF dipeptide motifs can produce amyloid-like hydrophobic supramolecular assemblies.[206] A recently published study involving aromatic dipeptides joined by hydrophilic AAs that formed coacervates has even demonstrated an increase in the rate of reactions that are prebiotically relevant.[168] However, unlike in modern LLPS systems, the role of aromatic hydrophobic AAs in primitive coacervate formation remains to be explored in-depth. One reason may be that aromatic AAs evolved later than other AAs, and so aromatic residues have rarely been a part of primitive coacervates, potentially leading some primitive coacervate researchers to eschew the inclusion of large amounts of aromatic residues in their systems.[31] However, recent research has pointed to the fact that basic AA residues may have been incorporated into primitive peptides much later than initially thought.[207] This suggests that the role of aromatic AAs and their interactions with other AAs that contribute to primitive phase separation warrants further investigation.

Comparison of peptide length

Peptide length is also an important factor for the formation of ordered structural proteins. Structural water-soluble proteins have a hydrophobic interior and a hydrophobic exterior, resulting in a rigid configuration and fixed shape, which is only possible when a protein is sufficiently long.[208] This may not have been possible on early Earth, as shorter peptides were more abundant.[209] However, it is not impossible for some long peptides to have formed on early Earth, such as abiotically on mineral surfaces, although their ability to fold into a stable structure is still unclear.[210] Nevertheless, it is much more likely that functional short disordered proteins might have emerged before longer ones,[188] while longer structural proteins are thought to have evolved later from these short primitive disordered proteins.[200,201] Given that long-folded proteins

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are not required for phase separation, as short RGG domains of IDPs in isolation.[181,183] can coacervate *in vitro* upon RNA binding, it is relevant to further examine shorter proteins and peptides with respect to both primitive and modern phase separation, in hopes of connecting the two.

Indeed, shorter prebiotically relevant peptides can also form coacervates, some of which can accumulate RNA and maintain a distinct pH more efficiently than coacervates formed by their larger counterparts.[197] Applications of coacervates composed of short peptides have also been found beyond OOL studies. For example, adhesive coacervates composed of short peptides and polyoxometallates have been developed to form an underwater adhesive compound on-site,[211] suggesting that potential synergies between OOL and applied research should be further explored. Finally, the complexity of modern coacervates and membraneless organelles makes it difficult to find quantitative similarities between modern and primitive phase separation. Therefore, minimalistic approaches to determine the shortest peptides capable of phase separation have also been developed. For example, a computational study of all 400 canonical dipeptides showed that peptides with medium aggregation capabilities can phase separate, and suggested that phase separation is a general property of peptides and proteins and does not depend on sequence length.[212] Short synthetic peptides composed of two dipeptide stickers joined by a hydrophilic spacer, resembling the modern IDP sticker-spacer arrangement, self-coacervated and even enhanced the rate of prebiotically relevant anabolic reactions.[168] Although the spacers used in this study may not have been prebiotically relevant, the fact that a polymer containing simple dipeptides could achieve such complex phase separation and function can help to support the theory that large polymeric molecules are not necessarily required for LLPS, indicating the plausibility of phase separation on early Earth from more-abundant short peptides. These are examples of bottom-up approaches which can further be applied to elucidate how simple coacervates of minimum complexity formed and how they gave rise to modern cells/modern membraneless organelles of higher complexity. By combining such bottom-up approaches with approaches to minimize sequence-length of modern IDPs, we may be able to reconcile and more closely connect modern with primitive phase separation.

Conclusion and Prospective

Was a membrane strictly required at the origins of life?

After having reviewed and investigated a number of different aspects of primitive and modern LLPS, a clear question that remains to be answered is: What is the relevance of primitive LLPS to modern cells? In particular, one major difference between LLPS and modern cells is the existence of a lipid bilayer

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membrane, which offers stability to modern cells and sustains a complex, out-of-equilibrium chemical network inside the cells, while simultaneously preventing the dilution of its contents to the surrounding environment, among other life-sustaining functions. Such functions are crucial to cell survivability, and thus it is likely that such a membrane is a prerequisite of biology. So, perhaps membraneless primitive LLPS eventually acquired a membrane at some point in the history of evolution?

Protocells are theoretical or experimental models that consist of a compartment that separates chemical reactions taking place inside it, separated from the rest of the environment. They are studied to decipher the enigmatic origin of the first cells and life on Earth. Like cells, they are generally accepted to have been bound by a lipid bilayer membrane at some point in history. Such a membrane is semi-permeable and only allows hydrophobic molecules to pass through and enter the compartment, thereby allowing stable compartmentalization of important genetic or catalytic molecules within an evolving protocell. However, membranes could also project a barrier in exchange of certain nutrients and ions, especially for polar biomolecules like primordial nucleic acids, peptides, minerals, ions, *etc*.[213] Moreover, it is thought that conditions conducive to the formation of lipids as chemically complex as phospholipids did not exist on early Earth, as complex biological reactions, non-existent at that time, are needed to synthesize such chemically complex molecules.[214] Thus, it has been proposed that primitive cell membranes were composed of simpler amphiphiles such as fatty acids/alcohols more abundant on primitive Earth (likely due to the simplicity in their abiotic synthesis). Fatty acid membranes have increased lipid dynamics,[215] and the presence of a lower number of acyl chains has been shown to increase membrane permeability.[216] Thus, membranes formed by fatty acids containing single acyl chains are more permeable to polar molecules than membranes composed of phospholipids that contain two acyl chains. Higher permeability of primitive fatty acid membranes can result in the more potential exchange of life-relevant molecules between the interior and the exterior of the cell, leading to the chemical evolution of metabolic networks or genetic polymers inside the cell; however, too much permeability may result in diffusion/loss of encapsulated catalytic or genetic materials, suggesting that a balance is required. Although fatty acid monomers could potentially be synthesized on early Earth, the formation of fatty acid vesicles requires specific conditions of pH, salt and fatty acid concentrations, which may have been challenging in certain environments on primitive Earth.[31] Therefore, if complex lipids could not have been synthesized to a significant extent and simple amphiphiles like fatty acids could not have adequately assembled into vesicles on early Earth due to suboptimal environmental synthetic conditions, then one must consider other types of

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compartments that could have formed on early Earth before the advent of membrane-based compartments, such as membraneless compartments, which could have eventually gained a membrane.

Chemical evolution in/of membraneless protocells

Semi-permeable phospholipid membranes provide selective permeability of molecules inside the cell, and also inside membraneless organelles in modern cells. However, phase-separated systems like coacervates[21,29] and membraneless organelles[217] also have selective partitioning activity without a membrane due to a clear chemical boundary between the condensed interior and the dilute exterior. The partitioning property leads to the maintenance of an out-of-equilibrium state inside the coacervate, resulting in the emergence of chemical gradients (facilitating the movement of molecules from their interior to exterior and driving electrochemical processes) in coacervates and also enabling control of different encapsulated biochemical reactions, similar to both modern cells and membrane-bound protocells.[31] Inside coacervates, selective partitioning of molecules has also been shown both to accelerate the rate of reactions by concentrating solutes and reactants and to inhibit reactions by selectively compartmentalizing enzymatic/reaction substrates and separating them from each other;[168] similar chemical processes also take place in membraneless organelles.[217]

Coacervates can also assemble and disassemble readily and completely in the presence or absence of stimuli like pH,[192] temperature,[166,167] etc. Assembly of coacervates themselves can promote/inhibit certain reactions, and the disassembly of these coacervates will lead to a complete reversal of the reaction kinetics. Stimuli-driven coacervates are quite similar to modern stress granules in terms of how they assemble/disassemble. Stress granules are formed when cells are subjected to extremes of pH, temperature, ionic stress, etc. to protect cellular proteins and RNA from degradation and prevent the formation of misfolded proteins, which could lead to aberrant cellular function. When these environmental stresses are not present, the stress granules are disassembled. The assembly and disassembly of stress granules are controlled by the phosphorylation/dephosphorylation of translation-associated proteins in response to external stresses. eIF2a, a protein complex involved in translation initiation, is phosphorylated by eIF2α kinase. This prevents the initiation of translation and leads to the encapsulation of RBPs, ribosomal subunits, translation initiation factors and translationally arrested mRNAs into stress granules. A decrease in stress leads to the dephosphorylation of eIF2α, which leads to the disassembly of stress granules and the resumption of the initiation of translation.[218]

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Similar to the assembly and disassembly mechanism of stress granules, some simple *in vitro* coacervates have been synthesized which can assemble and disassemble due to phosphorylation and dephosphorylation, respectively. A study showed that phosphorylation and dephosphorylation of serines in a peptide sequence that formed a particular type of coacervate led to the assembly and disassembly of the coacervate, respectively.[72] Peptide/nucleotide coacervates which could be assembled by phosphorylating ADP to ATP and vice-versa were also synthesized.[191] However, the fact remains that *in vitro* coacervates are much simpler in function and composition as compared to membraneless organelles, and these studies investigated only direct phosphorylation/dephosphorylation- they do not analyze whether these reactions take place as a result of changes in environmental stress factors. Nevertheless, the fact that simple *in vitro* coacervates can be regulated by enzymatic reactions that also regulate stress granule assembly/disassembly during stress hints towards the possibility that the assembly/disassembly of primitive coacervates could have been regulated by phosphorylation/dephosphorylation type reactions (possibly non-enzymatic as well). In particular, non-enzymatic phosphorylation has been shown to be thermodynamically feasible in prebiotic Earth conditions,[219] while phosphorylation of molecules has been shown to occur abiotically in phase separated aqueous microdroplets.[220] This suggests that phosphorylative control of primitive coacervate assembly/assembly could have been possible *via* primitive environmental fluctuations, which themselves could have allowed components encapsulated within coacervates to access cyclical changes in environmental conditions (*i.e.*, stressors). Thus, assembly/disassembly could lead to chemical evolution even in membraneless compartments.

These properties of membraneless protocells thus show their ability to facilitate early chemical reactions and evolution. However, at first inspection, one may assume that membraneless protocells can do all of the things that membrane-bound protocells can, there may be certain properties in which membraneless protocells were less proficient at than membrane-bound ones (such as generating stronger chemical gradients due to a physical membrane vs. a simple boundary), necessitating the eventual emergence of membrane-bound compartments during the emergence of early life.

Can coacervates give rise to membrane-bound cells?

Thus, although membranes might not have been a strict requirement at the very early stages of the origin of life, they were certainly important to maintain and sustain it at some point during life's development, as membranes may be more effective at certain essential primitive functions. For example, as chemical evolution within non-membrane-bound compartments leads to the formation of more complex chemical

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networks, it became more important to separate the concentrated internal milieu from the dilute exterior, maintain gradients between inside and outside, and to generate energy. For example, ATP synthase, a molecular machine embedded in membranes of modern cells, is responsible for the synthesis of ATP in the form of energy.[221] If some simple cross-boundary ATP-generating machinery were required in primitive cells, then the chemical gradient required for ATP generation might have been larger in membrane-bound components.

Additionally, could coacervate systems have somehow contributed to the emergence of a fatty acid-bound membrane as a mechanism to transition from membraneless to membrane-bound protocells? In particular, coacervate systems containing fatty acid monomers that can readily assemble and form a fatty acid membrane outside the coacervate when a specific pH[222] or ionic strength[223] is present have been developed. Other membraneless protocell models (in this case, a poly(ethylene glycol)-dextran aqueous two-phase system) have shown the capability to host fatty acid membranes as well.[29] Further efforts have also been taken to study the plausibility of phospholipid-bound (which would have evolved from fatty acids at some point in the origin of life) membraneless compartments.[224,225] In fact, "membranization" of coacervates, that has led to coacervates with non-lipid-based membranes,[226] such as yeast cell wall fragments,[227] is another avenue of current research relevant to understanding the emergence of membranes on early Earth. Therefore, although coacervates lack membranes, the conversion of membrane-free to membrane-bound compartmentalization could have occurred through the scaffolding of lipid-based membranes by a primitive coacervate-based "cytoplasm".

Viruses and Primitive Coacervates

While we have discussed the connection between primitive coacervates and both membraneless organelles as well as as primitive cytoplasms, other types of modern biological structures could also be relevant to understanding primitive coacervates. One in particular are viruses, which we have discussed above in the context of their contribution to primitive nucleic acid-based systems and evolution (*i.e.*, the RNA and DNA worlds). There are three generally accepted hypotheses on the origins of viruses themselves:[144,228] regressive, escape theory,[144] and virus-first.[144,229] While we do not go into detail into these theories (please check the cited articles for more details), we do note that viruses are thought to be one of the earliest organisms to inhabit life on Earth, possibly predating the emergence of cells.[230–232]

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However, there is still more to be gleaned about viral origins and their role in the origins of life. For example, one current mystery is that viruses and extracellular vesicles (a modern, non-replicating biological compartment) have a number of known overlapping points, as viruses can use the same endocytic pathway as extracellular vesicles,[233] while extracellular vesicles can even contain proteins and genetic materials of viruses.[234] Thus, there may be some historical connection between viruses and simple compartments such as extracellular vesicles, which suggests that if viruses existed on early Earth, then they could have interacted with contemporaneous primitive compartments, including both vesicles and/or coacervates. In particular, the ability of viruses to transfer genetic material between cells and their potential to influence the evolution of organisms has led to speculation about their possible involvement in the evolution of life, especially with respect to the RNA world as described above.[228,235] Furthermore, viruses have played a crucial role in the evolution of cells and organisms,[236] with the first cells possibly emerging through a process of symbiosis, as viruses potentially provided the genetic diversity and innovation necessary for the emergence of new functions and structures in primitive systems.[228]

Thus, viruses may also have been capable of interacting with primitive compartments through the exchange of genetic material.[235] Given that primitive coacervates may have allowed facile exchange of encapsulated genetic components, it is plausible that horizontal gene transfer between primitive coacervates and viruses could have taken place (similar to that observed between viruses and eukaryotes[237] or even LUCA[238]), potentially providing a possible input for coacervates to adapt to the surrounding environment and fostering coacervate evolution. However, very few studies linking viruses with primitive compartments have taken place, save for a small number of examples with synthetic protocells.[239–241] Thus, more studies elucidating the interaction between primitive compartments (not only coacervates, but other systems as well) and viruses are needed. As a starting point, we note a number of facts which point to the plausibility of viral-coacervate connections. First, viruses can be encapsulated within coacervates, resulting in their longer term viability and delivery in biotechnological applications.[242,243] This suggests that primitive coacervates could have compartmentalized primitive viruses, possibly for protective purposes. Next, LLPS of viral proteins and other components within the cytoplasm of infected cells supports viral replication.[244–246] In fact, assembly of certain viruses within infected cells must occur within phase separated compartments,[247] suggesting that primitive coacervates could have facilitated primitive viral assembly. Finally, viruses even possess IDPs, which themselves may be able to phase separate;[248,249] the connection of viral IDPs (which could be more ancient in origin) to other known IDPs within membraneless organelles could reveal more about the composition of primitive IDPs and coacervates.

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Future Prospects in Primitive LLPS Research

This review attempts to bridge the gap between primitive and modern LLPS systems, although the gap still remains large (**Fig. 5**). For example, even though bottom-up approaches to study the formation of primitive coacervates exist, primitive phase separation has been rarely explored in the context of primitive disordered peptides, while coacervates formed using bottom-up approaches have hardly been compared with membraneless organelles in modern cells. Similarly, studies involving synthetic proteins mimicking modern IDPs that form *in vitro* coacervates are rarely correlated with disordered peptides that formed primitive LLPS systems on early Earth. Therefore, computational and experimental studies to explore these aspects along with other similarities/dissimilarities of primitive coacervates with modern membraneless organelles are necessary to connect primitive phase-separation with modern biology. In the context of OOL, ancestral reconstruction of ancient proteins is an emerging field that can help researchers understand primitive Earth conditions and the origin of the structure/function of modern proteins.[250]Ancestral reconstruction of primitive LLPS-forming IDPs to produce prebiotic pre-LUCA disordered proteins that can phase separate is one approach that can be beneficial to explore this idea further. More closely comparing primitive and modern LLPS in such a systematic way may empower researchers to understand both these systems at greater depth, not only providing further answers on the mechanism both of life's origins and essential cellular processes, but also potentially facilitating an increase in the applicability of such systems in biotechnology, nanotechnology, and nanotherapeutics.

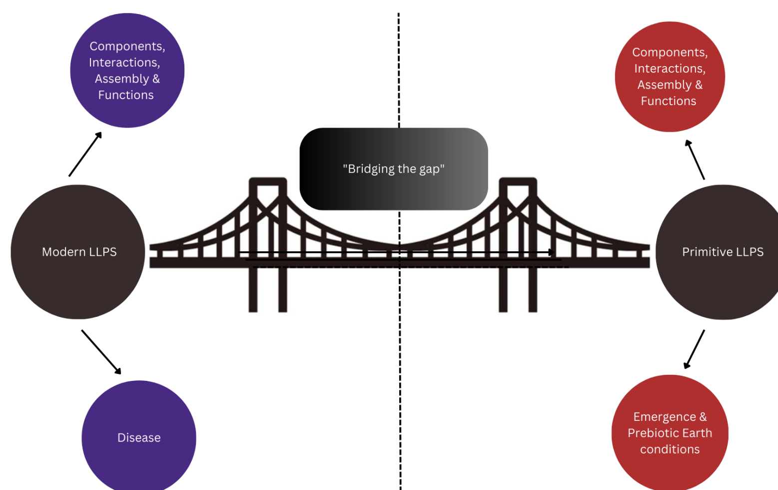

Figure 5. Bridging the gap between modern and primitive LLPS.

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Author Contributions

All authors wrote the manuscript.

Conflicts of Interest

No conflicts of interest.

Data Availability

No new data was generated.

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