SPOTLIGHT

Liquid–liquid phase separation drives herpesvirus assembly in the cytoplasm

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Liquid–liquid phase separation (LLPS) has emerged as a fundamental mechanism to compartmentalize biomolecules into membraneless organelles. In this issue, Zhou et al. (2022. _J. Cell Biol._ https://doi.org/10.1083/jcb.202201088), report that MHV-68 ORF52 undergoes LLPS to form cytoplasmic virion assembly compartments, regulating the spatiotemporal compartmentalization of viral components.

Compartmentalization of biomolecules is a key mechanism in efficiently regulating numerous biological processes in the densely packed cellular environment. Eukaryotic cells contain both membranous and membraneless organelles as subcellular compartment structures. Membraneless organelles, such as nucleoli and nuclear speckles in the nucleus and P-bodies and stress granules in the cytoplasm, are biomolecular condensates with liquid properties and mostly formed via liquid–liquid phase separation (LLPS). LLPS drives the formation of various intracellular condensates, where a single, uniform liquid phase separates into two compositionally distinct liquid phases, like an example of oil droplets in water. LLPS-driven biomolecular condensates are induced by phase-separating proteins containing multiple folded domains and intrinsically disordered regions (IDRs). They are also triggered by nucleic acids, such as DNA and RNA, or multivalent proteins via heterotypic interactions. LLPS-induced compartmentalization makes a biological process more efficient as it concentrates reaction factors together, increases reaction efficiency and kinetics, and excludes inhibitors. Furthermore, these membraneless organelles are highly dynamic and can rapidly undergo remodeling in response to various changes in the cell.

Viruses are obligate intracellular parasites that can invade various cellular systems at each step of their life cycle. A virus concentrates all essential factors in proximity to maximize the efficiency of genome replication and virion assembly in the milieu of cellular factors. Viruses reorganize the subcellular compartments to concentrate viral genomes and proteins and exclude other competing factors. While membrane-associated subcellular structures are used as viral factories in some RNA viruses, LLPS-mediated liquid condensates have recently been reported as replication compartments (RCs) or inclusion bodies in other RNA viruses. LLPS also regulates genome packaging and contributes to viral budding (1). Moreover, LLPS can suppress the host innate immunity by sequestering antiviral sensors into condensates or disrupting condensates harboring antiviral sensors. However, the investigation of liquid condensates in DNA viruses has only recently begun.

Herpesviridae is a large family of DNA viruses that replicate their genomes in the nucleus and produce mature virions in the cytoplasm. While they are subdivided into three subfamilies (α, β, and γ) based on their genome organization and pathogenesis, herpesviruses may share strategies for genome replication and virion assembly. After viral DNA replication, capsid assembly and viral DNA packaging occur in the nucleus. When the newly formed nucleocapsid is transported to the cytoplasm, the capsid acquires a protein coat with dozens of viral proteins called tegument. In a mature herpesvirus particle, a layer of tegument proteins is then wrapped around with the lipid bilayer of envelope containing viral glycoproteins. Therefore, in herpesviruses, DNA replication and nucleocapsid formation occur in the nuclear RCs, while virion assembly acquiring the most tegument proteins and the envelope takes place in cytoplasmic virion assembly compartments (cVACs). LLPS has been reported to drive RC formation of human cytomegalovirus (a β-herpesvirus) in the nucleus via UL112-113 proteins, which contain self-interaction domains and IDRs, with the viral DNA genome serving as a trigger (2). Herpes simplex virus 1 (an α-herpesvirus) ICP4 protein containing IDRs also contributes to RC formation via LLPS (3). Although all three subfamilies of herpesviruses are shown to form cVACs, the mechanisms underlying cVAC formation remain unknown.

In this issue, Zhou et al. (4) investigated the mechanism of virion assembly of a γ-herpesvirus, murine gammaherpesvirus 68 (MHV-68), in the cytoplasm using recombinant viruses expressing a fluorescently

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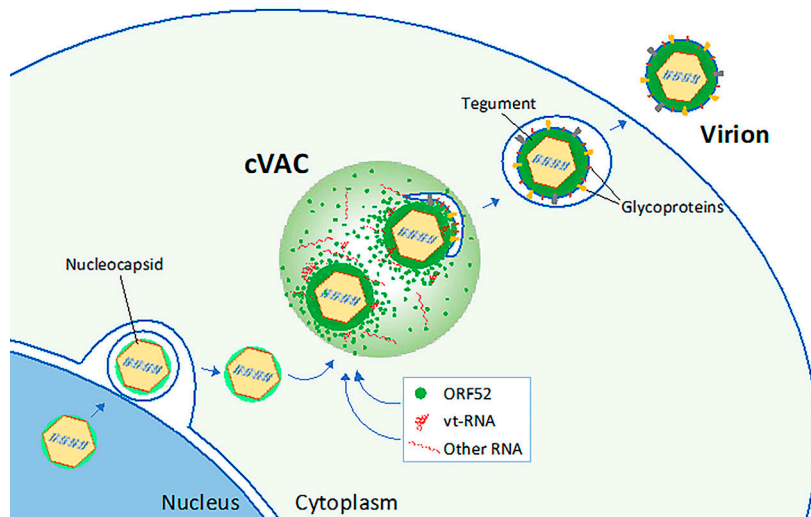Correspondence to Moon Jung Song: moonsong@korea.ac.kr.

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Figure 1. Herpesvirus tegument protein ORF52 undergoes LLPS to form cVACs. During γ-herpesvirus replication, the tegument protein ORF52, together with RNA including vt-RNAs, drives LLPS to form liquid condensates in the cytoplasm. When a nucleocapsid packaged with viral DNA is transported to the cytoplasm, the ORF52–liquid condensates act as cVACs. Other tegument proteins are concentrated to cVACs and virion maturation takes place. The mature infectious virions egress out of the cell and are ready to infect other host cells.

tagged tegument ORF52 protein (mCherry- or mEosEM-ORF52), which allowed the authors to track the kinetics of cVAC formation in real time or under electron microscopy (4). The live-cell imaging and correlative light and electron microscopy (CLEM) results demonstrated that MHV-68 infection induced the formation of cVACs, recruiting other tegument proteins. The dynamic nature of cVACs as membraneless organelles with liquid properties were confirmed in photobleaching experiments. The authors also showed that cVACs disappeared after treatment with 6% propylene glycol (PG), an aliphatic alcohol known to disrupt LLPS, but recovered following the removal of PG. This is the first report that demonstrates LLPS-driven formation of cVACs during herpesvirus infection.

What makes LLPS drive cVAC formation during MHV-68 assembly? The authors identified ORF52 among the tegument proteins, which formed spherical structures with liquid properties in cells. Without ORF52 expression, other viral tegument proteins were not concentrated together and no cVACs were formed, indicating that ORF52, the most abundant tegument protein, is required for LLPS-mediated cVAC formation during MHV-68 infection (Fig. 1). Although ORF52 contains IDRs, similar to

other phase-separating proteins, ORF52 alone was not sufficient to induce phase separation, and additional triggers, such as DNA or RNA, were required. The authors and others recently reported that ORF52 homologs of other herpesviruses undergo LLPS via self-oligomerization when mixed with DNA, disrupting the liquid condensates of a cytosolic DNA sensor, cyclic GMP-AMP synthase, with DNA, thereby serving as a viral immune evasion mechanism (5, 6). Although both DNA and RNA induced ORF52 droplets in phase separation assays in vitro, it was cytosolic nascent RNA rather than DNA that induced ORF52 phase separation in cVACs of infected cells. This is consistent with the notion that viral DNAs are packaged and less likely to be found in the cytosol during the late stage of infection. Total RNA extracted from uninfected or infected cells was able to induce ORF52-mediated phase separation. Interestingly, virion-associated viral tRNAs (vt-RNAs) were sufficient to cause ORF52 droplet formation in vitro (Fig. 1).

To further delineate the ORF52 domains essential for LLPS, the authors tested the ability of mutant constructs to form puncta in transfected cells and found that both oligomerization domains and IDRs, presumably mediating multivalent interactions, were important for phase separation.

When the same mutations were introduced into the viral genome, the formation of cVACs and mature virus particles was found to be correlated with their LLPS properties. Furthermore, ORF52 homologs of other γ-herpesviruses were able to induce phase separation and transcomplement the phenotype of the ORF52 mutant. The conserved IDR of ORF52 homologs was also critical for their phase separation, reinforcing the hypothesis that ORF52 homologs of γ-herpesviruses are essential and functionally conserved in inducing LLPS to form cVACs during virion assembly.

In summary, this study demonstrates that ORF52 builds up fence-free factories in the cytoplasm, where virion assembly takes place. This work raises a question regarding viral morphogenesis in herpesviruses. When cellular RNAs concentrate in cVACs, will they be packaged together into the virion? It will be intriguing to explore how the virus selectively recruits virion components yet excludes unnecessary cellular transcripts, which may be colocalized in cVACs. Overall, taking advantage of sensitive live-imaging and CLEM techniques together with various recombinant viruses, this work deepens our understanding of how herpesviruses exploit liquid condensates to efficiently replicate and produce mature virus particles. The beauty of this work lies in the comprehensive investigation of the biophysical properties of ORF52-induced cVACs via LLPS not only in transfection or in vitro assay systems but also in the context of viral infection, providing deeper insights into viral morphogenesis. These findings, together with other recent reports (7, 8), suggest that targeting LLPS may be a novel effective antiviral strategy that may work in two ways: by direct suppression of virus replication and assembly and by indirect activation of the host innate immunity through releasing the harness of antiviral sensors.

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