Trends in
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Review

Let's phase it: viruses are master architects of biomolecular condensates

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Viruses compartmentalize their replication and assembly machinery to both evade detection and concentrate the viral proteins and nucleic acids necessary for genome replication and virion production. Accumulating evidence suggests that diverse RNA and DNA viruses form replication organelles and nucleocapsid assembly sites using phase separation. In general, the biogenesis of these compartments is regulated by two types of viral protein, collectively known as antiterminators and nucleocapsid proteins, respectively. Herein, we discuss how RNA viruses establish replication organelles and nucleocapsid assembly sites, and the evidence that these compartments form through phase separation. While this review focuses on RNA viruses, accumulating evidence suggests that all viruses rely on phase separation and form biomolecular condensates important for completing the infectious cycle.

Highlights

Recent insights from biophysics have revealed that viruses harness phase separation to mediate replication organelle biogenesis and nucleocapsid assembly.

Replication organelle biogenesis is mediated by viral antiterminator proteins (sometimes with the aid of an additional phosphoprotein), which bind to, and condense, the viral RNA.

Nucleocapsid assembly is mediated by nucleocapsid proteins, which bind to, and condense, the viral RNA within the virion.

Across RNA viruses, the phosphoprotein, antiterminator, and nucleocapsid activities may be provided by a single modular protein or several distinct viral proteins.

Collaborations between virologists and biophysicists promise to accelerate discovery in both fields.

A new phase in molecular virology

Viruses are obligate infectious intracellular parasites. For successful viral infection, all viruses must express viral proteins, which orchestrate viral genome replication and the production of progeny infectious viral particles. To accomplish this, viruses concentrate their replication and assembly machinery within specialized compartments, hereon referred to as **replication organelles** (see Glossary) and **nucleocapsid** assembly sites, respectively. Viruses are grouped using the **Baltimore Classification System**, which classifies viruses according to their path to making mRNA [1]. This takes into consideration the nucleic acid content of their genome (i.e., DNA or RNA), whether their genome is single- or double-stranded (ssRNA and dsRNA, respectively), as well as the polarity of ssRNA (i.e., positive- or negative-sense). While we focus here on RNA viruses, including positive-sense RNA, negative-sense RNA, and dsRNA viruses, the concepts discussed herein can also be applied to the life cycles of DNA and retroviruses.

In negative-sense RNA (as well as dsRNA) viruses, these specialized compartments are typically cytoplasmic **membraneless organelles**, commonly referred to as inclusion bodies [2–6]. In many cases, these inclusion bodies are used as histological proof of infection, and recent studies in members of the cytoplasmic-replicating *Rhabdoviridae* (rabies virus and vesicular stomatitis virus), *Filoviridae* (Ebola virus), *Pneumoviridae* (respiratory syncytial virus), *Paramyxoviridae* (Nipah virus and measles virus), and *Reoviridae* (rotavirus) or the nuclear-replicating *Orthomyxoviridae* (influenza virus) suggest that viral RNA synthesis, as well as nucleocapsid assembly, occurs at these sites [2–13]. As such, inclusion bodies can be regarded as viral replication organelles or factories [2,4–6,8–11,13–15]. By contrast, for positive-sense RNA viruses, both replication organelles and nucleocapsid assembly sites are intimately associated with modified cellular membranes [16,17].

For both negative- and positive-sense RNA viruses, accumulating evidence suggests that replication organelles and nucleocapsid assembly sites are formed via **phase separation**, mediated by viral **antiterminator** or nucleocapsid proteins, respectively. In this review, we introduce

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virologists to the concepts of phase separation and **biomolecular condensates** and, in turn, provide biophysicists with tractable examples from the viral life cycle that present opportunities to test and further develop quantitative models of phase separation. First, we define biomolecular condensates and phase separation, describe the key molecular features of proteins and RNA molecules that participate in these processes, and discuss the diverse functions of biomolecular condensates. We then discuss antiterminator and nucleocapsid proteins, describing the key features and functions of these viral proteins and summarize the evidence to date that suggests that viral replication organelle biogenesis and genome packaging are mediated by phase separation.

Biomolecular condensates

Cells contain a variety of specialized compartments that organize physiological processes in space and time, coordinating the efficient flow of energy and information. While both membrane-bound and membraneless organelles were first described during the 1830s [18–20], renewed interest in the latter has exploded over the past decade (reviewed in [21–23]). The current renaissance was sparked by breakthroughs in our understanding of the physical mechanisms and molecular interactions that govern membraneless organelles [24–27]. This diverse set of compartments, now referred to as biomolecular condensates, includes nucleoli, Cajal bodies, germ (P) granules, stress granules, processing bodies, and signaling compartments.

Phase separation

Biomolecular condensates maintain a local concentration of specific macromolecules in the absence of an enclosing membrane [22]. In many cases, condensates form through a process called 'phase separation', in which the cytoplasm or nucleoplasm separates into two (or more) coexisting phases: a dense phase that is enriched for certain proteins and nucleic acids and a dilute phase that is depleted of those molecules (reviewed in [21–23]).

Phase separation, and, ultimately, the composition and material properties of the dense phase (i.e., the condensate), is determined by the number and strength of intermolecular interactions among components (Figure 1). If they are few and weak, then the molecules remain dissolved in the cellular milieu and phase separation does not occur. However, if they are intermediate in number and strength, then phase separation generates a liquid-like dense phase. These condensates are often characterized by rapid condensation and dissolution; internal rearrangement and external exchange of components; and an ability to fuse and relax into spherical droplets. If the intermolecular interactions are numerous and strong, then phase separation results in a solid-like dense phase, which maintains its shape, resists deformation, and lacks molecular rearrangement and exchange. Here, we use the general term 'phase separation', rather than the commonly used 'liquid–liquid phase separation', to encompass not only solid-like condensates, but also alternative physical mechanisms, such as polymer–polymer phase separation, microphase separation, and gelation, which can each underly the assembly of biomolecular condensates [28–31].

Intermolecular interactions depend on the intrinsic properties of the molecules themselves (discussed in the next section), as well as on the local environment. As such, phase separation can be induced, or suppressed, by changes in temperature, pH, salt concentration, or other chemical stimuli [21–23]. Accordingly, phase separation provides a mechanism by which cells can respond and adapt to environmental conditions.

Features of proteins and RNAs that undergo intracellular phase separation

A central feature of phase-separating molecules is their **multivalency**, or their ability to form multiple weak intermolecular interactions; simply put, these molecules tend to be 'sticky' [21–23,32]. A major class of proteins that are known to phase separate are the RNA-binding

Glossary

Antiterminator: proteins that bind to the viral genome and condense it into a replication organelle. This is an expansion of their traditional definition as viral proteins that prevent transcription termination of the viral RdRp. As described herein, the latter is typically accomplished through interactions with the RdRp, viral RNA, and, in some cases, an additional phosphoprotein, and is at least partially achieved through phase separation of viral proteins and RNA.

Baltimore Classification System: system used to classify viruses based on their path to mRNA. Seven Baltimore groups are described based on whether their genome is DNA or RNA, single-stranded (ss) or double-stranded (ds), and the polarity of their RNA (positive- or negative-sense): (i) dsDNA; (ii) ssDNA; (iii) dsRNA; (iv) (+)ssRNA; (v) (–)ssRNA; (vi) ssRNA with a DNA intermediate in its life cycle [ssRNA-reverse transcriptase (RT)]; and (vii) dsDNA with an RNA intermediate in their life cycle (dsDNA-RT).

Biomolecular condensates: intracellular compartments that maintain a high local concentration of specific molecules, such as proteins and nucleic acids, in the absence of a delimiting membrane. The term is often used synonymously with 'liquid–liquid phase separation' but is in fact agnostic to the underlying mechanism of assembly.

Capsid: from the Latin *capsa*, meaning case or box; refers to the protein coat or shell surrounding the viral genome.

Envelope: host cell-derived lipid bilayer decorated with the viral glycoprotein(s) that forms the outermost layer of many viruses.

Intrinsically disordered regions (IDRs): regions of proteins that lack a fixed or ordered 3D structure, typically in the absence of interaction partners, such as other proteins or nucleic acids. IDRs may be fully or partially unstructured, and sample a range of conformations with similar energies, determined by their primary sequence.

Low-sequence complexity domains: regions of proteins in which specific amino acids are overrepresented compared with their proportions in the proteome. These are typically enriched in a small subset of amino acids, including polar (glycine, glutamine, and serine) and aromatic (generally tyrosine) residues.

proteins, which achieve this through three main features: (i) **intrinsically disordered regions (IDRs)**; (ii) RNA-binding domains (RBDs); and (iii) post-translational modifications (PTMs) (Figure 2A) [21,22,33,34]. First, IDRs are protein domains that lack a defined 3D structure, but often contain repeated stretches of amino acids with **low-sequence complexity** (LC), typically enriched in polar and aromatic residues, but generally depleted in hydrophobic amino acids [22,33–37]. In the cellular environment, IDR-containing proteins can transiently interact with multiple binding partners (proteins and RNAs) and, thus, are able to coalesce into dense biomolecular condensates, likely arising from their ability to sample many conformational states [21,34]. A second feature that provides many RNA-binding proteins with the ability to undergo phase separation is their specific RBDs, which include RNA-recognition motifs (RRMs), K-homology (KH) domains, arginine-glycine-glycine (RGG) motifs, and zinc-finger domains [34,38,39]. Proteins that contain both IDRs and RBDs have a high propensity to phase separate, suggesting that they function cooperatively in the condensation process (Figure 2A) [34,37,40]. Finally, PTMs can modulate the propensity of proteins to undergo phase separation by altering the hydrophobicity, bulkiness, or charge of modified amino acids, thereby modulating the strength of intermolecular interactions [34,41,42]. In particular, RNA-binding proteins are commonly modified by phosphorylation, which can alter local charge density and drive the assembly or dissolution of biomolecular condensates (Figure 2A) [21,43–45].

Notably, interactions between positively charged protein domains (e.g., arginine- and lysine-rich motifs) and the negatively charged phosphate backbone of nucleic acids can dramatically increase the likelihood of phase separation through increased multivalency and favorable electrostatic interactions (Figure 2A) [33,43,46]. Thus, RNA is a uniquely potent scaffold for phase separation, due partly to its high negative charge density and because it mimics the IDRs and LC domains of proteins. Its single-stranded and flexible nature, as well as its ability to form different structural conformations, enable it to interact specifically and nonspecifically with many RNA-binding proteins and tune the properties of biomolecular condensates [23,47,48]. Moreover, the length of an RNA molecule correlates with its ability to interact with multiple inter- and intramolecular binding partners, thereby increasing multivalency, which is critical for phase separation [47,49]. As such, RNA can seed or nucleate biomolecular condensates, impact their temporal and spatial formation, regulate the recruitment of RNA-binding partners, and serve as the key element that defines specific biomolecular condensates [22,34,48,50].

Diverse functions of biomolecular condensates

The biochemical properties of biomolecular condensates suggest that they mediate a variety of biological functions. First, the high local concentration of molecules in condensates can activate biochemical reactions, signaling, or the assembly of cellular structures [21,48,51]. Similarly, the chemical environment within condensates can stabilize certain conformations of biomolecules that may influence their activity, potentially stabilizing enzyme interactions with their substrates [52]. By contrast, selective sequestration of certain molecules, to the exclusion of others, enables condensates to prevent, slow, or buffer reactions or signaling events [21,51]. Likewise, condensates exhibit a range of material properties, from liquid-like fluids to solid-like gels, that confer mechanical functions. For example, the nuclear pore complex may act as a mechanical filter, while other condensates can generate mechanical forces that help shape and move cellular structures, including endosomes and membrane-bound organelles [53–55]. Interestingly, since molecules can passively exchange between condensates and the surrounding environment, the composition of biomolecular condensates can be regulated in a more flexible manner than those of classic membrane-bound organelles, which often require specific signals for active import and export [21,22]. Given their rapid and reversible assembly, tunability, and the functional flexibility of condensates, it is not surprising that viruses, which are relatively simple biological machines, would make use of phase separation to carry out key steps of their infectious life cycle.

Matrix: viral structural proteins that link the envelope with the nucleocapsid.
Membraneless organelles: distinct cellular compartments that are not enclosed by a traditional lipid membrane.
Multivalency: ability to form multiple intra- or intermolecular interactions simultaneously.
Nonstructural protein: viral protein that is not a structural component of the viral particle.
Nucleocapsid: capsid of the virus with the enclosed nucleic acid genome (i.e., the nucleic acid–protein assembly within the virion).
Nucleoprotein: proteins that complex with nucleic acids. In negative-sense RNA viruses, the nucleoprotein often participates in both antitermination (replication organelle biogenesis) and assembly (forms the nucleocapsid).
Phase separation: thermodynamically driven, reversible phenomenon whereby biomolecules demix into coexisting phases, each with a distinct chemical composition and physical properties.
Phosphoprotein: protein that is post-translationally modified by the attachment of one or more phosphate groups. In eukaryotic cells, the target amino acid is most often serine, threonine, or tyrosine.
Replication organelles: specialized subcellular compartments in which replication of the viral genome occurs. Replication organelles maintain dynamic interactions with the cytosol (and sometimes also cellular organelles) to obtain metabolites, lipids, proteins, and RNAs required for efficient viral replication.
RNA-dependent RNA polymerase: enzyme that catalyzes RNA synthesis from an RNA template.
Virions: infectious viral particles.

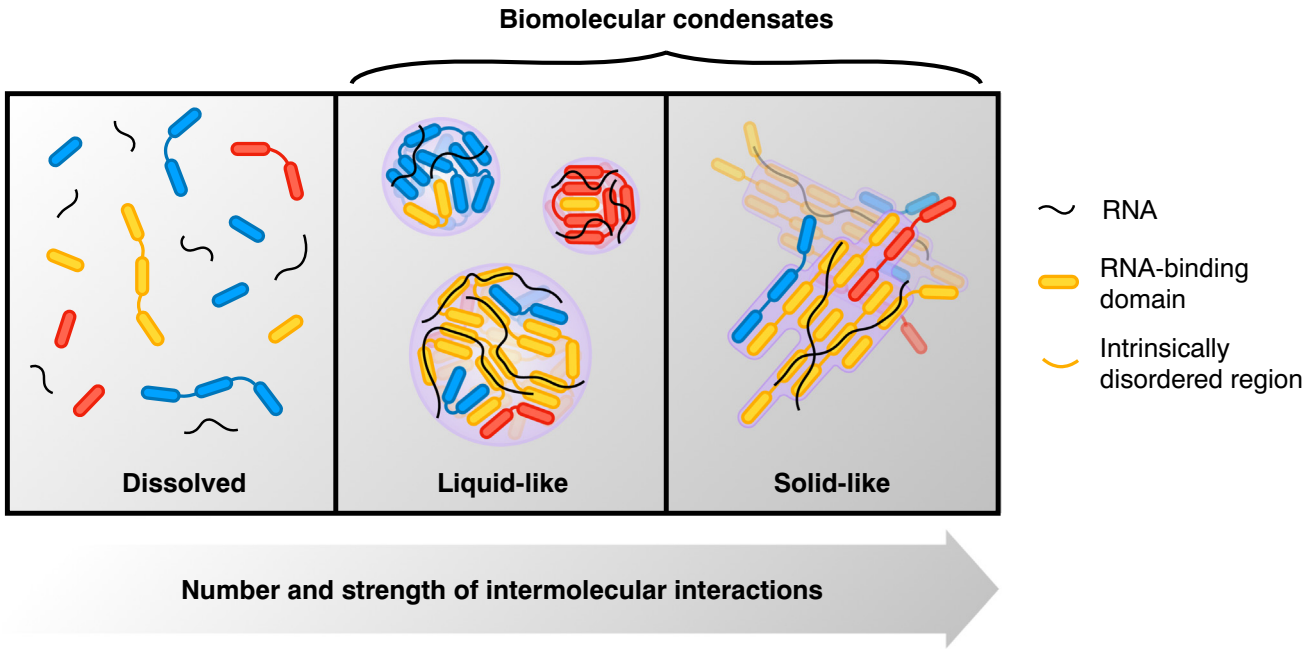

Figure 1. Phases of intracellular biomolecules. Proteins and nucleic acids can form a spectrum of phases with distinct composition, density, and dynamics. The properties of a particular phase are determined by the number and strength of intermolecular interactions within that phase. When intermolecular interactions are few and weak, biomolecules remain dissolved. However, when the number and strength of interactions are greater and exceed a threshold, biomolecules separate into a dense phase with a high local concentration and a dilute phase with low concentration. The dense phase, or biomolecular condensate, is liquid-like when the interactions are weak and transient, allowing biomolecules to move within and to enter/exit the phase. Alternatively, the dense phase is solid-like when the interactions are strong and long-lived, restricting biomolecular mobility.

Viral biomolecular condensates

While it has long been recognized that viruses form biomolecular condensates to mediate viral replication and nucleocapsid assembly, the concept that this occurs through phase separation is relatively new [3–6,8–11,13,15]. Consistent with this, the RNA virus proteome is enriched in IDR-containing proteins with respect to the eukaryotic proteome [56]. Based on similarities to components of cellular biomolecular condensates, at least two types of viral protein, namely antiterminators and nucleocapsid proteins, have features and roles consistent with their ability to phase separate with viral RNA molecules. As such, accumulating evidence suggests that viruses are truly masters of phase separation. In the following text, we describe the key features of viral antiterminators and nucleocapsid proteins, and the benefits of biomolecular condensates in the life cycles of both positive- and negative-sense RNA viruses.

Viral antiterminator proteins condense viral RNAs to form replication organelles

For an RNA virus to persist and propagate, the genomic RNA must be copied from end to end with no loss of nucleotide sequence. Additionally, since all viruses are parasites of the cellular protein synthesis machinery, they must produce viral mRNAs that can be efficiently translated by host cell ribosomes. However, the path to mRNA differs in positive- and negative-sense RNA viruses. In positive-sense RNA viruses, the genomic RNA is an mRNA and, hence, viral replication proteins, including the viral **RNA-dependent RNA polymerase** (RdRp), can be produced immediately upon entry into the cell (Box 1) [1]. Once the viral replication proteins accumulate, replication organelles can be established, and replication proceeds through a negative-strand replicative intermediate to produce progeny positive-sense viral genomic

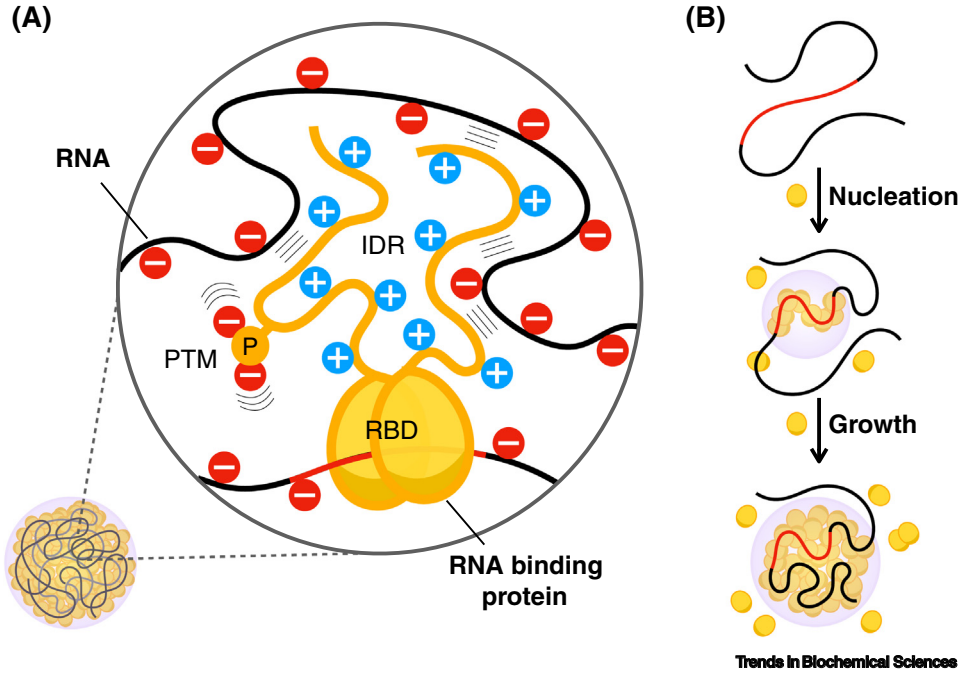

Figure 2. Structure and formation of biomolecular condensates. (A) RNA-binding proteins have a high propensity to undergo phase separation with their substrate RNAs. Multivalency is achieved through both specific and nonspecific interactions with substrate RNAs, mediated by both RNA-binding domains (RBDs) and intrinsically disordered regions (IDRs). These interactions are also modulated by post-translational modifications (PTMs), commonly phosphorylation (P). Straight lines indicate attraction, while curved lines indicate repulsion. (B) Biomolecular condensates are often nucleated by high-affinity interactions between RNA-binding proteins and specific sequences or structures on the substrate RNA (red). Increasing valency is provided through protein–protein (e.g., dimerization or higher-ordered oligomerization) and low-affinity protein–RNA interactions (e.g., electrostatic, hydrophobic, π–cation, and π–π interactions), resulting in the formation of a biomolecular condensate.

RNAs. By contrast, negative-sense RNA viruses cannot synthesize the replication proteins immediately upon entry into the cell (Box 2) [1]. Instead, they must first produce mRNAs from the negative-sense genomic RNA, which can be translated by host ribosomes to generate the viral replication proteins. Replication organelles can then be established, and replication proceeds through a full-length antigenomic viral RNA intermediate, leading to the production of progeny negative-sense viral genomic RNAs.

Antiterminator proteins are a major constituent of viral replication organelles and are often used as markers to visualize these replication compartments in infected cells [5,8]. Viral replication organelles are typically detectable a few hours post infection and, in most negative-sense RNA viruses, they initially appear as small spherical cytoplasmic inclusions, which become larger as infection progresses [5,8–10]. Members of the *Orthomyxoviridae* (e.g., influenza viruses) and at least some members of the *Bunyavirales* order (formerly the *Bunyaviridae*, e.g., Bunyamwera virus) are a notable exception, because they establish replication organelles in the nucleus or in association with the Golgi complex, respectively [13,57,58]. Antiterminator proteins typically interact with the viral RdRp and, in some cases, an additional viral **phosphoprotein**, and they bind to, coat, and condense the viral genomic RNA. As recent literature suggests, the multivalent interactions between antiterminator proteins and the viral RNA drive phase separation of the viral genome and promote viral RNA replication [5,8,11,13,15,59–63]. In negative-sense RNA viruses, this activity is often provided by the **nucleoprotein**, a multifunctional protein that generally has

Box 1. The dilemma of positive-sense RNA viruses

In positive-sense RNA viruses (e.g., *Picornaviridae* and *Flaviviridae*), the viral genome and the mRNA are one and the same. In some cases, subgenomic mRNAs are also produced through temporally controlled gene expression (e.g., *Bromoviridae*, *Nodaviridae*, *Togaviridae*, and *Tombusviridae*) or discontinuous transcription (e.g., *Coronaviridae*) mechanisms. Regardless, in all positive-sense RNA viruses, since the genomic RNA is the positive strand, the viral replication proteins, which are responsible for viral replication organelle biogenesis, including RdRp, can be produced immediately upon entry of the viral RNA into the cell cytoplasm (Figure I). However, as the viral proteins begin to accumulate, the virus is met with a dilemma: since the ribosome travels in the 5′ to 3′ direction and the RdRp travels in the 3′ to 5′ direction, translation and viral RNA replication cannot occur simultaneously on a single RNA molecule. As such, there must be a mechanism to switch off translation for viral RNA replication to be initiated [96].

As discussed herein, phase separation mediated by replication organelle biogenesis proteins (herein referred to as antiterminator proteins) may allow positive-sense RNA viruses to collectively solve this dilemma. Phase separation of the viral genomic RNA promotes replication organelle biogenesis and results in ribosome exclusion, thereby allowing viral replication and the production of progeny positive-sense genomic RNA, through the generation of a negative-strand replicative intermediate. This process is typically asymmetric, leading to more positive-sense genomic RNAs than negative-strand replicative intermediates. In positive-sense RNA viruses, the viral replication biogenesis protein (or antiterminator) typically contains a membrane association (transmembrane or amphipathic) domain or interacts with another viral protein that anchors it to a cellular membrane, thereby creating a vesicular (membrane-associated) replication organelle [16,17].

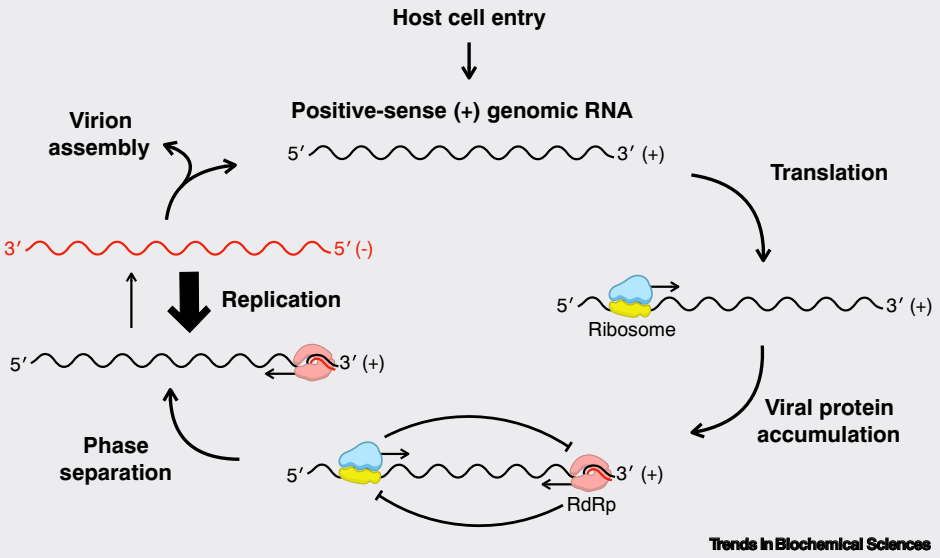

Figure I. Phase separation may mediate the switch from translation to replication in positive-sense RNA viruses. After host cell entry, positive-sense RNA viruses can immediately serve as templates for translation by host cell ribosomes. Translation of the viral genome leads to accumulation of the viral replication proteins, including the viral RNA-dependent RNA polymerase (RdRp), as well as a viral protein(s) with antiterminator and phosphoprotein activities. Given that translation and replication cannot occur simultaneously on the same RNA template, a mechanism must exist to switch off translation to allow initiation of RNA replication. This switch is likely mediated by host and/or viral proteins, including the antiterminator and phosphoprotein, which bind to, and phase separate, the viral RNA to facilitate ribosome exclusion and replication organelle biogenesis. Viral RNA replication can then proceed through a negative-strand RNA intermediate, which gives rise to progeny positive-sense genomic RNAs. These progeny genomic RNAs can then be assembled into viral particles or can engage in subsequent rounds of translation and replication.

roles in both replication organelle biogenesis and nucleocapsid assembly. As such, we prefer the term ‘antiterminator’ herein, which better captures the activity of these proteins in replication organelle biogenesis, and can be universally applied across RNA viruses, in which the antiterminator, phosphoprotein, and nucleocapsid functions can be provided by one or several distinct viral proteins (Figure 3).

Box 2. The dilemma of negative-sense RNA viruses

In negative-sense RNA viruses (e.g., *Rhabdoviridae*, *Filoviridae*, *Pneumoviridae*, *Paramyxoviridae*, and *Orthomyxoviridae*), the genomic RNA is complementary to the coding strand, and does not itself encode any protein. As such, negative-sense RNA viruses must carry their RdRp with them in the viral particle so that they can produce mRNAs that can be read by host ribosomes when they arrive in the host cell. Transcription of the first subgenomic mRNAs occurs in the partially uncoated nucleocapsid, where the genome is associated with the nucleoprotein. However, this presents the virus with a dilemma: the mRNAs produced are subgenomic and, thus are not suitable templates for the production of genome-length progeny RNAs (Figure I). Therefore, negative-sense RNA viruses must have a mechanism to switch from transcription (the synthesis of viral mRNAs) to replication (the synthesis of genome-length positive- and negative-sense RNAs).

Antiterminator proteins, also referred to as nucleoproteins, allow negative-sense RNA viruses to overcome this dilemma. Antiterminators are often the first and most abundant viral proteins produced, and they interact with the viral RdRp, sometimes also an additional phosphoprotein, and coat or condense the viral genomic RNA and nascent mRNA transcripts. At low concentrations, antiterminator proteins permit transcription, while, at high concentrations, they promote phase separation, stabilizing the RdRp and preventing termination (antitermination), thereby enabling genome-length viral RNA synthesis [1,97]. Replication can then proceed within the replication organelle through the generation of a full-length antigenomic replicative intermediate. Similar to positive-sense RNA viruses, this process is typically asymmetric, leading to more negative-sense genomic RNAs than antigenomic replicative intermediates. Since transcription of the first mRNAs after infection occurs in the partially uncoated virion, where the genomic RNA is likely to be in a condensed state, phase separation may be required for both transcription and replication.

Notably, dsRNA viruses (e.g., *Reoviridae*) must overcome the same dilemma as negative-sense RNA viruses, because their genome segments (being dsRNA) are not a suitable substrate for the ribosome. As such, similar to negative-sense RNA viruses, dsRNA viruses must first transcribe mRNAs that can be translated by host cell ribosomes. However, in contrast to negative-sense RNA viruses, positive-sense viral mRNAs are suitable templates for subsequent synthesis of full-length complementary negative-sense RNAs. Interestingly, both transcription and genome replication are associated with viral particles or progeny cores, such that the viral dsRNA is always in a condensed state.

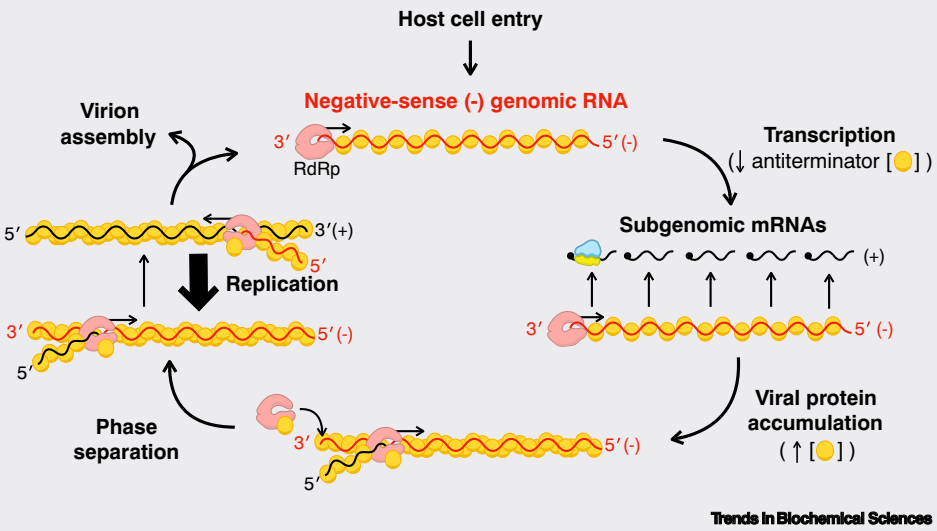

Figure I. Phase separation may mediate the switch from transcription to replication in negative-sense RNA viruses. After host cell entry, negative-sense RNA viruses must first produce viral mRNAs that can be translated by host cell ribosomes. The viral RNA-dependent RNA polymerase (RdRp) is physically carried in the viral particle, such that transcription of viral mRNAs can occur immediately upon entry into the host cell. Notably, this activity is associated with the partially uncoated nucleocapsid. Given that the viral mRNAs are too small to serve as templates for genomic RNA synthesis, a mechanism must exist to switch from mRNA transcription to genome replication. This is typically mediated by the accumulation of the viral replication proteins, including the antiterminator, typically in association with the phosphoprotein and RdRp. The antiterminator and phosphoprotein bind to, and phase separate, the viral genomic RNA to facilitate replication organelle biogenesis and 'antitermination' by the viral RdRp. Viral RNA replication can then proceed through a full-length positive-strand RNA intermediate, which gives rise to negative-sense genomic RNA progeny. These progeny genomic RNAs can then be assembled into viral particles or can engage in additional rounds of transcription and replication.

Genome	Family	Genus	Phosphoprotein	Antiterminator	Nucleocapsid
(+)ssRNA	***Bromoviridae***	*Bromovirus*	1a		CP
	Coronaviridae	*Betacoronavirus*	P	N	
	Flaviviridae	*Hepacivirus, Pestivirus*	NS5A		Core
		Flavivirus	NS5?	NS2A,NS2B/3?	Capsid
	Nodaviridae	*Alphadenovirus*	Protein A		Capsid
	Picornaviridae	*Enterovirus*	VpG?3D?	2C,3CD?	VP1-4
	Togaviridae	*Alphavirus*	Nsp3	Nsp4	Capsid
	Tombusviridae	*Tombusvirus*	p33		p41
(-)ssRNA	***Arenaviridae***	*Mammarenavirus*	NP		
	Filoviridae	*Ebolavirus*	VP30,VP35	NP	
	Orthomyxoviridae	*Alphainfluenzavirus*	NP		
	Paramyxoviridae	*Morbillivirus*	P	N	
	Peribunyaviridae	*Orthobunyavirus*	N		
	Pneumoviridae	*Metapneumovirus*	P	N	
	Rhabdoviridae	*Lyssavirus, Vesiculovirus*	P	N	
dsRNA	***Reoviridae***	*Reovirus*	σNS	μNS	λ1/λ2/λ3?
		Rotavirus	NSP5	NSP2	VP1/VP2/VP3?

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Figure 3. Differential distribution of phosphoprotein, antiterminator, and nucleocapsid activities across RNA viruses. Across the positive-sense (+) and negative-sense (–) single-stranded RNA (ssRNA) viruses, as well as the double-stranded RNA (dsRNA) viruses, phophosprotein, antiterminator, and nucleocapsid activities are separated across one or more viral proteins. Known and/or putative phosphoprotein (orange), antiterminator (yellow), and nucleocapsid (green) activities and the viral protein(s) associated with these activities for several major RNA virus families and genera are indicated. Abbreviations: CP, capsid protein; N, nucleocapsid (*Betacoronavirus*, *Morbillivirus*, and *Orthobunyavirus*); NP/N, nucleoprotein (*Alphainfluenzavirus*, *Lyssavirus*, *Mammarenavirus*, *Metapneumovirus*, and *Vesiculovirus*); NS, nonstructural; NSP, nonstructural protein; P, phosphoprotein; VP, viral protein; VpG, viral protein genome-linked.

Interestingly, while it is recognized that the formation of biomolecular condensates is a common feature of negative-sense RNA (and dsRNA) virus replication organelles [3–6,8–11,13,15], this concept has not been widely applied to positive-sense RNA viruses, the replication organelles of which are vesicular in nature [16,17]. Moreover, the term 'antiterminator', describing the specific activity of this protein in genome replication and replication organelle biogenesis, is not typically used in positive-sense RNA viruses and, instead, these proteins are referred to as 'replication organelle biogenesis' proteins [16,17]. However, the vesicular nature of their replication organelles does not preclude their formation via phase separation [59–63]. Rather, the replication organelle biogenesis protein, hereon referred to as an antiterminator, typically either contains an amphipathic or transmembrane domain that directly targets it to a cellular membrane (e.g., the endoplasmic reticulum or Golgi complex), or it interacts directly with another viral replication protein that is membrane associated [16,17,59,60,64–67]. In both positive- and negative-sense RNA viruses, antiterminator interactions are typically nucleated by high-affinity binding sites on the viral RNA, and condensation of the viral RNA(s) is likely promoted by low-affinity interactions across the remainder of the viral genome (Figure 2B) [60,64,67–71]. In some cases, this is likely facilitated by short- and long-range RNA–RNA interactions, which aid in condensation of the viral genome [23,48,72].

Whether derived from positive- or negative-sense RNA viruses, viral antiterminator proteins have all the common hallmarks of proteins that undergo intracellular phase separation. First, many

antiterminators contain positively charged IDRs and LC domains. In some cases, these unstructured domains are provided by the associated phosphoprotein (Figure 3) [5,8–11,15]. Second, they are RNA-binding proteins, typically containing specific RBDs. Moreover, antiterminator proteins (as well as their associated phosphoproteins) characteristically form dimers and/or higher-order oligomers, increasing valency. This is elegantly exemplified by studies in negative-sense RNA viruses, including the N and P proteins of rabies virus, respiratory syncytial virus, vesicular stomatitis virus, and measles virus, and the NP protein of influenza virus; as well as dsRNA viruses, including the NSP2 and NSP5 proteins of rotavirus [5,6,8,11,13,15]. However, it is also beginning to be recognized that antiterminator proteins promote phase separation in positive-sense RNA viruses too, as exemplified by studies of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) N protein [59–63]. Interestingly, while the functional role of phosphorylation is not always clear, there is some evidence to suggest that it may aid in the transition from initiation to elongation during RNA synthesis, or in the movement of progeny RNAs from viral replication organelles to assembly sites [73–75]. The former may be a similar process to eukaryotic transcription, in which phosphorylation of the RNA polymerase II C-terminal domain regulates shuttling between promoter and gene body condensates [76,77]. Indeed, phosphorylation of the P protein is required for transcriptional activation and recruitment of the RdRp to the N-protein:RNA assembly in vesicular stomatitis virus infection [74]. Additionally, the latter function is exemplified by recent studies of the phosphorylation and dephosphorylation of the SARS-CoV-2 N protein, which may allow it to localize to or shuttle from replication and assembly sites [59–62]. Similarly, phosphorylation of the influenza virus NP protein regulates oligomerization, with the oligomeric form required for genome replication, and the monomeric state implicated in nucleocapsid assembly [75,78,79].

Antiterminator-mediated replication organelle biogenesis generally requires RNA and, at least for some positive-sense RNA viruses, the size of the replication organelle is defined by the length of the viral genomic RNA [28,29,66,67,70,80–84]. Notably, in some cases, cellular expression of specific viral **nonstructural proteins** in the absence of infection was shown to be sufficient for replication organelle biogenesis; thus, it was assumed that RNA was not required for this process [84–88]. However, in the absence of viral substrate RNA(s), it is possible that viral antiterminator protein(s) can condense cellular or plasmid-derived RNAs to form replication organelle-like structures, as previously shown for the antiterminator protein (1a) of brome mosaic virus [64,87]. This would explain the heterogenous size and nature of the replication organelle-like structures observed when viral nonstructural proteins are expressed in isolation, in contrast to the relatively uniform size distribution observed during infection (at least in positive-sense RNA viruses), and is consistent with the idea that RNA is important for viral replication organelle biogenesis [66,67,81–84]. By contrast, the rotavirus (a dsRNA virus) antiterminator (NSP2) and phosphoprotein (NSP5) have been demonstrated to phase separate in the absence of RNA *in vitro*; however, this process is likely facilitated by RNA within cells [6]. Finally, besides the antiterminator and the viral RNA, in some cases, active viral replication has been shown to be necessary for replication organelle biogenesis [66,70,80]. Nonetheless, while it is clear that the length of the viral RNA can define the size of the replication organelle, it is likely that additional inter- and intramolecular interactions between viral nonstructural proteins and/or host proteins or lipids also contribute to the size and form of viral replication organelles [66]. Nonetheless, viral antiterminator proteins are key to the establishment of viral replication organelles and accumulating evidence suggests that they mediate their biogenesis via phase separation with viral RNA(s).

Nucleocapsid proteins condense viral genomes inside virions

For a virus to persist and infect new cells, the viral genome must be specifically packaged into a viral particle and released from the host cell. How do RNA viruses, from the smallest picornavirus

(~7 kb) to the largest coronavirus (~32 kb), squeeze all that genomic RNA into a tiny (22–500 nm) viral particle? Similar to viral replication organelle biogenesis, accumulating evidence suggests that genome packaging or nucleocapsid assembly is also mediated by phase separation, through the action of viral nucleocapsid proteins [9,11,13,59–63,89]. The term 'nucleocapsid' refers to the nucleic acid genome surrounded by the protein coat (or **capsid**) of the virus [1]. Nucleocapsid proteins, also known as coat or capsid proteins, are the viral proteins responsible for binding to, and condensing, the viral genome inside the **virion** (Figure 2B). As described for viral antiterminator proteins, nucleocapsid proteins have all the classical molecular features that drive phase separation. First, they are modular proteins with positively charged IDRs, LC domains, and RBDs, which allow them to package the viral genomic RNA, through both high- and low-affinity interactions [1]. Notably, some viral nucleocapsid proteins only interact with viral genomic RNAs via nonspecific hydrophobic or charged interactions [90–95]. As such, genome specificity is provided by another viral protein, sometimes the viral antiterminator protein [68,69]. Second, nucleocapsid proteins form dimers and/or higher-order oligomers, providing multivalency for nucleocapsid assembly. Indeed, several recent studies in both positive- and negative-sense RNA viruses, including flaviviruses, coronaviruses, paramyxoviruses, and orthomxyoviruses, lend support to the notion that nucleocapsid assembly sites are biomolecular condensates [9,11,13,59–63,89]. Finally, nucleocapsid proteins are typically regulated by phosphorylation, which can aid in nucleocapsid assembly by regulating contacts between nucleocapsid proteins or with other viral structural proteins (e.g., **matrix** or **envelope** glycoproteins) [75]. Alternatively, it is also possible that phosphorylation facilitates the disassembly or uncoating reaction upon nucleocapsid delivery into a newly infected host cell [75]. Taken together, these findings suggest that viral nucleocapsid proteins, similar to antiterminator proteins, perform viral genome packaging by phase separation with viral genomic RNAs.

Fascinatingly, and in further reinforcement of their parallel roles and activities, the antiterminator and nucleocapsid functions in some viruses are performed by the same viral protein, with examples in both positive- and negative-sense RNA viruses (Figure 3) [5,8–10,13,15,59–63]. For example, the coronavirus N protein is both the antiterminator, responsible for replication organelle biogenesis, and the nucleocapsid protein, which coats the viral genome to create the nucleocapsid during virion assembly [59–63]. Interestingly, while the antiterminator and nucleocapsid functions are typically provided by the action of the nucleoprotein in mammalian negative-sense RNA viruses, these roles in positive-sense RNA or dsRNA viruses are generally separated across two (or more) viral proteins (coronaviruses being a notable exception) [16,17,59–63,89]. Whether this separation of function is a result of convergent or divergent evolution remains an open question.

Benefits of viral biomolecular condensates

With increasing evidence that viral antiterminators and nucleocapsid proteins can phase separate, a major question that remains is what the benefits are of forming biomolecular condensates in the viral life cycle. Based on the known roles of biomolecular condensates, as well as the tasks that viruses must accomplish to complete their life cycles, we can envision several benefits (Figure 4, Key figure). First, biomolecular condensates can promote the colocalization of both the viral RNA and the replication or assembly proteins for replication organelle biogenesis and/or nucleocapsid assembly, respectively. Second, biomolecular condensates can serve as barriers to large molecular complexes, such as ribosomes, and thereby inactivate reactions, such as translation (helping to solve the dilemma of positive-sense RNA viruses; Box 1) [21,53,96]. Condensates can also activate enzymatic or nucleation reactions by concentrating enzymes and their substrates in close proximity, while the chemical properties of condensates may also favor reactions by stabilizing active conformations [21,23,48]. These benefits are particularly advantageous for cytoplasmic RNA synthesis, which poses a unique challenge for RNA viruses

Key Figure

Functional roles of viral biomolecular condensates

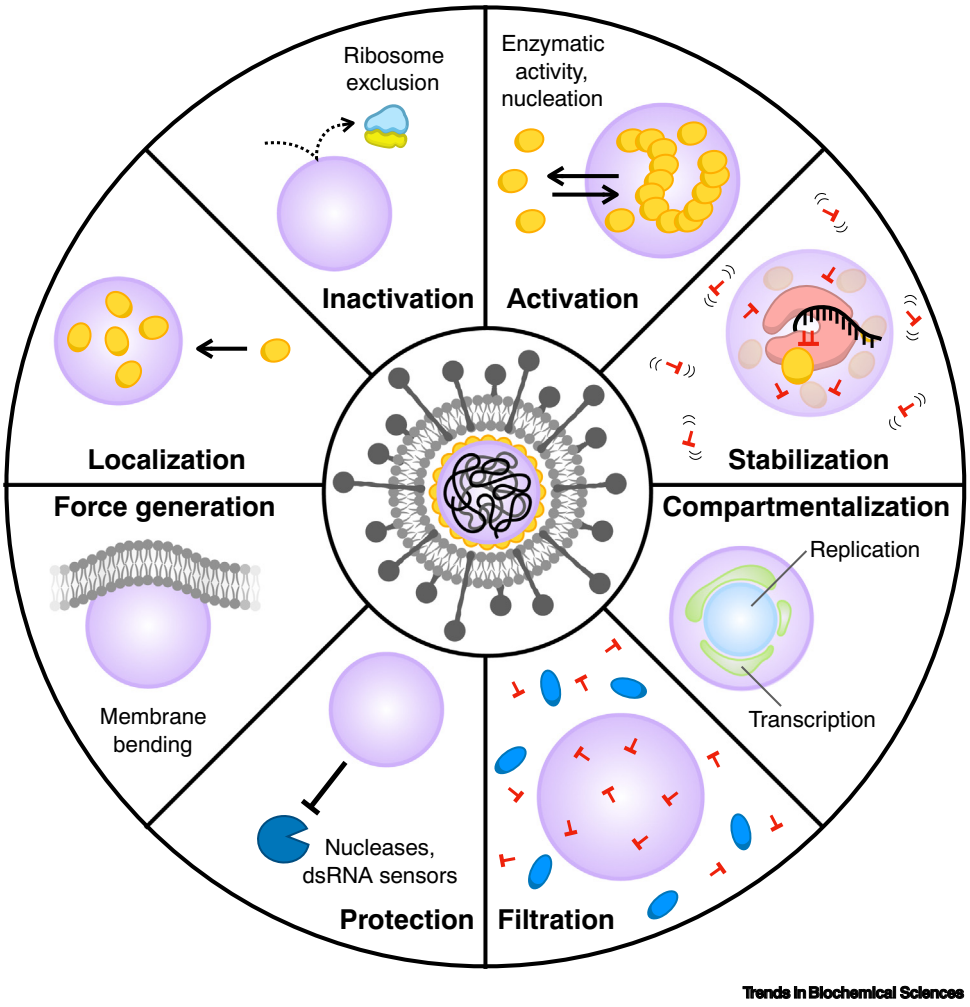

Figure 4. Viral bimolecular condensates can mediate localization, inactivation, activation, stabilization, compartmentalization, filtration, protection, and force generation. Figure inspired by [21].

[97]. Initiation of RNA synthesis is a complex enzymatic process, in which the viral RdRp and the genomic RNA must stabilize and position two incoming ribonucleotide triphosphates sufficiently well to allow the formation of the first phosphodiester bond. This is especially challenging at the terminus of a linear RNA template because the RdRp has limited template with which it can associate [97]. Thus, phase separation may serve as a mechanism to stabilize to the initiating RdRp complex and can further aid in polymerase fidelity during RNA synthesis.

Some biomolecular condensates have internal organization or subcompartmentalization, with distinct subphases demixing to form ‘core’ and ‘shell’ architectures [98–100] (Figure 4). In this

way, viruses may use biomolecular condensates to spatially separate reactions, such as transcription, replication, and/or nucleocapsid assembly. For example, in the negative-sense RNA virus replication organelle, the more stable 'core' has been suggested to be the replication compartment, whereas the less concentrated 'shells' are thought to primarily be mRNA transcription/storage or nucleocapsid assembly compartments [5,8–11,15]. Moreover, it is possible that transcription, similar to replication, also requires phase separation. For negative-sense RNA (and dsRNA) viruses, the initial mRNA transcription events after viral entry occur within the partially uncoated virion, where the genomic RNA is still complexed with the antiterminator/nucleocapsid protein within a biomolecular condensate (Box 2).

Biomolecular condensates can also serve as a selective filter, allowing access to certain proteins or metabolites (e.g., ribonucleotide triphosphates), while excluding others (Figure 4). This is also beneficial for protection of the viral genomic RNA, because condensates can exclude nucleases or cellular sensors of RNA [48,101]. In support of this notion, a recent study in negative-sense RNA viruses suggests that depletion of the nucleoprotein, presumably reducing the ability to condense the viral genomic RNA, leads to an increase in spurious transcription products and innate immune activation [101].

In contrast to negative-sense RNA viruses, which have cytoplasmic replication organelles, positive-sense RNA viruses create vesicular or membranous replication organelles [16,17]. While this could be accomplished through the action of membrane-tethered antiterminator proteins, it is also possible that the viral biomolecular condensate itself generates force for membrane bending to aid in replication organelle biogenesis, as recently suggested for several positive-sense RNA viruses [67,81,83] (Figure 4). The same may be true for the assembly of enveloped viral particles, because the condensed nucleocapsid could generate force for viral budding and the acquisition of the viral envelope. Altogether, biomolecular condensates can provide viruses with numerous advantages that allow them to establish replication organelles, assemble viral particles, and even evade innate immune responses.

Concluding remarks

Taken together, there is accumulating evidence to suggest that viruses are truly masters of phase separation. Yet, numerous questions remain about the host and viral components of biomolecular condensates, the molecular grammar of viral phase separation, the properties of viral biomolecular condensates, and how we might exploit these properties to develop antivirals or improve packaging of mRNA vaccines (see Outstanding questions) [37]. While virologists have been studying viral replication organelles and nucleocapsid assembly for decades, it is only recently that we have started to appreciate the role of phase separation. By contrast, biophysicists have made great progress in characterizing the molecular and physicochemical basis of cellular biomolecular condensates, but, in many cases, their biological function(s) remains an active area of investigation. Importantly, viruses are simplified systems that have facilitated foundational discoveries in cell and molecular biology, including DNA replication, splicing, RNA polymerase II promoters, and oncogenes (among others) [102].

Moreover, a lot is known about viral antiterminator and nucleocapsid proteins. Virologists have performed extensive mutational and deletion analyses, identified their binding partners, and, in some cases, have even developed synthetic small molecules or antivirals that target these proteins. Thus, a wide variety of tools are available to help elucidate the mechanisms, processes, and functions of viral biomolecular condensates. Furthermore, polymer chemists have developed theoretical models, which generate quantitative predictions that can be used to rigorously distinguish among various mechanisms. Finally, biophysicists have defined the molecular

Outstanding questions

What is the molecular grammar that defines viral phase separation?

What are the material properties of viral biomolecular condensates?

What are the key host and viral factors that control the composition of viral biomolecular condensates?

What host and viral proteins localize to viral biomolecular condensates?

How do the activities of viral proteins and RNAs change by virtue of being in a biomolecular condensate, rather than being more uniformly distributed in the cell?

How do viruses regulate the size of their biomolecular condensates?

What dictates the internal organization of viral biomolecular condensates?

How do viruses spatially and/or temporally separate replication and assembly during viral infection?

What are the functional consequences of encoding the phosphoprotein, antiterminator, and nucleocapsid activities in multiple distinct proteins versus a single modular viral protein?

How do viral biomolecular condensates influence disease pathogenesis?

Can we develop antivirals that target or disrupt biomolecular condensates?

Can we exploit the features of viral phase separation for the specific packaging and delivery of therapeutic mRNAs?

grammar of phase separation and are actively designing experimental strategies to control phase separation in live cells. Thus, let this article serve as a call to action for further collaboration between virologists and biophysicists to accelerate discovery in both our respective fields. Ultimately, by working together, we will advance the mechanistic and functional understanding of biomolecular condensates and reveal novel therapeutic strategies to tackle both viral-derived and non-communicable diseases associated with aberrant phase separation.

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Declaration of interests

None declared by authors.

References

1. Flint, S.J. *et al.* (2020) Principles of Virology. Wiley & American Society for Microbiology
2. Heinrich, B.S. *et al.* (2010) Protein expression redirects vesicular stomatitis virus RNA synthesis to cytoplasmic inclusions. *PLoS Pathog.* 6, e1000958
3. Hoenen, T. *et al.* (2012) Inclusion bodies are a site of ebolavirus replication. *J. Virol.* 86, 11779–11788
4. Lahaye, X. *et al.* (2009) Functional characterization of Negri bodies (NBs) in rabies virus-infected cells: evidence that NBs are sites of viral transcription and replication. *J. Virol.* 83, 7948–7958
5. Rincheval, V. *et al.* (2017) Functional organization of cytoplasmic inclusion bodies in cells infected by respiratory syncytial virus. *Nat. Commun.* 8, 563
6. Geiger, F. *et al.* (2021) Liquid-liquid phase separation underpins the formation of replication factories in rotaviruses. *EMBO J.* 40, e107711
7. Kristensson, K. *et al.* (1996) Rabies: interactions between neurons and viruses. A review of the history of Negri inclusion bodies. *Neuropathol. Appl. Neurobiol.* 22, 179–187
8. Nikolic, J. *et al.* (2017) Negri bodies are viral factories with properties of liquid organelles. *Nat. Commun.* 8, 58
9. Ringel, M. *et al.* (2019) Nipah virus induces two inclusion body populations: identification of novel inclusions at the plasma membrane. *PLoS Pathog.* 15, e1007733
10. Zhou, Y. *et al.* (2019) Measles virus forms inclusion bodies with properties of liquid organelles. *J. Virol.* 93, e00948-19
11. Guseva, S. *et al.* (2020) Measles virus nucleo- and phosphoproteins form liquid-like phase-separated compartments that promote nucleocapsid assembly. *Sci. Adv.* 6, eaaz7095
12. Shaw, M.W. and Compans, R.W. (1978) Isolation and characterization of cytoplasmic inclusions from influenza A virus-infected cells. *J. Virol.* 25, 608–615
13. Alenquer, M. *et al.* (2019) Influenza A virus ribonucleoproteins form liquid organelles at endoplasmic reticulum exit sites. *Nat. Commun.* 10, 1629
14. Dolnik, O. *et al.* (2015) Marburg virus inclusions: a virus-induced microcompartment and interface to multivesicular bodies and the late endosomal compartment. *Eur. J. Cell Biol.* 94, 323–331
15. Heinrich, B.S. *et al.* (2018) Phase transitions drive the formation of vesicular stomatitis virus replication compartments. *mBio* 9, e02290-17
16. Harak, C. and Lohmann, V. (2015) Ultrastructure of the replication sites of positive-strand RNA viruses. *Virology* 479-480, 418–433
17. Reid, C.R. *et al.* (2015) The virus-host interplay: biogenesis of +RNA replication complexes. *Viruses* 7, 4385–4413
18. Brown, R. (1833) Observations on the organs and mode of fecundation in Orchidae and Asclepiadeae. *Trans. Linn. Soc. (Lond.)* 16, 685–743
19. Wagner, R. (1835) Einige Bemerkungen und Fragen über das Keimbläschen (vesicular germinativa). *Arch. Anat. Physiol. Wissenschaft. Med.* 8, 373–377
20. Valentin, G. (1836) *Repertorium Für Anatomie Und Physiologie*, De Gruyter
21. Alberti, S. *et al.* (2019) Considerations and challenges in studying liquid-liquid phase separation and biomolecular condensates. *Cell* 176, 419–434
22. Banani, S.F. *et al.* (2017) Biomolecular condensates: organizers of cellular biochemistry. *Nat. Rev. Mol. Cell Biol.* 18, 285–298
23. Rhine, K. *et al.* (2020) RNA droplets. *Annu. Rev. Biophys.* 49, 247–265
24. Brangwynne, C.P. *et al.* (2009) Germline P granules are liquid droplets that localize by controlled dissolution/condensation. *Science* 324, 1729–1732
25. Kato, M. *et al.* (2012) Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. *Cell* 149, 753–767
26. Li, P. *et al.* (2012) Phase transitions in the assembly of multivalent signalling proteins. *Nature* 483, 336–340
27. Weber, S.C. and Brangwynne, C.P. (2012) Getting RNA and protein in phase. *Cell* 149, 1188–1191
28. Peng, A. and Weber, S.C. (2019) Evidence for and against liquid-liquid phase separation in the nucleus. *Noncoding RNA* 5, 50
29. Erdel, F. and Rippe, K. (2018) Formation of chromatin subcompartments by phase separation. *Biophys. J.* 114, 2262–2270
30. Harmon, T.S. *et al.* (2017) Intrinsically disordered linkers determine the interplay between phase separation and gelation in multivalent proteins. *eLife* 6, e30294
31. Yamazaki, T. *et al.* (2021) Paraspeckles are constructed as block copolymer micelles. *EMBO J.* 40, e107270
32. Martin, E.W. and Holehouse, A.S. (2020) Intrinsically disordered protein regions and phase separation: sequence determinants of assembly or lack thereof. *Emerg. Top. Life Sci.* 4, 307–329
33. Maharana, S. *et al.* (2018) RNA buffers the phase separation behavior of prion-like RNA binding proteins. *Science* 360, 918–921
34. Wiedner, H.J. and Giudice, J. (2021) It's not just a phase: function and characteristics of RNA-binding proteins in phase separation. *Nat. Struct. Mol. Biol.* 28, 465–473
35. Oldfield, C.J. and Dunker, A.K. (2014) Intrinsically disordered proteins and intrinsically disordered protein regions. *Annu. Rev. Biochem.* 83, 553–584
36. Patel, A. *et al.* (2015) A liquid-to-solid phase transition of the ALS Protein FUS accelerated by disease mutation. *Cell* 162, 1066–1077
37. Wang, J. *et al.* (2018) A molecular grammar governing the driving forces for phase separation of prion-like RNA binding proteins. *Cell* 174, 688–699

38. Lunde, B.M. *et al.* (2007) RNA-binding proteins: modular design for efficient function. *Nat. Rev. Mol. Cell Biol.* 8, 479–490
39. Thandapani, P. *et al.* (2013) Defining the RGG/RG motif. *Mol. Cell* 50, 613–623
40. Gotor, N.L. *et al.* (2020) RNA-binding and prion domains: the Yin and Yang of phase separation. *Nucleic Acids Res.* 48, 9491–9504
41. Bah, A. and Forman-Kay, J.D. (2016) Modulation of intrinsically disordered protein function by post-translational modifications. *J. Biol. Chem.* 291, 6696–6705
42. Hofweber, M. and Dormann, D. (2019) Friend or foe—post-translational modifications as regulators of phase separation and RNP granule dynamics. *J. Biol. Chem.* 294, 7137–7150
43. Aumiller, W.M., Jr and Keating, C.D. (2016) Phosphorylation-mediated RNA/peptide complex coacervation as a model for intracellular liquid organelles. *Nat. Chem.* 8, 129–137
44. Jin, F. and Grater, F. (2021) How multisite phosphorylation impacts the conformations of intrinsically disordered proteins. *PLoS Comput. Biol.* 17, e1008939
45. Na, Z. *et al.* (2021) Phosphorylation of a human microprotein promotes dissociation of biomolecular condensates. *J. Am. Chem. Soc.* 143, 12675–12687
46. Boeynaems, S. *et al.* (2019) Spontaneous driving forces give rise to protein-RNA condensates with coexisting phases and complex material properties. *Proc. Natl. Acad. Sci. U. S. A.* 116, 7889–7898
47. Khong, A. *et al.* (2017) The stress granule transcriptome reveals principles of mRNA accumulation in stress granules. *Mol. Cell* 68, 808–820
48. Poudyal, R.R. *et al.* (2021) RNA sequence and structure control assembly and function of RNA condensates. *RNA* 27, 1589–1601
49. Sanchez-Burgos, I. *et al.* (2022) RNA length has a non-trivial effect in the stability of biomolecular condensates formed by RNA-binding proteins. *PLoS Comput. Biol.* 18, e1009810
50. Elbaum-Garfinkle, S. *et al.* (2014) Tau mutants bind tubulin heterodimers with enhanced affinity. *Proc. Natl. Acad. Sci. U. S. A.* 111, 6311–6316
51. Peeples, W. and Rosen, M.K. (2021) Mechanistic dissection of increased enzymatic rate in a phase-separated compartment. *Nat. Chem. Biol.* 17, 693–702
52. Nott, T.J. *et al.* (2016) Membraneless organelles can melt nucleic acid duplexes and act as biomolecular filters. *Nat. Chem.* 8, 569–575
53. Bergeron-Sandoval, L.P. *et al.* (2021) Endocytic proteins with prion-like domains form viscoelastic condensates that enable membrane remodeling. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2113789118
54. Schmidt, H.B. and Gorlich, D. (2016) Transport selectivity of nuclear pores, phase separation, and membraneless organelles. *Trends Biochem. Sci.* 41, 46–61
55. Shin, Y. *et al.* (2018) Liquid nuclear condensates mechanically sense and restructure the genome. *Cell* 175, 1481–1491
56. Tokuriki, N. *et al.* (2009) Do viral proteins possess unique biophysical features? *Trends Biochem. Sci.* 34, 53–59
57. Davies, K.A. *et al.* (2020) The RNA replication site of Tula orthohantavirus resides within a remodelled Golgi network. *Cells* 9, 1569
58. Fontana, J. *et al.* (2008) The unique architecture of Bunyamwera virus factories around the Golgi complex. *Cell. Microbiol.* 10, 2012–2028
59. Carlson, C.R. *et al.* (2020) Phosphoregulation of phase separation by the SARS-CoV-2 N protein suggests a biophysical basis for its dual functions. *Mol. Cell* 80, 1092–1103
60. Iserman, C. *et al.* (2020) Genomic RNA elements drive phase separation of the SARS-CoV-2 nucleocapsid. *Mol. Cell* 80, 1078–1091
61. Jack, A. *et al.* (2021) SARS-CoV-2 nucleocapsid protein forms condensates with viral genomic RNA. *PLoS Biol.* 19, e3001425
62. Savastano, A. *et al.* (2020) Nucleocapsid protein of SARS-CoV-2 phase separates into RNA-rich polymerase-containing condensates. *Nat. Commun.* 11, 6041
63. Wang, S. *et al.* (2021) Targeting liquid-liquid phase separation of SARS-CoV-2 nucleocapsid protein promotes innate antiviral immunity by elevating MAVS activity. *Nat. Cell Biol.* 23, 718–732
64. Sullivan, M.L. and Ahlquist, P. (1999) A brome mosaic virus intergenic RNA3 replication signal functions with viral replication protein 1a to dramatically stabilize RNA in vivo. *J. Virol.* 73, 2622–2632
65. Kopek, B.G. *et al.* (2007) Three-dimensional analysis of a viral RNA replication complex reveals a virus-induced mini-organelle. *PLoS Biol.* 5, e220
66. Kopek, B.G. *et al.* (2010) Nodavirus-induced membrane rearrangement in replication complex assembly requires replicase protein a, RNA templates, and polymerase activity. *J. Virol.* 84, 12492–12503
67. Cerikan, B. *et al.* (2020) A non-replicative role of the 3' terminal sequence of the dengue virus genome in membranous replication organelle formation. *Cell Rep.* 32, 107859
68. Barnard, T.R. *et al.* (2021) Molecular determinants of Flavivirus virion assembly. *Trends Biochem. Sci.* 46, 378–390
69. Foster, T.L. *et al.* (2010) All three domains of the hepatitis C virus nonstructural NS5A protein contribute to RNA binding. *J. Virol.* 84, 9267–9277
70. Spuul, P. *et al.* (2011) Assembly of alphavirus replication complexes from RNA and protein components in a novel trans-replication system in mammalian cells. *J. Virol.* 85, 4739–4751
71. You, S. and Rice, C.M. (2008) 3' RNA elements in hepatitis C virus replication: kissing partners and long poly(U). *J. Virol.* 82, 184–195
72. Davis, M. *et al.* (2008) Bioinformatic and physical characterizations of genome-scale ordered RNA structure in mammalian RNA viruses. *J. Virol.* 82, 11824–11836
73. Jakubiec, A. and Jupin, I. (2007) Regulation of positive-strand RNA virus replication: the emerging role of phosphorylation. *Virus Res.* 129, 73–79
74. Lenard, J. (1999) Host cell protein kinases in nonsegmented negative-strand virus (*Mononegavirales*) infection. *Pharmacol. Ther.* 83, 39–48
75. Hoover, H.S. and Kao, C.C. (2016) Phosphorylation of the viral coat protein regulates RNA virus infection. *Virus Adapt. Treat.* 8, 13–20
76. Cramer, P. (2019) Organization and regulation of gene transcription. *Nature* 573, 45–54
77. Guo, Y.E. *et al.* (2019) Pol II phosphorylation regulates a switch between transcriptional and splicing condensates. *Nature* 572, 543–548
78. Chan, R.W. *et al.* (2010) Influenza H5N1 and H1N1 virus replication and innate immune responses in bronchial epithelial cells are influenced by the state of differentiation. *PLoS ONE* 5, e8713
79. Ye, Q. *et al.* (2006) The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. *Nature* 444, 1078–1082
80. Egger, D. *et al.* (2000) Formation of the poliovirus replication complex requires coupled viral translation, vesicle production, and viral RNA synthesis. *J. Virol.* 74, 6570–6580
81. Kallio, K. *et al.* (2013) Template RNA length determines the size of replication complex spherules for Semliki Forest virus. *J. Virol.* 87, 9125–9134
82. Kovalev, N. *et al.* (2016) Role of viral RNA and co-opted cellular ESCRT-I and ESCRT-III factors in formation of Tombusvirus spherules harboring the Tombusvirus replicase. *J. Virol.* 90, 3611–3626
83. Ertel, K.J. *et al.* (2017) Cryo-electron tomography reveals novel features of a viral RNA replication compartment. *eLife* 6, e25940
84. Hellstrom, K. *et al.* (2017) Partially uncleaved alphavirus replicase forms spherule structures in the presence and absence of RNA template. *J. Virol.* 91, e00787-17
85. Berger, C. *et al.* (2014) Daclatasvir-like inhibitors of NS5A block early biogenesis of hepatitis C virus-induced membranous replication factories, independent of RNA replication. *Gastroenterology* 147, 1094–1105
86. Romero-Brey, I. and Bartenschlager, R. (2014) Membranous replication factories induced by plus-strand RNA viruses. *Viruses* 6, 2826–2857
87. Schwartz, M. *et al.* (2002) A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. *Mol. Cell* 9, 505–514

88. Yau, W.L. *et al.* (2019) Model system for the formation of tick-borne encephalitis virus replication compartments without viral RNA replication. *J. Virol.* 93, e00292-19
89. Ambroggio, E.E. *et al.* (2021) Dengue and Zika virus capsid proteins bind to membranes and self-assemble into liquid droplets with nucleic acids. *J. Biol. Chem.* 297, 101059
90. Fromentin, R. *et al.* (2007) A method for in vitro assembly of hepatitis C virus core protein and for screening of inhibitors. *Anal. Biochem.* 366, 37–45
91. Kofler, R.M. *et al.* (2002) Capsid protein C of tick-borne encephalitis virus tolerates large internal deletions and is a favorable target for attenuation of virulence. *J. Virol.* 76, 3534–3543
92. Kofler, R.M. *et al.* (2003) Spontaneous mutations restore the viability of tick-borne encephalitis virus mutants with large deletions in protein C. *J. Virol.* 77, 443–451
93. Patkar, C.G. *et al.* (2007) Functional requirements of the yellow fever virus capsid protein. *J. Virol.* 81, 6471–6481
94. Samsa, M.M. *et al.* (2012) Uncoupling cis-Acting RNA elements from coding sequences revealed a requirement of the N-terminal region of dengue virus capsid protein in virus particle formation. *J. Virol.* 86, 1046–1058
95. Schrauf, S. *et al.* (2009) Extension of flavivirus protein C differentially affects early RNA synthesis and growth in mammalian and arthropod host cells. *J. Virol.* 83, 11201–11210
96. Barton, D.J. *et al.* (1999) Translating ribosomes inhibit poliovirus negative-strand RNA synthesis. *J. Virol.* 73, 10104–10112
97. Noton, S.L. and Fearns, R. (2015) Initiation and regulation of paramyxovirus transcription and replication. *Virology* 479-480, 545–554
98. Pak, C.W. *et al.* (2016) Sequence determinants of intracellular phase separation by complex coacervation of a disordered protein. *Mol. Cell* 63, 72–85
99. Wheeler, J.R. *et al.* (2016) Distinct stages in stress granule assembly and disassembly. *eLife* 5, e18413
100. Swain, P. and Weber, S.C. (2020) Dissecting the complexity of biomolecular condensates. *Biochem. Soc. Trans.* 48, 2591–2602
101. Nilsson-Payant, B.E. *et al.* (2021) Reduced nucleoprotein availability impairs negative-sense RNA virus replication and promotes host recognition. *J. Virol.* 95, e02274-20
102. Enquist, L.W. and Editors of the Journal of Virology (2009) Virology in the 21st century. *J. Virol.* 83, 5296–5308