

ABSTRACT

Title of Dissertation: THE COMPOSITION, PROPERTIES, AND PHASE SEPARATING BEHAVIOR OF INFECTIOUS BURSAL DISEASE VIRUS (IBDV) VIRUS FACTORIES

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Infectious bursal disease virus (IBDV), a member of the family *Birnaviridae*, is a significant agricultural pathogen of chickens and turkeys, and is the causative agent of infectious bursal disease (Gumboro disease) in chickens. As a double-stranded RNA (dsRNA) virus, IBDV exhibits molecular biology similar to other dsRNA viruses, such as reovirus and rotavirus, including the formation of cytoplasmic inclusions during infection. For all these viruses, the cytoplasmic inclusions are sites of certain aspects of viral replication; in the case of IBDV, they are termed virus factories (VFs). Previous work has shown that VFs form via liquid-liquid phase separation (LLPS), a biophysical phenomenon in which biomolecules segregate into distinct phases without a membranous barrier. The LLPS is also the mechanism by which well-studied cellular structures such as P-bodies, stress granules, and the nucleolus are formed, and is exploited by viruses from multiple families as part of their replicative strategies.

IBDV, and birnaviruses more broadly, are understudied pathogens. As such, the molecular biology of IBDV infection, including the formation, properties, and behavior of VFs, is not fully understood. The objective of this work was to resolve several unanswered questions

regarding the composition and properties of IBDV VFs – namely, I aimed to develop a suite of biochemical and analytical tools for the quantitative measurement of VFs in infected cells, to identify the minimal set of biomolecules required to induce VF formation, and to identify how the physical properties of the structures are modulated. I successfully developed a biological and computational toolkit for the quantitative analysis of VFs and discovered that IBDV VFs exhibit increased liquidity at late time points, a behavior markedly different from that of analogous inclusions formed by rotavirus. Furthermore, I have identified VP3, VP1, and viral RNA as the minimal components for IBDV VF phase separation, which will be useful for VF phase separation research. Finally, our study of VP3 revealed an intrinsically disordered region (IDR) at the carboxy terminus of the protein, which is frequently identified as essential for phase separation. Interestingly, the VP3 IDR is both an important *driver* of VF phase separation and contributes to the liquidity of VFs, although it is not essential to their formation. These findings address several gaps in the understanding of IBDV molecular virology and IBDV VFs, thereby contributing to research on viral replicative structures and LLPS biology.

THE COMPOSITION, PROPERTIES, AND PHASE SEPARATING BEHAVIOR OF
INFECTIOUS BURSAL DISEASE VIRUS (IBDV) VIRUS FACTORIES

by

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Preface

Approximately one in every ten biological entities on Earth is a cell – the rest are viruses. The soils, waters, and organisms of the Earth teem with viruses, around one million for every star in the observable universe.

There is some debate in academic circles as to the proper classification of viruses with respect to the “tree of life”: do viruses merit a full branch, like *Eukarya*, *Archaea*, and *Bacteria* as proposed by Woese, Kandler and Wheelis? Viruses, after all, encode hereditary material in nucleic acids, evolve as populations, and replicate in living cells. While they do not metabolize independently, is this merely intracellular parasitism taken to its logical extreme? And if so, is the taxonomy of life not flawed if an entity could specialize so completely as to no longer be considered life?

I do not propose to answer this question definitively, particularly within the narrow scope of this research. I do, however, wish to proffer my admittedly oblique answer to where viruses belong in the “tree of life”: viruses are not a branch on the tree, but rather a vine that winds around it.

The relationship between viruses and their hosts is an example of remarkable biological intimacy, and given their propensity for borrowing each other’s genetic material, it is no great surprise that strategies and phenomena are shared between them as well. The study of viruses is of interest not only in its own right, but as a window into the strategies and phenomena of life as a whole – my work concerning liquid-liquid phase separation is but one example.

I have loved the study of life from the moment I was introduced to the topic, and I can think of no more fitting way to celebrate that love than this investigation into the macabre ballet between virus and host.

This document was written by a Human, without the use of generative artificial intelligence.

Dedication

To Mom, Dad, Lia, and Alex – for everything.

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List of Abbreviations

(-)ssRNA: Negative sense single-stranded RNA
(+)ssRNA: Positive sense single-stranded RNA
1,3-PD: 1,3-Propanediol
1,6-HD: 1,6-Hexanediol
AU: Airy unit
BSA: Bovine serum albumin
C-: Carboxy
C_{Crit}: Critical concentration
CDS: Coding sequence
CM: Convolute membrane
CMV: Cytomegalovirus
CPE: Cytopathic effect
C_{Sat}: Saturation concentration
DENV: Dengue virus
DFT: Discrete Fourier transform
DMEM: Dulbecco's modified Eagle's medium
DMV: Double membrane vesicle
dsRNA: Double-stranded RNA
EBOV: Ebola virus
eGFP: Enhanced green fluorescent protein
ER: Endoplasmic reticulum
FFT: Fast Fourier transform
MOI: Multiplicity of infection
FG: Phenylalanine-glycine
FRAP: Fluorescent recovery after photobleaching
GFP: Green fluorescent protein
h.p.i.: Hours post infection
h.p.t. Hours post transfection
HCV: Hepatitis C virus
HDBSCAN: Hierarchical Density-Based Spatial Clustering of Applications with Noise
HVR: Hypervariable region
IB: Inclusion body
IBDV: Infectious bursal disease virus
IBV: Infectious bronchitis virus
IDP: intrinsically disordered protein
IDR: Intrinsically disordered region
IPNV: Infectious pancreatic necrosis virus
LD: Lipid droplet
LLPS: Liquid-liquid phase separation
MD: Molecular dynamics
MERS: Middle East respiratory syndrome coronavirus

MF: Mobile fraction
mNG: mNeonGreen
mRNA: Messenger RNA
MSD: Mean squared displacement
MV: Measles virus
N-: Amino
NEB: New England Biolabs
NPC: Nuclear pore complex
ORF: Open reading frame
OVT: Oncolytic viral therapy
P14P: Phosphatidylinositol-4-phosphate
P2: Patch two
PAE: Predicted aligned error
PBC: Periodic boundary conditions
PBS: Phosphate buffered saline
PDI: Protein disulfide isomerase
PFA: Paraformaldehyde
PRR: Pattern recognition receptors
PVA: Paracrystalline virus array
RdRp: RNA-dependent RNA polymerase
ReoV: Reovirus
RG: Reverse genetics
RLR: RIG-I-like-receptor
RNP: Ribonucleoprotein
ROI: Region of interest
rRNA: Ribosomal RNA
SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2
SEM: Standard error of the mean
SG: Stress granule
SMV: Single membrane vesicle
TC: Tetracysteine
TCID₅₀: Tissue culture infectious dose 50
TEM: Transmission electron microscopy
UMAP: Uniform manifold approximation and projection
UTR: Untranslated region
VF: Virus factory
vRNA: Viral RNA
vRNP: Viral ribonucleoprotein
ZIKV: Zika virus

Chapter 1: Introduction

Viral Replication Complexes

Viral genome replication can be conceptually considered to be a complex biochemical reaction. As viruses must co-opt cellular machinery to replicate, the essential biomolecules involved in replication may include both viral and cellular components sequestered at the site of viral replication. These biomolecules can be considered as “reactants” in the replication reaction, and the rate of viral replication is therefore dependent on the nature and concentration of the reactants. For eukaryotic cellular biochemical reactions, concentration control and reactant selectivity can be achieved by membranous compartmentalization into organelles whose internal composition and chemistry are tightly regulated. As the majority of viruses face substantial selective pressure for gene and protein economy, they typically appropriate existing organelles or portions thereof to facilitate replication, rather than encoding the machinery to create their own organelles outright. The induced reorganization of the subcellular environment can be profound, and the resultant structures where viral replication proceeds are typically termed “viral replication complexes”.

Viruses with a genome comprised of positive-sense (+), single-stranded (ss) RNA are known to remodel the membranous compartments of the cells they infect to facilitate viral replication. For example, the *Flaviviridae*, including Dengue virus (DENV), Zika virus (ZIKV), and hepatitis C virus (HCV) exhibit remodeling of the endoplasmic reticulum (ER)[1][2] [3]. Viral proteins induce abnormal curvature in the ER membrane, causing invaginations into the ER lumen, which can include inward-facing “spherules” connected to the cytosol by a narrow “neck” region, as

well as convoluted membranes and dilated ER cisternae. The membrane itself can then be remodeled, for example, by the recruitment of phosphatidylinositol-4-phosphate (PI4P) and cholesterol, and can be invaded by lipophilic viral proteins, such as NS1 in the case of DENV and ZIKV [4][5]. As the canonical replication compartment, ER spherules create an environment that can be chemically modulated (leveraging the selective permeability inherent to the ER membrane), can be enriched in advantageous biomolecules such as ribonucleotides, and by virtue of the physically constrained “neck” region connecting the spherule to the cytoplasm, shield immunogenic biomolecules such as the double stranded (ds) RNA intermediates of ssRNA replication from cytoplasmic immune surveillance [1]. In contrast, the *Picornaviridae* family induces the formation of both single membrane vesicles (SMVs) and double membrane vesicles (DMVs) [6]. In the case of poliovirus, vesicular structures accumulate throughout the course of infection, appearing to “mature” at later stages, progressing from predominantly single-layered to double and multi-layered at late stages [7]. These vesicles are structurally and compositionally akin to autophagosomes, though unlike true autophagosomes, fusion with lysosomes is blocked, and their formation is induced virally, in the case of poliovirus, by the action of proteins including 3AB and 3BC [8][9]. Similar to the spherules induced by members of the *Flaviviridae*, *Picornaviridae* vesicles segregate potentially immunogenic biomolecules from the cytosol and concentrate replicative machinery and substrates to facilitate efficient replication [10].

Similar to the members of the *Picornaviridae* and *Flaviviridae*, viruses of the *Coronaviridae*, including Middle East respiratory syndrome coronavirus (MERS), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and infectious bronchitis virus (IBV), induce a variety of membranous structures, including invaginated ER spherules, convoluted membranes (CMs),

and DMVs [11]. In the case of SARS-CoV-2, DMVs are thought to be the sites of viral RNA (vRNA) synthesis, with transport between the synthesis site within the DMV lumen and the cytosol being facilitated by a proteinaceous channel composed of viral proteins NSP3 and NSP4, which form a membrane-spanning RNA-specific transporter, structurally reminiscent of the nuclear pore complex (NPC) [12,13].

Viruses with a negative (-) sense ssRNA genome within the *Orthornavirae* Kingdom, including *Mononegavirales* such as Lyssaviruses, measles virus (MV), and Ebola virus (EBOV) generate replicative complexes by an entirely different biochemical mechanism from the membrane reorganization of the (+)ssRNA viruses. The replication complexes induced by these viruses have no encompassing membrane, and are instead a type of biomolecular condensate: a collection of biomolecules with relatively weak average intermolecular interactions, but distinct bulk physical and chemical properties [14–17]. The nomenclature surrounding virus-derived structures is somewhat confusing, as in different contexts, it may be advantageous to name and describe these bodies by their appearance, their physical properties, or their function. The terms “inclusion”, “puncta”, “body”, or variants thereof are based on their appearance in micrographs [18][19][20][21][22]. However, appearance is often insufficient to describe the properties and behaviors of the structures, and so physical and biochemical descriptors such as “biomolecular condensates” or “viroplasms” have also been used, which may be appropriately applied once experiments have been performed validating the physicochemical nature of the structures [23][24]. Finally, a well-studied structure may be named for its function, such as “virus factories”, “replication complexes”, or “replicative bodies” [25][26][27].

Utilization of biomolecular condensates as replication complexes offers a notable complexity advantage: they tend to form spontaneously or semi-spontaneously when the minimal component set is present at sufficient concentration globally, or around nucleation sites when the minimal component set only reaches sufficient concentration locally. The minimal component set can include as little as a single protein [28–30], but more often includes a small set of proteins and potentially nucleic acids as well [31–33]. Despite their minimal component sets, biomolecular condensates can adopt nuanced compositions and coordinate complex chemistry through the recruitment of additional components. Once the condensate has formed from the minimal component set, biochemical “affinity” leads to the selective recruitment of some biomolecules and the exclusion of others, effectively generating a higher-order biochemical complex while requiring only a small set of starting materials.

Among Lyssaviruses, MV, and EBOV, condensate replication complexes are generally composed of viral RNA, alongside three proteinaceous components: N, L, and P (VP35 in the case of EBOV) [15,34,35]. Among the best-characterized of the (-)ssRNA replication complexes are the inclusion bodies (IBs) of MV infection. Cells infected with MV exhibit numerous small, near-spherical IBs throughout the cytoplasm, which diffuse rapidly and readily coalesce. The IB formation phenomenon is so robust in MV that the process can readily be recapitulated in cell free systems: a combination of N and P proteins is sufficient to drive the formation of “droplets” which take up genomic viral RNA, the amount of which influences the measured viscosity of the droplets [15]. Functionally, vRNA replication likely takes place within the interior of MV IBs, with transcription (and dynamic exchange with the cytosol) indicated to be localized to the periphery, although the composition of MV IBs is relatively homogenous throughout [14] [15].

Though compositionally similar, the IBs formed by Lyssaviruses, termed “Negri bodies” (a typical pathognomonic sign of rabies virus infection), show evidence of internal biochemical organization. Negri bodies are also composed of N, P and L proteins, along with genomic and antigenomic RNA [36], and imaging studies have revealed spatial segregation of functions such as translation and replication into biochemically distinct subdomains. This contrasts with the “well mixed” nature of MV IBs, where, although functions are spatially discrete, that discretization is largely a function of position rather than composition [14,37]. In other words, although different regions of MV IBs have different functions, the composition is largely uniform, and *location* within the IB determines function rather than arising from multiphasic organization.

Conceptually bridging the behavior of Lyssavirus and MV replication complexes are those induced by EBOV. EBOV IBs, which are known to coordinate nucleocapsid assembly, are composed of viral RNA, N, L, VP35 (an EBOV analog for P), and viral transcription factor VP30. Although relatively large, EBOV IBs exhibit liquid-like behavior at early time points, including coalescence [16,19,38]. Interestingly, EBOV IBs appear to undergo “maturation”, both chemically and physically. Chemically, an additional viral protein, VP24, is increasingly recruited at later timepoints, and physically, EBOV IBs exhibit a greater degree of subdomain organization at later timepoints, suggesting maturation into more structured condensates [16,19]. Biomolecular condensate-based replication complexes are therefore capable of coordinating and regulating replication chemistry both spatially and temporally, while arising from a relatively simple component set.

Replication complexes comprised of biomolecular condensates are also found in viruses with double-stranded RNA (dsRNA) genomes, including families such as the *Reoviridae* and *Birnaviridae*. The *Reoviridae* family is the best-characterized of the dsRNA viruses, and the replication strategies of multiple member taxa are subjects of substantial research interest. Of the *Reoviridae*, rotavirus and mammalian reovirus (ReoV) are perhaps the most comprehensively studied, and both notably use biomolecular condensates as replication complexes.

In the case of Rotavirus, these biomolecular condensates are termed “viroplasms”, and like the IBs of *Mononegavirales*, have a multi-protein minimal component set – though rather than the tripartite N, P, and L set characteristic of *Mononegavirales*, rotavirus condensates are induced by the combination of NSP2 and NSP5 [23,39–42]. NSP5 is a flexible, non-structural protein that can assemble into several higher-order oligomers, and the carboxy (C-) terminal region of NSP2 is flexible, allowing it to participate in domain-swapping interactions that may contribute to viroplasm formation. In ReoV, the component set is more minimal still: a single nonstructural protein, μ NS, is sufficient to induce the formation of biomolecular condensates [43,44].

Although the utilization of biomolecular condensates as a strategy to assemble replication complexes is implemented by diverse means across viruses, the phenomena share a common chemical basis: The formation of these condensates is largely driven by liquid-liquid phase separation (LLPS), a biophysical and biochemical phenomenon that facilitates the emergence of higher-order structures from simple intermolecular interactions, and imbues the resultant condensates with physical properties advantageous to viral replication.

Liquid-Liquid Phase Separation

LLPS as a phenomenon is not restricted to viruses, and is of notable and increasing research interest in cell biology, in part due to its ubiquitous nature throughout the cell and across taxa. This ubiquity may in part be explained by the relatively simple physical underpinnings of the phenomenon: LLPS is driven by the formation of molecular assemblages from biomolecules capable of multivalent interactions [31,45]. It is distinguished from aggregation by the characteristically weak strength of the interactions, which permit diffusion of the biomolecules, while the cumulative effect of the interactions maintains the energetic favorability of phase separation. In such a system, “solute:solute” interactions are favored and “solute:solvent” interactions are disfavored, with the energetic gulf widening as the concentration of solute increases. Consequently, for a given set of environmental conditions, a saturation concentration (C_{Sat}) exists where interactions between solute and solvent are so unfavorable that the mixture will spontaneously “de-mix”, changing from a single homogenous phase to a biphasic system where a “heavy” or “dense” (solute rich) and “light” or “dilute” (solvent rich) phase co-exist but do not co-localize (Figure 1.1).

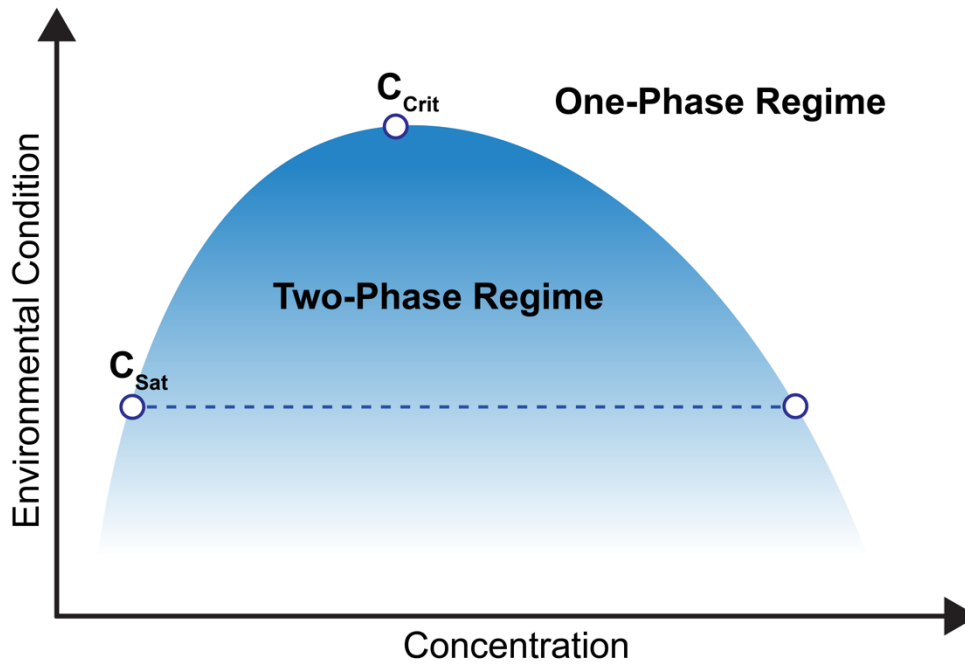


Figure 1.1 Representative phase transition diagram. A representative phase transition diagram for a single-component phase separating system. Component concentration on the X axis (unitless) is plotted against environmental condition (unitless) on the Y axis. Environmental conditions are multivariate and include temperature and osmolarity. The saturation concentration is denoted C_{Sat} , and the critical concentration is denoted C_{Crit} . Note that at some extreme environmental conditions such as very low temperatures, “phase separation” is poorly defined, reflected here by the lack of a precisely defined lower bound to the two-phase region.

Macroscopically, this typically takes the form of distinct “droplets” of heavy phase within the light phase. Because the heavy phase remains liquid and exhibits viscoelastic properties, these droplets relax towards shapes that minimize interfacial surface area, hence the emergence of the spherical or rounded structure typical of condensates formed by LLPS [46].

Constituent components of biomolecular condensates formed through LLPS can be broadly categorized as “scaffold” or “client” biomolecules. Scaffold biomolecules are essential for phase separation to occur and contribute to the requisite multivalency that drives the phenomenon, whereas client biomolecules are recruited and phase separate, but would not do so in the absence

of the scaffolds [47]. The requirement of multivalent and weak interactions for phase separation means that intrinsically disordered proteins (IDPs) and proteins with intrinsically disordered regions (IDRs) are commonly found in LLPS structures, as these proteins and domains lack a single well-defined secondary structure, enabling them to participate in numerous weak or transient interactions as they explore their conformation space [47][48]. However, the requisite multivalency for phase separation can also be achieved through structured protein domains, meaning IDPs or IDRs are not absolutely required for LLPS.

Eukaryotic cells make use of same advantages of the LLPS phenomenon as do viruses: LLPS condensates tend to assemble spontaneously and often quickly, concentrate biomolecules, and because they can selectively recruit client molecules or exclude others, enable relatively tight chemical control without the energetic and material costs of membrane-bound organelle assembly. They therefore permit the local dynamic compartmentalization required for the cell to respond to different conditions. Typical examples of cellular LLPS condensates include processing (P)-bodies, stress granules (SGs), and the nucleolus, but structures including the NPC have been found to exhibit LLPS characteristics.

P-bodies are cytoplasmic ribonucleoprotein (RNP) granules that serve as dynamic sites for mRNA translational repression, storage, and turnover [49]. The “scaffold set” of P-bodies includes RNA, along with RNA-binding proteins including Dcp1, Dcp2, and Lsm1-7 [50–53]. Moreover, the mRNA undergoing processing within the P-bodies is a critical driver of the phenomenon – non-translating mRNA must accumulate for de-mixing to occur. This direct linkage of P-body formation to mRNA concentration illustrates an advantage of the LLPS

phenomenon: because P-bodies are a direct *consequence* of mRNA concentration, there is no need for mRNA feedback or sensing machinery, the P-bodies themselves are an emergent phenomenon of the condition they address. Moreover, the “solubility” of the RNA within a P-body is determined by translational status, as mRNAs undergoing translation are unable to enter the heavy phase, thus substrate specificity is achieved as a simple emergent physical property rather than requiring complex regulation [54].

SGs are cytoplasmic RNP structures formed by LLPS in response to conditions that impede translation initiation. These structures are composed of untranslated mRNAs along with their associated proteins and are also associated with global translation repression and mRNA triage. During periods of oxidative stress, mRNA translation is frequently stalled by mechanisms including the phosphorylation of eIF2 α [55–57]. This leads to a relatively rapid rise in the concentration of stalled mRNPs in the cytoplasm. In concert with a set of RNA binding proteins, including G3BP1/2, TIAR, and FMRP, the stalled mRNPs phase separate into SGs, physically sequestering the stalled transcripts and allowing cells to prioritize the translation of stress response genes [58–60]. Several of the RNA-binding proteins, particularly G3BP1, exhibit a high degree of intrinsic disorder, as is frequently found in proteins associated with LLPS [61]. Therefore, although nucleic acids themselves can provide the flexibility and multivalency required to drive the LLPS phenomenon, they may also do so in concert with IDPs or proteins with IDRs.

Although the NPC was previously viewed as a proteinaceous pore, more recent research has illuminated the role of LLPS in the NPC. The interior of the pore is rich in phenylalanine-glycine

(FG) repeat nucleoporins, which form a phase separated matrix within the pore, and biophysical compatibility with the FG LLPS network contributes to the NPC's selectivity [62,63]. Moreover, physical disturbances like the passage of large cargoes do not permanently deform the matrix, and it is able to elastically restore the pore. Compatible materials face minimal energetic barriers when transiting the pore, as they freely enter the NPC's heavy phase and therefore transit quickly without needing to transit a rigid matrix, but incompatible cargoes face a nearly insurmountable energetic barrier and are thus excluded efficiently [64].

One of the earliest and best-characterized examples of cellular LLPS is the nucleolus, the nuclear site of ribosomal biogenesis. The nucleolus is composed largely of ribosomal RNA (rRNA) and associated ribosomal proteins, where nascent rRNA transcripts provide the requisite multivalency for LLPS to occur [65]. The nucleolus is therefore similar to SGs and P-bodies in that they are emergent phenomena driven by the cumulative effect of a network of weak interactions coordinated by RNA, but differs substantially in function [66]. The nucleolus also exhibits notable functional inhomogeneity. The center of the nucleolus is notably fibrillar, and coexists with a dispersed granular phase. These “subphases” are liquid and coexist within the larger “heavy phase” of the nucleolus as a whole, but are materially and functionally distinct. The nucleolus is therefore not only an example of the LLPS phenomenon in cells, but an example of the higher-order spatial and functional discretization achievable by the phenomenon even in the absence of membranes [33].

In addition to the examples described above, there are many less well characterized incidences of the LLPS phenomenon that contribute to cellular function and viral replication, which present

opportunities to increase our understanding of LLPS itself. For example, among the aforementioned dsRNA viruses, Rotavirus and ReoV are the best studied, and by consequence, so too are the LLPS condensates they form. Yet a related dsRNA virus family exists, which forms notable biomolecular condensates, but has comparatively poorly understood LLPS behavior: the *Birnaviridae*.

Birnaviridae

The *Birnaviridae* is a family of viruses that have a single protein capsid, and a bipartite dsRNA genome [67] (Figure 1.2).

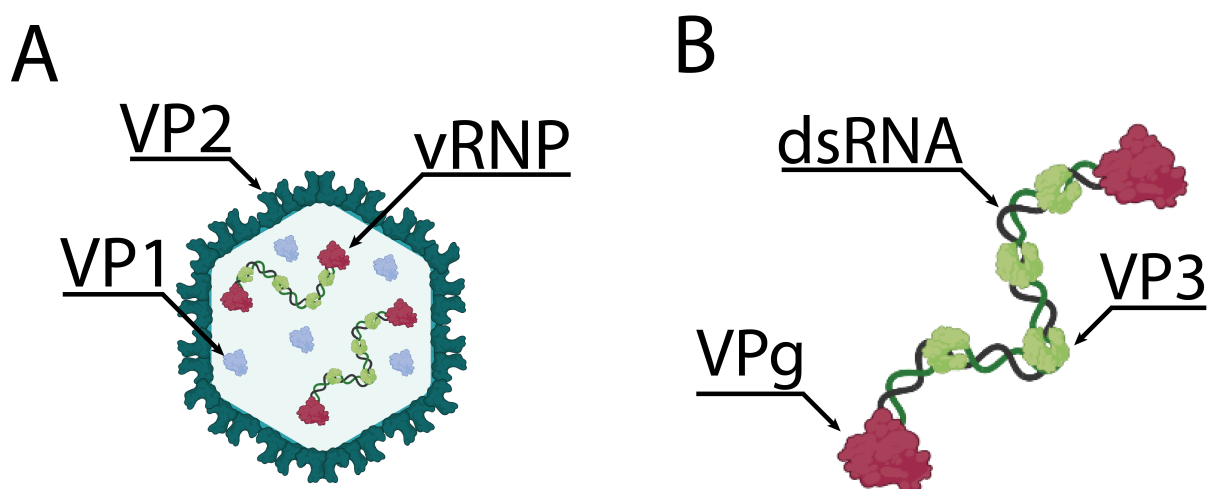


Figure 1.2 The IBDV virion and vRNP. Schematic of the IBDV virion composed of units of viral protein (VP)2 organized into a single-layered T=13 icosahedral capsid (dark blue), packaging the bipartite genome as 2 segments of vRNP (A). The vRNP segments consisting of a dsRNA genome segment (dark and light green helix), coated with VP3 acting as an RNP (light green), with the free 5' end of each strand associated with VP1 acting as VPg (maroon) (B).

Birnaviruses have been isolated from birds, fish, rotifers, mollusks, and insects such as mosquitoes and flies, and the sequences of birnaviruses have also been found in pigs, porcupines, and the feces of bats [67][68][69][70][71][72]. Of these, infectious pancreatic necrosis virus

(IPNV) of the salmonid fishes, and infectious bursal disease virus (IBDV) of phasianid birds are of the most significant economic impact to aquaculture and the poultry industry respectively, with considerable investment being made into disease control, for example through the use of vaccines or the engineering of more disease-resistant animals [68][67]. Despite their importance, little is known concerning the replication cycle of the *Birnaviridae* compared to the more well-studied *Reoviridae* family. Traditionally, much of our understanding of birnavirus replication was extrapolated from work with reoviruses, however, more recently, studies have suggested that birnaviruses have some features more akin to (+)ssRNA viruses.

The name “birnavirus” is a portmanteau of “bisegmented RNA virus”, and as such, the genome is comprised of two segments of dsRNA (Segments A and B), which are contained within a single-layered icosahedral capsid with a T=13 symmetry [73][74]. The larger of the two segments (Segment A) encodes a polyprotein which undergoes co-translational autocatalytic cleavage by the viral protein VP4 protease into proteins pre- (p-)VP2 (VP2 ultimately forms the capsid), VP4, and VP3, which is a protein with multiple functions [75][76][77]. Segment A also contains an overlapping secondary open reading frame (ORF) encoding VP5, which may play a role in modulating virulence, and non-lytic egress of virions [78][47][48]. The smaller of the two segments (Segment B) encodes VP1, the viral RNA-dependent RNA polymerase (RdRp) [79][80] (Figure 1.3).

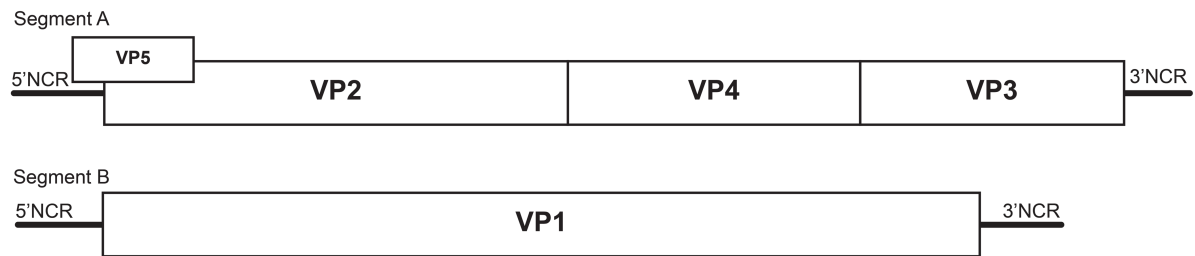


Figure 1.3 IBDV genome schematic. A schematic representation of the IBDV genome. Segment A (top) encodes a polyprotein comprised of pVP2, VP4 and VP3, which is cleaved by VP4 into individual components, and pVP2 is proteolytically processed into VP2 (A). Segment B (bottom) encodes only VP1.

Within the virion, the birnavirus genome segments exist in the form of a viral ribonucleoprotein (vRNP) complex, associated with packaged VP1 and VP3, the latter of which binds to the genome by virtue of a sequence-independent RNA-binding domain [78][81]. VP3 also associates with the inner face of the VP2 capsid. Moreover, while one full genome is comprised of one Segment A and one Segment B, *Birnaviridae* particles have been recovered from infected cells that contain between 0 and 4 genome segments, and it is thought that birnaviruses undergo so-called “random packaging” where newly synthesized vRNP complexes nucleate encapsidation, rather than being packaged into pre-formed or partially formed capsids [82][81].

The surface of the birnavirus capsid is comprised of pentamers and hexamers of VP2 homotrimers, arranged so as to externally project a hypervariable region (HVR) which is known to contain major epitopes of antibodies [83][84]. As the name of this region implies, mutations are common, likely the result of immune selection pressure driving antigenic drift, and the HVR sequence serves to distinguish between strains [85]. This propensity for mutation has hampered the development of broadly cross-protective vaccines. In addition, the bipartite birnavirus

genome is capable of reassortment in co-infected cells, further complicating epidemiology and disease control [86][87].

IBDV

IBDV reproductive cycle

The IBDV reproductive cycle begins with attachment of the viral particle to the host cell. Our knowledge of the molecules IBDV particles bind to on the surface of host cells is incomplete, although HSP90, IgM, CD74, and CD44 have been implicated [88][89][90][91]. Following attachment, the virus is taken up into an endosome, whereupon the gradually decreasing pH and concentration of calcium ions promote the disassembly of the capsid, releasing a small peptide, pep46, which promotes pore formation in the endosomal membrane, granting the vRNP complexes access to the cytoplasm [92][93]. Cytoplasmic puncta, or inclusions, appear shortly after, notably enriched in viral proteins VP1 and VP3, as well as viral dsRNA. These structures, termed “virus factories” (VFs), are visible by fluorescence microscopy from approximately 6 hours post infection (h.p.i), and are initially associated with endosomal membranes [93]. Over the course of infection, these structures grow both due to viral component synthesis and coalescence [94]. Although it has been observed that VFs are both enriched in dsRNA and are the sites of *de novo* RNA synthesis, unlike the replicative bodies of related reo- and rotaviruses, IBDV VF have not been observed with virus particles inside, and synthesis of vRNPs does not require the capsid [95][96]. Instead, transmission electron microscopy (TEM) reveals so-called paracrystalline virus arrays (PVAs), composed of tightly packed assembled virions in the cytoplasm, and it remains unknown how a VF structure leads to the formation of a PVA. It is thought that, upon the death of the host cell, virions within the cytoplasm are released to the

extracellular space during cell lysis. However, IBDV infected cells can also shed virus prior to lysis, in a process that has been reported to be mediated by protein VP5, and work by Méndez *et al.* demonstrated the VP5-dependent appearance of membrane-wrapped PVAs, as well as a network of single-membrane vesicles containing viral particles, which may be exported from the cell [97].

IBDV VF Formation

Following viral uncoating, and the extrusion of the vRNP complexes into the cytoplasm, Gimenez *et al.* demonstrated a colocalization of VP3 with the early endosomal marker protein Rab5, a behavior further confirmed by colocalization of VP3 with Rab5-Q79L, a constitutively active Rab5 mutant that, due to overstimulated vesicular fusion, results in enlarged vesicles around which the distribution of VP3 could be readily observed [98]. Furthermore, the authors revealed that VP3 associated with the cytoplasmic leaflet of the endosomal membrane. After treatment with saponin, which permeabilizes the plasma membrane and internal membranes, both VP3 and the ER resident protein disulfide isomerase (PDI), which serves as a positive control for internal membrane permeabilization, were detected. However, permeabilization with digitonin, which can be made to selectively permeabilize only the plasma membrane, ablated PDI staining but did not affect VP3 detection, indicating that VP3 is exposed to the cytoplasm. This same investigation provided evidence that a VP3 region referred to as “patch 2” (P2) (residues 159, 168, 198, 200) comprised a surface-exposed positively charged motif, which regulated the membrane associating behavior of VP3. In cells expressing VP3 with the P2 residues mutated to the negatively charged aspartic acid, colocalization of VP3 with Rab5 was lost, with the VP3 signal assumed a ubiquitous cytoplasmic distribution. This led to the hypothesis that IBDV replication complex could be seeded on endoplasmic membranes [92].

Of the early endosome (EE) population with which VP3 was observed to colocalize, there was a particular affinity of VP3 for phosphatidylinositol-3-phosphate (PI3P) enriched EEs, and VP3 appeared to form a non-uniform coating around PI3P enriched EEs. This behavior was further confirmed by the treatment of VP3 expressing cells with PI3P production inhibiting drugs LY294002 and Vps34-IN1, both of which prevented the appearance of VP3 puncta. Furthermore, treatment with rapamycin induced the detachment of VP3 puncta from EEs by recruiting FKBP-MTM1 to EEs, which dephosphorylated PI3P. Although VP3 puncta remained visible following such treatment, there was a notable reduction in average puncta diameter. Taken together, these data support a mechanism for IBDV VF formation based upon nucleation at PI3P enriched EEs, and the discontinuous VP3 “coating” of these EEs may serve as a region of enriched VP3 for the nucleation of other VF components, and ultimately the formation of the VF [98]. Recent work by Zanetti *et. al.* uncovered the mechanism by which IBDV VP3 is seeded onto PI3P-enriched EE membranes in greater detail – the interaction is initially mediated by nonspecific adsorption of the C-terminus of VP3 to the membrane driven by electrostatic forces, which is later reinforced by the interaction of P2 with PI3P, with an arginine at position 200 (R₂₀₀) being essential both to the interaction and to IBDV replication [99].

This reliance on host membranes classifies IBDV VF formation as a heterogeneous nucleation event, a strategy shared by other members of the *Reoviridae* family but utilized differently. For instance, rotavirus viroplasms are intimately associated with lipid droplets (LDs), where rotavirus proteins NSP2 and NSP5 recruit LDs to the viroplasm. Although the nucleation behavior has not been characterized in detail, the association is notable and may influence phase

separation dynamics [100]. In the heterogenous model, the platform—whether the PI3P-rich endosome for IBDV or the LD for rotavirus—may act as a “wetting” surface that stabilizes the early condensate [41,101,102].

In contrast, ReoV factories, driven by the μ NS protein, form via phase separation in the cytosol without a strict requirement for membrane enclosure or heterogenous nucleation. Unlike the membrane-anchored factories of IBDV and rotavirus, ReoV inclusions appear to form autonomously in the bulk cytoplasm or in association with the cytoskeleton, highlighting a divergence in how segmented dsRNA viruses spatially organize their replication machinery.

IBDV VF properties

The properties of LLPS-induced biomolecular condensates can be characterized by quantifying the dynamics of movement, the fluorescence recovery after photobleaching (FRAP), and condensate break-down in response to treatment with aliphatic diols such as 1,6-Hexanediol (1,6-HD). These properties have therefore been measured for IBDV VFs, to evaluate whether they were formed through LLPS [103][104][105].

First, IBDV VFs were fluorescently tagged *in-vivo* by the generation of recombinant IBDV viruses. Utilizing a 2-plasmid reverse genetics system encoding the lab-adapted IBDV strain PBG98, a “split-green fluorescent protein (GFP)”-tagged virus was rescued, with the GFP-11 domain fused to the C-terminus of VP1, and a tetracysteine (TC)-tagged virus was also rescued, with the TC motif fused to the same location. VFs containing VP1-GFP11 or VP1-TC could be

detected in cells either expressing GFP1-10, or in cells stained with biarsenical dyes, respectively. Timelapse microscopy revealed that IBDV VFs were highly mobile within the cytoplasm, exhibiting movement on both small and large scales, with large-scale movement reduced upon treatment with either nocodazole, or cytochalasin-D, suggesting that microtubule trafficking and the actin cytoskeleton may actively or passively (by collision) drive translocation of the structures. Furthermore, VFs were observed to undergo fission and fusion events, where a single VF splits into two or more smaller bodies, or two or more smaller VFs collide and merge into a single structure, respectively. Notably, the dynamics of VF populations caused by multiple simultaneous infections were found to be initially independent. Cells simultaneously infected with split-GFP labeled IBDV and TC labeled IBDV initially developed separate populations of VFs positive for each tag. Over time, fusion of the VFs within the cell lead to the emergence of large, “double-positive” VFs. The role (if any) of such initially segregated VF populations and subsequent cross-strain VF fusion in genome mixing and recombination events remains unknown [94].

FRAP assays demonstrated that recovery of bleached regions of split-GFP- labeled VFs in GFP1-10 expressing cells occurred on relatively short timescales (approximately 140 seconds for maximum recovery), suggestive of an internal liquid state. This property distinguished the IBDV VFs from protein crystals or aggregates. Moreover, IBDV VFs also exhibited sensitivity to 1,6-HD treatment. 1,6-HD is a small aliphatic diol, which typically causes the rapid breakdown of LLPS structures, likely by perturbing the hydrogen bonding environment. As such, reactivity to this molecule is considered a standard test for LLPS, and split-GFP IBDV-infected cells treated

with 4% 1,6-HD exhibited near-complete loss of VF structure within 90 seconds of treatment [95].

Taken together, the observations that IBDV VFs moved rapidly in the cytoplasm, had a rapid FRAP, and were dissolved by 1,6-HD treatment are consistent with the VFs being formed by LLPS. As LLPS structures require the establishment of an interaction network, a sufficient concentration of scaffold components must be present for the structures to nucleate. If one or more condensate scaffolds have an affinity for a cellular structure or compartment, they will accumulate in the vicinity, until the critical concentration is reached for phase separation to occur, whereupon the LLPS structure could form at that site. In the context of IBDV, it is hypothesized that the affinity of VP3 for PI3P-containing EE membranes causes the nucleation of the IBDV VFs at these sites, which form and grow by means of LLPS. However, whether the VFs remain associated with these membranes throughout the reproductive cycle, or whether they dissociate and associate with other compartments is poorly understood. Recently, it has been shown that in IBDV-infected cells, VP3 structures associated with the Golgi complex, and even remained associated with Golgi stacks after nocodazole treatment of the cells caused disruption to the native distribution of the Golgi apparatus [106]. Furthermore, these authors discovered that a Rab1b-GBF1-ARF secretory pathway axis was essential for IBDV replication, demonstrating that the IBDV VF structures can contact cellular membrane compartments to perform critical roles in viral replication. This interaction of LLPS structures with cellular membranes remains an understudied area of cell biology [93].

IBDV VF processes

As previously described, virus derived replicative structures are given diverse names, such as “puncta” which reflect physical appearance, “biomolecular condensates” which reflect the mechanisms of formation, and “virus factories” which reflect function. In the case of IBDV, all of these classes of descriptor can be meaningfully applied to some extent: IBDV VFs have a punctate appearance in infected cells, exhibit properties of LLPS, and are the site of at least one step of the viral replication process, RNA synthesis. Experiments performed by Reddy *et. al.* involved pulse-treatment with 5-ethynyl uridine (EU), a reactive RNA nucleoside analogue. RNA synthesized during the pulse period incorporates this analogue and may be detected using the Click-IT staining system [107]. Such treatment of split-GFP tagged IBDV in infected DF-1 cells revealed a strong colocalization of the *de novo* RNA signal with the split-GFP VF marker, suggesting the VFs to be the site of RNA synthesis [95]. While the *de novo* synthesized RNA detected by this protocol could be replicative intermediates, or genomic strands, they could also be molecules resembling mRNA that are destined for translation. As previously discussed, ribosomal proteins have been shown to co-localize with VP3, and it is possible that translation also occurs within or proximal to the VFs, although this remains to be experimentally verified [108]. The role of the VFs in viral assembly also remains poorly understood. As previously described, IBDV virions form tightly packed PVAs in infected cells. However, unlike reo- and rotaviruses, these PVAs are separate from the VFs, and virions are not observed within VFs of IBDV, raising questions as to the location of their assembly, and the molecular mechanisms underpinning this process [95][109]. Since the viral dsRNA genome is detectable within VFs, as are viral structural and nonstructural proteins, the necessary materials are present within VFs to assemble virions. Three potential mechanisms readily present themselves from the available

data: off-site assembly, assembly and ejection, and simultaneously assembly. The off-site assembly hypothesis posits that viral components are translocated to another cellular site, where they are assembled into completed virions. The assembly and ejection hypothesis posits that virions are assembled within or proximal to VFs, but are incompatible with the VF's liquid phase, and are therefore ejected into the cytoplasm. The third, simultaneous assembly, would suggest that an entire VF may reach a critical concentration of viral components, at which time virions would rapidly and spontaneously form throughout, forming a group of virions and “converting” the VF from a condensate to a group of assembled viral particles. Experimental evidence favoring one of these models is, at present, lacking.

IBDV VF interaction with cellular structures

The edges of IBDV VFs have been observed to colocalize with the microtubule network and the actin cytoskeleton, and nocodazole or cytochalasin D treatment inhibited VF coalescence, consistent with these structures playing a role in VF movement in the cytoplasm [94]. In addition, IBDV VFs have been found in close association with the Golgi network, even after disruption of the Golgi distribution by nocodazole treatment [110]. However, the localization of IBDV VFs with other cellular structures, for example, LDs, mitochondria, SGs, etc., has not been investigated to date.

IBDV VF Composition

It is known that viral dsRNA, and the proteins VP3 and VP1 strongly colocalize with VFs, however, the IBDV VP2 capsid protein was found to have a substantially lower colocalization, with an average Mander's coefficient of colocalization with VP1-GFP of 0.6, compared to that of

VP3, which was 0.9, consistent with the VFs being discrete structures to the PVAs seen by TEM [111][95]. It is known that VP3 is critical to VF formation, as in its absence, IBDV proteins do not show a tendency to form inclusions, however, the molecular mechanisms by which VP3 forms a VF remain poorly understood [95]. It is possible that VP3 drives LLPS, however, the domains on the VP3 responsible for this phenomenon, and the mechanism by which this is achieved, have thus far remained unknown. In the terminology of LLPS, VP3 represents the condensate scaffold, and other viral components incorporated into the VFs are client biomolecules [112]. The range of possible client molecules recruited to a VF is not limited to those derived from the virus however, and numerous condensate-forming viruses incorporate host -cell proteins into their replication complexes, for a variety of biological goals [36],[114]. The IBDV VF likely provides an environment enriched in the biomolecules and cellular proteins required for viral replication. In addition, the IBDV VF likely shields viral components from triggering cellular innate antiviral responses and may sequester signaling pathway proteins away from their intended interaction partners, preventing pathway activation, in a manner similar to the strategies employed by other viruses [115][21]. It is also reasonable to hypothesize that biomolecules which normally associate with known client molecules may themselves be recruited as clients [116]. To this end, the cellular interactome of VP3 has been assayed by Co-immunoprecipitation (Co-IP) and mass spectrometry (MS). In one instance of this assay, 137 interacting proteins were identified, giving an indication as to the breadth of possible VP3 clients that may be recruited to VFs during infection. Protein-protein interaction (PPI) analysis of 121 of these proteins revealed several functionally connected groups, for example numerous mutually-interacting ribosomal subunit proteins, clusters of metabolically active proteins, regulatory proteins of the actin cytoskeleton, and cardiomyocyte adrenergic signaling molecules, indicating

the breadth of possible functions of the VP3 scaffolded VF interaction network [108]. However, while defining the VP3 interactome by Co-IP and mass-spectrometry provides valuable information for follow-up studies, the necessary cell lysis steps in the protocol means any LLPS structures would be lost, and different approaches are required for defining the IBDV VF composition explicitly.

In addition to defining the VP3 interactome, several studies have characterized the interaction of VP3 with individual cellular proteins: VP3 undergoes post-translational modification, including ubiquitination, and of the E3 ubiquitin ligases, TRAF6 has been observed to directly interact with VP3 and cause a significant enhancement of VP3 K11- and K33- linked ubiquitination. This enhances the stability of VP3, markedly extending the protein's half-life [117][118]. VP3 exerted the opposite effect on TRAF6 itself: the presence of VP3 significantly reduced the level of TRAF6 detectable by western blot, while the level of TRAF6 transcription remained unchanged, suggesting that VP3 destabilized TRAF6 by promoting its autophagic degradation. As a consequence, TRAF6-mediated NF κ B activation was impaired, attenuating downstream immune responses [117]. This contrasts with the effect of TRIM25, a U3 ubiquitin ligase which targets VP3 for degradation [119]. Overexpression of TRIM25 has been observed to inhibit IBDV replication, by ubiquitination at Lys854. Recombinant viruses encoding a mutant VP3 that cannot be ubiquitinated at this position (VP3 K854R) exhibited enhanced replication, indicating that TRIM25 ubiquitination modulated IBDV replication through VP3 destabilization [120]. As TRIM25 was observed to colocalize with VP3 enriched VFs, the impact of VF concentration remains an unanswered question. It is possible that the high concentration of VP3 within VFs

reduces the degradation induced by K854 ubiquitination as compared to diffuse VP3, however, experiments investigating this possibility have yet to be performed.

The dsRNA genome is also sequestered to VFs during IBDV infection, due to the sequence-independent interaction with VP3 [121][122]. This likely prevents the activation of chMDA5, a chicken expressed RIG-I like receptor (RLR) that binds dsRNA and activates the interferon signaling pathway [123][124][125]. By recruiting dsRNA to VFs, VP3 creates an environment where dsRNA-chMDA5 interactions are energetically unfavorable, and therefore shields the genome from surveillance [126]. The sequestration of dsRNA to the VF also enables additional RNA-binding proteins to be recruited to the VFs. As an example, Staufen1 (STAU1), a host RNA-binding protein, was found to co-immunoprecipitate with VP3, and its VF localization was confirmed by immunofluorescence microscopy [127][128]. STAU1 not only bound IBDV dsRNA, but promoted viral replication by reducing chicken interferon beta (chIFN- β) promoter activity due to competition against chMDA5 for dsRNA binding, resulting in inhibition of the RLR signaling pathway [129].

Some modifications may be removed from proteins though interacting with VF proteins. For example, apoptosis inhibitor 5 (API5) is recruited to VFs as a client during infection, and the interaction between VP3 and API5 was observed to reduce API5 SUMOylation, by inducing UBC9 degradation. SUMOylated API5 enhanced MDA5 mediated IFN- β activity, so the VP3-mediated reduction in API5 SUMOylation suggested an immune evasion role for this behavior. Consistent with this hypothesis, viral replication was enhanced in API5 knockdown cells, as well as cells expressing a mutated API5 (API5 K404R) lacking its SUMOylation site [130]. In

another example, one component of the IFN- β pathway, TRAF3, was found to colocalize with VP3 during IBDV infection, which reduced the formation of a TBK1-TRAF3 complex, by blocking ubiquitination of TRAF3 at K155. This interaction was mediated by a VP3 region termed “coiled-coil region 1” (CC1), comprising residues VP3 residues 12-24, a region also responsible for the dissociation of the PIK3C3-BECN1 complex, and subsequent inhibition of autophagosome formation [131]. As the ubiquitination of TRAF3 is a critical component of the IFN- β signaling pathway, the VP3-mediated TRAF3 ubiquitination blockade during IBDV infection is analogous to the immune evasion strategies of other viruses [132][133][134].

Hypothesis

Based on the existing body of IBDV research, the apparent role of VP3 in early VF formation dynamics and analogous systems in related dsRNA viruses, I hypothesized that VP3 acted as a proteinaceous scaffold for LLPS of VFs, analogous to ReoV μ NS.

Aims and Objectives

Overall Aim

The overall aim of this research was to define the molecular basis of the formation of IBDV VFs, and to evaluate the role of VP3 in driving the LLPS phenomenon in birnavirus infection. This was achieved by the investigation of three sub-objectives:

Objective 1: Developing a system for LLPS characterization

We developed a system for the characterization of IBDV VFs, leveraging microscopy and image analysis. I developed methodologies to label VFs in both live and fixed cells, facilitating the

analysis of liquidity by FRAP, the measurement of condensate dynamics, and the quantification of morphological properties with computer vision.

Objective 2: Defining the minimal component set for LLPS of VFs

As VP3 represents a major component of IBDV VFs, I hypothesized that it was a key driver of the phenomenon. I aimed to characterize the minimal set of components required to induce phase separation, which I determined to include VP3, VP1, and viral RNA. I therefore determined that while VP3 was critical to phase separation, it does not drive the phenomenon independently, a key distinction between VP3 and ReoV μ NS from which its behaviors have previously been inferred.

Objective 3: Evaluating the contribution of the VP3 C-terminus to the formation of IBDV VFs and driving the LLPS phenomenon

Utilizing AlphaFold modeling and molecular dynamics (MD) simulations, I robustly characterized the IBDV C-terminus as a highly dynamic IDR. Although IDRs are frequently involved in LLPS, they are not absolutely required for the phenomenon to occur, and I therefore aimed to narrowly define the role of the C-terminal IDR in the formation of VFs. I found that while the C-terminus was not, in fact, absolutely required for LLPS to occur, it significantly enhanced their production and modulated their physical properties.

Chapter 2: Materials and Methods

2.1 Computational Methods

2.1.1 AlphaFold. Structural modeling with AlphaFold2 was accomplished using the AlphaFold2 Colab notebook published by Deepmind

(<https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb?pli=1>), based on the amino acid sequence of the VP3 protein from IBDV strain PBG98, obtained from GenBank Accession MT010364. Processing was performed using the monomer model, with relaxation performed in GPU mode. Structural modeling with AlphaFold3 was accomplished using the AlphaFold Server (<https://www.alphafoldserver.com>). Structures were visualized with UCSF ChimeraX v.1.17.1 (UCSF, CA) [135,136].

2.1.2 Predicted Aligned Error (PAE). AlphaFold's predicted error in the relative position of each pair of residues, measured in Angstroms, was extracted from the .zip archives generated by the AlphaFold Colab notebook by ingesting the "predicted_aligned_error.json" file with Python's JSON module, and values were plotted as a heatmap with Plotly.

2.1.3 IUPred3 Disorder Plot: IUPred3 was accessed through the Eötvös Loránd University web portal (<https://iupred3.elte.hu/>) and run in short disorder mode with medium smoothing. Results were downloaded in JSON format and loaded using Python's JSON module, and plotted as a line plot with Plotly [137].

2.1.4 Sequence Entropy. The sequence entropy of VP3 was determined and plotted as a line plot with Plotly. Sequence entropy was calculated using a sliding-window approach, where for each

residue at position i in a sequence of length n , the bounds of a window with size w are defined as:

$$W_i = \left[\max\left(0, i - \frac{w}{2}\right), \min\left(n, i + \frac{w}{2} + 1\right) \right]$$

[138] This formulation allowed for the window size w to be set to 20 residues and be resized dynamically when a 20-residue window centered on i was not possible due to the position of i . For each residue a within W_i , the probability $p(a)$ was given by the equation:

$$p(a) = \frac{F(a)}{\text{length}(W_i)}$$

Where $F(a)$ is the frequency of residue a . The Shannon entropy H at each position i was then calculated from the values of $p(a)$ by the equation:

$$H_i = - \sum_{a \in W_i} p(a) \log_2 p(a)$$

These values were then normalized to give an entropy score E by the equation:

$$E_i = \frac{H_i}{\max(H)}$$

2.1.5 Molecular dynamics (MD) simulations. To run the MD simulations and generate movies, the software GROMACS was installed on a fresh installation of Ubuntu 22.04, running on an AMD 3800X processor with 32GB of RAM. The GROMACS binary was compiled with the (-DGMX_GPU = ON) flag, with 3,584 CUDA cores provided by an Nvidia GTX 1080Ti graphics processor (Nvidia, CA). Each simulation was 20 nanoseconds in duration and performed using the OPLS-AA/L all-atom force field with a 2 femtosecond (fs) timestep. Energy minimization and equilibration were carried out before each simulation. The trajectories of every alpha carbon

atom in VP3 were determined, and the absolute point positions were extracted from the trajectory data. Periodic boundary condition effects were corrected algorithmically, and the alpha carbons of VP3 were visualized as animated three-dimensional scatter plots of the atomic coordinates.

2.1.6 Periodic Boundary Condition Correction As PBC were employed in MD simulations, the absolute positions of atoms in trajectories were corrected by an algorithm. This algorithm ensured that in a simulation box d with dimensions d_x, d_y, d_z , for each atom i of M total atoms, at each time step t of N total time steps, no translation of $r_i(t + 1)$ in x , y , or z by an integer multiple of d_x, d_y , or d_z respectively results in a position $r_i'(t + 1)$ where $|r_i'(t + 1) - r_i(t)| < |r_i(t + 1) - r_i(t)|$. If such a translation is found, it is applied. The coordinate space for each frame was then normalized by uniformly translating the positions r such that the centroid of all points on each frame lay at $(\frac{d_x}{2}, \frac{d_y}{2}, \frac{d_z}{2})$.

2.1.7 B factor calculations. The B factor was calculated from the MD simulations, and the mean and standard error of the mean (SEM) plotted as a line plot with Plotly. The B factor was calculated from the MD trajectories by determining the mean position of each alpha-carbon across all frames of the simulation, given by the equation:

$$\langle r_i \rangle = \frac{1}{N} \sum_{t=1}^N r_i(t)$$

Where N is the total number of time steps for the simulation, and $r_i(t)$ is the position of alpha carbon r_i at time t . The mean squared displacement (MSD) was then calculated for the simulation on a per-atom basis, given by the equation:

$$\langle (r_i - \langle r_i \rangle)^2 \rangle = \frac{1}{N} \sum_{t=1}^N (r_i(t) - \langle r_i \rangle)^2$$

This MSD value was then converted to a B factor value for the i^{th} atom by the equation:

$$B_i = \frac{8\pi^2 \langle (r_i - \langle r_i \rangle)^2 \rangle}{3}$$

To perform residue:residue statistical analysis of the B factor values, the B factor value B_i for the alpha carbon of each residue i was calculated as described for each of 6 independent simulations. For each residue, the B factor values were tested for normality by a Shapiro-Wilk test, with a significance threshold α of 0.05. As not all values p_i met the significance threshold, the non-parametric Kruskal-Wallis test was performed across the groups, with a significance threshold α of 0.05. Given that $p_{kruskal} \ll 0.001$, *post-hoc* pairwise testing was performed using Dunn's test, with Benjamini-Hochberg correction.

2.1.8 Atomic motion correlation analysis. For each MD simulation, the Pairwise Pearson's product-moment correlation coefficients (r_{xy}) were calculated for the fluctuations of all alpha carbons about their mean positions. For statistical comparison, the mean absolute value of the correlation coefficients for each pair of alpha carbons (excluding self-comparisons) in the C-terminus was compared to that of an equally-sized (36 residue) range centered on each residue of the structure where such a window was possible. A Shapiro-Wilk test was performed on each range and the null hypothesis strictly failed to be rejected at a significance level $\alpha = 0.05$, and Levene's test was performed between each range and the C-terminus, however, the null hypothesis was rejected for some ranges. As an assumption of normality but not of homoscedasticity was supported, mean correlation coefficients were compared by a one-tailed Welch's unequal variances t -test with the alternate hypothesis $H_a: \overline{|r_{ab}|} > \overline{|r_{cd}|}$ $a, b \in$

$range_{test}$ and $c, d \in range_{c_{term}}$. The null hypothesis was rejected at a significance level $\alpha = 0.05$ for all ranges except those centered on residues 74 – 96 and 149-163, both of which correspond to predicted unstructured regions of the protein, and the latter of which was previously found to be unstructured by X-ray crystallography. For completeness, this analysis was also performed using a dynamically sized test window to permit analysis of windows centered on residues at the N and C extrema. Although statistical tests should be considered less reliable as the window, and therefore sample size decreases, the conclusions of the analysis were unchanged.

2.2 Cell Culture

2.2.1 Cell culture. The chicken DF-1 fibroblast cell-line was obtained from the American Type Culture Collection (ATCC) (VA, USA) and the HD11 cell line was a kind gift from Dr. Mark Parcells, University of Delaware. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (ThermoFisher Scientific, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, ThermoFisher Scientific, MA, USA) and incubated in an atmosphere of 37°C and 5% CO₂.

2.3 Plasmids, Cloning and Virus Culture

2.3.1 Plasmids. Plasmids expressing the following were used in this study: IBDV VP3 (untagged), mNeonGreen (mNG), IBDV VP3 fused to mNG, TC, or enhanced GFP (eGFP) reporters at the N-terminus (mNG::VP3, TC::VP3, eGFP::VP3), IBDV VP3 lacking the C terminus fused to mNG at the N-terminus (mNG::VP3 Δ C) IBDV VP3 C terminus fused to mNG at the N-terminus (mNG::Cterm), IBDV VP1 fused to FLAG at the C-terminus (VP1::FLAG), IBDV reverse genetics (RG) plasmid encoding segment A (SegA), IBDV RG plasmid encoding

segment B (SegB), segment A lacking VP3 (SegAΔVP3), segment A lacking the C terminus (SegAΔC), SegB with an HA tag inserted between the 5' untranslated region (UTR) and the coding region of VP1 (HA::SegB), and SegB with mNG fused at the C-terminus (SegB::mNG). Each of the inserts was cloned into the pSF-CAG-KAN vector (Sigma-Aldrich, DE), and the IBDV sequences of segment A, B, VP1, and VP3 were designed based on the cell-culture adapted strain, PBG98 (GenBank accession numbers MT010364 and MT010365).

2.3.2 Mutagenic PCR. Mutagenic PCR was performed by the generation of mutagenic primers (either featuring 5' overhangs or mismatched nucleotides) and extension was performed using the Q5® high-fidelity DNA polymerase master mix (New England Biolabs (NEB) (MA, USA)) in accordance with manufacturer protocols, using the NEB Tm calculator to determine appropriate cycling conditions based on the mutagenic primer sequence. Crude PCR product was analyzed by gel electrophoresis and excised from the gel and purified for downstream cloning using the Monarch® spin DNA Gel extraction kit (NEB) in accordance with manufacturer protocols. DNA was quantified by Nanodrop after purification.

2.3.3 Molecular Cloning. The vector and insert DNA were digested with appropriate restriction nucleases (NEB) in accordance with manufacturer protocols for each enzyme. The reactions were then separated by gel electrophoresis and the Monarch® spin DNA Gel Extraction kit (NEB) was utilized to extract DNA from gel slices. The DNA was quantified by nanodrop and the backbone and insert DNA were ligated with T4 DNA ligase (ThermoFisher Scientific), in accordance with manufacturer protocols, at 16°C overnight. The ligation product was then used to transform chemically competent E-coli (NEB), according to manufacturer's protocols. After

transformation and outgrowth in SOC media (ThermoFisher Scientific), the mixture was streaked onto agar plates with the appropriate antibiotic selection marker (ThermoFisher Scientific). Plates were incubated at 37°C overnight to allow colonies to develop. Colonies were selected and grown overnight in LB broth (ThermoFisher Scientific), in the presence of selection antibiotic, at 37°C with shaking at 200rpm. A 1mL aliquot of the broth was reserved from each of these outgrowths, and the remainder was processed using the Spin miniprep kit (Qiagen, MD), and screened by an analytical restriction digest. Once a positive sample was identified, additional LB broth supplemented with the appropriate antibiotic selection was inoculated with the 1mL reserved aliquot of the positive outgrowth and incubated overnight at 37°C with shaking at 200rpm and processed using the Spin Maxiprep kit (Qiagen), with final elution in molecular biology grade water. The DNA was quantified by nanodrop, diluted with molecular biology grade water to 1,000ng/μL, and stored at -20°C.

2.3.4 Transfection. DF-1 cells were transfected with the relevant plasmids using Lipofectamine 2000 transfection reagent (ThermoFisher Scientific), according to the manufacturer's recommendations for the size of the culture vessel. Briefly, the lipofectamine 2000 reagent and DNA were diluted in Opti-MEM media (Gibco, ThermoFisher Scientific), mixed, and incubated for 20 minutes at room temperature (RT) before application to adherent cells dropwise into the media.

2.3.5 Virus Rescue. A molecular clone of the cell-culture adapted IBDV strain PBG98 was rescued by transfecting DF-1 cells with the RG plasmids Segment A and Segment B, and monitoring cells for cytopathic effect (CPE). The resulting virus was titrated in DF-1 cells and

the titer quantified by the tissue culture infectious dose-50 (TCID₅₀) method described by Reed and Muench. Unless otherwise stated, DF-1 cells were infected at a multiplicity of infection (MOI) of 5 in downstream experiments.

2.3.6 TCID₅₀. IBDV titration was performed by the TCID₅₀ method in DF-1 cells. DF-1 cells were seeded in 24-well attachment-treated tissue culture plates, and allowed to grow to 80% confluency. Wells were then infected in quadruplicate with serially diluted aliquots of virus stock, and the TCID₅₀ point was determined as the dilution at which two of the four wells exhibited CPE.

2.4 Imaging

2.4.1 Immunofluorescence microscopy. 12-well tissue culture treated plates (Corning, AZ) were populated with 18mm No.1 glass cover slips and seeded with DF-1 cells and maintained as described (see cell culture). Cells were washed with sterile phosphate-buffered saline (PBS), fixed in 4% (v/v) paraformaldehyde (PFA) in PBS for fifteen minutes at RT, and washed again with PBS. Cells were then permeabilized with 0.1% (v/v) Triton-X 100 in PBS for 15 minutes, and incubated with a blocking buffer of 4% (w/v) of bovine serum albumin (BSA) in PBS for one hour at RT. The primary antibody was then diluted in blocking buffer, and cells were incubated in this solution at RT for one hour with rocking. After incubation, the primary antibody solution was aspirated and the cells were washed with PBS. The secondary antibody conjugated to a fluorophore was then diluted in blocking buffer, and cells were incubated in this solution for one hour at RT with rocking. Cells were washed with PBS, counterstained with DAPI and cover slips were mounted onto slides prior to imaging.

2.4.2 Microscopy and Image Processing

Fluorescence micrographs were acquired with either a Nikon Ti-2 widefield fluorescent microscope with a 60x oil immersion objective, a Zeiss LSM980 scanning confocal microscope with a 63x oil immersion objective, or a Leica Stellaris 8 scanning confocal microscope with a 63x oil immersion objective. To capture mosaic images with the Leica Stellaris 8, a grid of evenly spaced focal points was established manually, and the tiled acquisition and mosaic assembly proceeded automatically via the integrated LAS X software suite, with mosaics exported as .TIF files. Images of individual cells were obtained by cropping the mosaic images directly produced by the LAS X software. Image processing (cropping, scale bars, pseudocolor) was performed in Fiji.

2.4.3 Live cell imaging. Cells were seeded in confocal dishes with 20mm No.1 glass bottoms (MatTek, MA) at a density of 2×10^6 cells per dish. Cells were imaged in a live-cell imaging chamber of a Zeiss LSM 980 laser scanning confocal microscope and maintained in a 5% CO₂ environment at 37 Celsius during imaging. The microscope was configured to minimize laser power (and thus photobleaching) without introducing excess noise, and with Z-drift compensation configured to run every 5 frames to maintain a constant imaging plane.

2.4.4 Aliphatic Diol Treatment. DF-1 cells were seeded in glass-bottom confocal dishes with 20mm No.1 glass bottoms at a density of 2×10^6 cells per dish. Immediately prior to imaging, the media was removed from the cells and 100 μ l of fresh media added. Cells were imaged with a Zeiss LSM 980 laser scanning confocal microscope configured to minimize laser power (and thus photobleaching) without introducing excessive noise, and with Z-drift compensation configured to run every 5 frames. An open-ended time-lapse acquisition was then configured.

Thirty seconds after acquisition began, 900µl of media containing 4.44% (v/v) of the specified aliphatic diol (Beantown Chemical, NH) was carefully added to the dish by pipette, for a final concentration of 4% (v/v), with continual imaging. Image acquisition was continued until photobleaching, cell drift or toxicity became excessive.

2.4.5 FRAP. FRAP was performed using a Zeiss LSM 980 laser scanning confocal microscope.

The imaging area was cropped to maximize the proportion of the scanned area occupied by the puncta of interest, and a circular measurement region of interest (ROI) was established, centered about a point ROI. The system was configured to capture a 256x256 pixel image every 75.95 milliseconds with a 1 Airy unit (AU) pinhole. The experiment was configured to capture 5 pre-bleach frames, followed by a single-frame 488nm bleaching pulse at 10% power at the point ROI, followed by continuous acquisition of frames thereafter. Extraction of intensity data was performed in Fiji (v2.3.0/1.53f, open source). Unmodified files from the Zeiss microscope (.czi files) were loaded with BioFormats importer, and 3 ROIs were established on the images:

“Bleach”, an ROI encompassing the region bleached in the first frame after the bleaching pulse, “puncta”, an ROI encompassing the entire puncta, and “background”, an ROI outside the puncta.

The multi-measure function was then used to capture mean intensity data for each ROI across every frame of the experiment, the results of which were exported as comma-separated value (.csv) files for analysis. This analysis was performed in Python, with a script available at (<https://github.com/LadInTheLab/vp3-llps>). The script was based on easyFRAP-web (42).

Background fluorescence intensity was compensated for in the ROI and whole puncta by subtracting it at each time point t , as given by the equations:

$$I(t)_{ROI}' = I(t)_{ROI} - I(t)_{BG}$$

$$I(t)_{VF}' = I(t)_{VF} - I(t)_{BG}$$

The “double normalized” fluorescence intensity was then calculated using the equation:

$$I(t)^{double} = \left(\frac{\frac{1}{n_{pre}} \cdot \sum_{t=1}^{n_{pre}} I(t)_{VF}'}{I(t)_{VF}'} \right) \cdot \left(\frac{I(t)_{ROI}'}{\frac{1}{n_{pre}} \cdot \sum_{t=1}^{n_{pre}} I(t)_{ROI}'} \right)$$

Where n_{pre} is the number of pre-bleach frames. The “full-scale normalized” intensity was then calculated using the equation:

$$I(t)^{fullscale} = \frac{I(t)^{double} - I(t_{postbleach})^{double}}{1 - I(t_{postbleach})^{double}}$$

Where $t_{postbleach}$ is the first post-bleach timepoint. Curve parameters were then determined from this data by least-squares curve fitting, with the equations:

$$I_{single} = I_0 - a \cdot e^{-\beta t}$$

$$I_{double} = I_0 - a \cdot e^{-\beta t} - \gamma \cdot e^{-\delta t}$$

Which represent single and double term exponential curves respectively. As part of least-squares curve fitting, initial values of $I_0 = 0.85$, $\alpha = 0.5$, $\beta = 0.563$, $\gamma = 0.316$, and $\delta = 0.36$ were used. As full-scale normalization was employed, mobile fraction mf was determined directly from the curve parameters, as $mf = I_0$ for full-scale normalized data. The half-maximal recovery time t_{half} or $t_{1/2}$ for a single-exponential fit is given by the equation:

$$t_{half} = \frac{\ln 2}{\beta}$$

Whereas for double-exponential fits, t_{half} is computed numerically. Choice of exponential equation was made by comparing coefficients of determination (R^2), as determined by the equation:

$$R^2 = 1 - \frac{\sum_t^n (I(t)^{fullscale} - f(t))^2}{\sum_t^n \left(I(t)^{fullscale} - \left(\frac{1}{n} \sum_t^n f(t) \right)^2 \right)}$$

Where $f(t)$ is the value of the chosen exponential equation f with the calculated curve parameters at time t .

2.5 Image Processing

2.5.1 Puncta quantification and measurement of morphology. Puncta were quantified by

acquiring mosaic images with a Leica Stellaris 8 scanning confocal microscope. Cells were selected at random from the fields and puncta were counted manually. Fifty cells were analyzed for each of 3 independent trials for each condition. Measurement of puncta morphology in cells was accomplished by automatic segmentation. Briefly, images of 50 single cells were captured with a Zeiss LSM980 Airyscan scanning confocal microscope and processed using a custom Python script which performed watershed segmentation to isolate puncta. Segmentation was manually reviewed prior to measurement. The script is available on Github (<https://github.com/LadInTheLab/vp3-llps>), and the algorithm utilizes the Scikit-image library for segmentation and measurement. Statistics were calculated by grouping puncta by condition (wild type or ΔC) and, after a Shapiro-Wilk test determined that the assumption of normality was not valid, a Mann-Whitney U Test was performed between the groups for each metric.

2.5.2 Watershed Segmentation VFs were extracted from images by watershed segmentation.

Foreground and background markers were defined by percentile levels. Specifically, background markers were defined as pixels with intensities below a lower percentile threshold P_{BG} , while

foreground markers were defined as pixels with intensities above an upper percentile threshold

P_{FG} :

$$Background = \{I(x, y) \mid I(x, y) \leq P_{BG}\}$$

$$Foreground = \{I(x, y) \mid I(x, y) \leq P_{FG}\}$$

where $I(x, y)$ denotes the grayscale intensity at pixel location (x, y) . Pixels with intensities between these thresholds were resolved during the watershed flooding process.

The percentile-based approach permitted adaptive thresholding across images with varying contrast and illumination. The watershed algorithm was then applied to the inverted intensity image, such that higher-intensity regions were treated as catchment basins. The output of the watershed segmentation was a labeled image in which each connected foreground region corresponded to a segmented object.

2.5.3 Contour Extraction For each segmented object, a binary mask was constructed from the labeled image. Contours were then identified as the set of pixels at the border between foreground and background regions within this mask. When multiple contours were detected for a given object, such as interior holes, only the outermost contour was retained for analysis. Contour extraction was performed by functions from the Scikit-image library.

2.5.4 Fourier Analysis Fourier-based shape analysis was performed on two-dimensional closed contours to characterize global and local shape properties in the frequency domain. To enforce invariance to translation, scale and orientation, contours were normalized and resampled prior to

Fourier analysis. Each contour consisting of N ordered points (x_i, y_i) was represented as a complex-valued sequence:

$$z_i = x_i + iy_i$$

where i denotes the imaginary unit. The discrete Fourier transform (DFT) of the contour was then computed using the Fast Fourier Transform (FFT), normalized by the number of points N :

$$Z_k = \frac{1}{N} \sum_{n=0}^{N-1} z_n e^{-2\pi i k n / N}$$

The zero-frequency (DC) component (corresponding to the absolute position of the contour) was excluded from further analysis. To account for both clockwise and counterclockwise rotational components of the shape, positive and negative frequency coefficients were extracted symmetrically. For a contour of length N , harmonics were considered up to a maximum index $k_{max} = \lfloor N/2 \rfloor$. For each harmonic k , the combined magnitude spectrum M_k was defined as:

$$M_k = |Z_k| + |Z_{-k}|$$

This formulation captures the total energy contribution of each harmonic irrespective of rotational direction.

Several Fourier-derived metrics were computed from the magnitude spectrum. The total spectral energy E_{total} was defined as:

$$E_{total} = \sum_{k=1}^{k_{max}} M_k^2$$

Spectral slope was calculated by fitting a linear model to the log–log relationship between harmonic index and magnitude:

$$\log(M_k) = \alpha \log(k) + \beta$$

where the fitted coefficient α represents the spectral slope and quantifies the rate at which higher-frequency contributions decay.

The high-frequency energy ratio was defined as the fraction of the total spectral energy contained within the highest 20 harmonics:

$$E_{HF} = \frac{\sum_{k_{max}-19}^{k_{max}} M_k^2}{E_{total}}$$

The normalized energy probability distribution p_k was defined as:

$$p_k = \frac{M_k^2}{E_{total}}$$

and the spectral entropy H was calculated as:

$$H = - \sum_k p_k \log(p_k)$$

Finally, Fourier circularity was defined as the proportion of total energy contained in the first harmonic:

$$C_{Fourier} = \frac{M_1^2}{E_{total}}$$

As a perfect circle concentrates all Fourier energy in the first harmonic, this metric provides a frequency-domain measure of shape circularity.

2.5.5 Laplacian Energy Calculation Laplacian-based shape metrics were computed to quantify local curvature variation and contour roughness. As with Fourier analysis, Laplacian analysis was performed on normalized and uniformly resampled contours.

For a contour represented by ordered points $P_i = (x_i, y_i)$, the discrete Laplacian operator L_i at point i was approximated using second-order central differences:

$$L_i = P_{i+1} - 2P_i + P_{i-1}$$

with periodic indexing applied to enforce contour closure. The magnitude of the Laplacian vector at each point,

$$\ell_i = \|L_i\|$$

serves as a local measure of curvature-related roughness, with larger values indicating sharper local deviations from linearity.

Summary statistics of the Laplacian magnitude distribution were computed, including the mean μ_ℓ , standard deviation σ_ℓ , minimum, maximum, and range. Laplacian variation was defined as the coefficient of variation:

$$V_\ell = \frac{\sigma_\ell}{\mu_\ell}$$

The Laplacian energy was defined as the variance of the Laplacian magnitude:

$$E_{Lap} = Var(\ell)$$

which quantifies the overall heterogeneity of curvature along the contour.

To assess spatial alternation between regions of above- and below-average roughness, the Laplacian magnitude was mean-centered:

$$\tilde{\ell}_i = \ell_i - \mu_i$$

and the number of zero crossings was determined by counting sign changes between adjacent values of $\tilde{\ell}_i$. This metric reflects the frequency of transitions between smoother and rougher contour segments.

Finally, a smoothness index was defined as the inverse of the Laplacian energy:

$$S = \frac{1}{E_{Lap}}$$

such that higher values correspond to smoother, more uniform contours.

2.6 Statistics

2.6.1 Statistics and Plotting. Statistics and plotting were performed in Python unless otherwise specified. Statistical calculations were performed using the SciPy package, and plotted using the Plotly package.

Chapter 3: Developing a System for the Characterization of IBDV VFs

3.1 Introduction.

Although their punctate morphology and the intrinsically “concentrated” nature of their components frequently contribute to ready visualization of LLPS structures by microscopy, measurement of the physical and biochemical properties of these structures is somewhat more complicated than that of the membranous organelles typical of eukaryotic cells. These challenges are a consequence of the physicochemical nature of LLPS and the mechanics of LLPS structure formation: because LLPS structures form as the result of multiple classes of intermolecular interactions (solvent-solvent, solvent-solute, and solute-solute), their compositions and properties are intrinsically tied to their local microenvironments. Furthermore, because they are defined physically not by a membrane but a phase transition barrier (effectively an unusually steep concentration gradient), they are fragile. Attempts to isolate the structures for study, since they typically involve physically disruptive actions and inherently involve changes to the local microenvironment, are liable to fail or yield impertinent data. It is therefore best practice to study LLPS structures *in situ* – but the methodologies employed for this mode of investigation must also be informed by the unique biochemistry of LLPS, since the phenomenon presents both opportunities and challenges here as well [139,140].

At rest, LLPS structures are not typically geometrically complex – though this is a direct result of their physicochemical nature. LLPS structures tend to exhibit viscoelastic properties, meaning they exhibit both viscous and elastic characteristics. They are thereby elastically deformable when force is applied to them, and the rounded, low-complexity shape typical of LLPS is a consequence of energy minimization – by assuming the shape of lowest surface area, the

unfavorable solvent-solute interactions are minimized. It follows therefore that the morphology of LLPS structures, by virtue of being driven by the very intermolecular interactions that drive the phenomenon as a whole, is a meaningful biochemical probe in and of itself [141,142]. The greater challenge is in extracting meaningful data from these morphologies. Valid and pertinent metrics must be chosen, and the simplicity of the shapes in question necessitates efficient measurement of hundreds or thousands of structures in different conditions to measure subtle differences with confidence.

Typically, the shapes of LLPS structures are assessed using scientific image analysis tools such as ImageJ or Fiji, where manual selection or techniques such as thresholding are used to segment and measure structures. In the context of LLPS, “sphericity” is a frequently cited shape metric, because as previously described, LLPS structures are expected to be rounded. However, sphericity is an imperfect metric for the roundness of condensates. Sphericity quantifies how closely a shape approaches a perfect sphere. Although perfect spheres exhibit the minimum surface area for a given volume, in the cellular environment, condensates large enough to form will frequently be unable to attain such an idealized shape due to cytoplasmic crowding or impingement by organelles or cell membranes. Other metrics, such as geometric solidity or turning number, which offer a more “shape agnostic” measure of roundness, may therefore be superior to simple sphericity measurements in practical applications.

Much as static analysis of LLPS structures is conceptually straightforward, so too are the techniques for labeling such structures in fixed cells. By their very nature, LLPS structures concentrate biomolecules within themselves – raising an antibody against a protein enriched in

the heavy phase is likely to generate a serviceable probe for visualization. However, the dynamic properties of LLPS structures are only visible in living cells, necessitating labeling methods that do not require fixation or permeabilization. Fortunately, several live-cell compatible reporters exist, including a large library of constitutively fluorescent proteins, split-fluorophore systems, and reactive tags. The question, therefore, is largely of the delivery system. Though direct conjugation of a reporter to a heavy-phase resident protein is an appealing strategy, viruses, particularly small RNA viruses, are frequently attenuated by or outright reject genomic insertions, especially large genomic insertions like fluorescent proteins [143]. There is therefore no guarantee that the generation of a virus encoding a reporter will be possible. Fortunately, as opposed to membrane-bound organelles whose composition is frequently dictated by complex trafficking mechanisms, integration of biomolecules into an LLPS structure is better framed as a question of compatibility with and affinity for the heavy phase. This molecular promiscuity facilitates a foundational method for studying LLPS structures: the “spike-in”.

Broadly, spike-in labeling is the labeling of LLPS structures by the expression of some amount of a reporter engineered to have a high affinity for the heavy phase of the structure under study. This is frequently achieved by conjugation of a reporter to the whole or partial sequence of a scaffold protein [144,145]. In principle, the affinity of this reporter-tagged species for the heavy phase causes it to preferentially partition into that phase – and the liquid nature of LLPS allows the reporter species to intermix freely with the structure, leading to homogenous labeling. This permits a wide variety of microscopy-based investigative modalities to be employed.

Just as I propose to indirectly measure the elastic aspect of viscoelastic LLPS structures by morphological analysis, measurement of the viscous characteristics of the structures can be achieved by measurement of internal diffusion. As implied by the name of the phenomenon, both the heavy and light phases in an LLPS system are liquid – the molecules within them slide relatively freely past each other, and at any temperature above absolute zero, random motions cause molecules to follow unpredictable paths – in an unconstrained case (as in a liquid system), this manifests as diffusion [146]. By measuring the diffusion of molecules within an LLPS structure, I measure the viscous aspect of its physical properties. To measure diffusion in subcellular compartments, FRAP is a frequently employed technique. In FRAP, the structure whose diffusive properties are to be measured is fluorescently labeled, and a confocal microscope is configured to deliver an extremely short, high-power pulse of laser energy to a small region of the structure. This pulse, by a combination of photochemical and thermal effects, irreversibly quenches the fluorescence of any label molecules within this “bleach area” [147]. The microscope continuously acquires data after the bleach pulse, and by measuring the recovery of fluorescence in the initially dark bleach area due to the diffusion of bleached molecules out of the bleach area and diffusion of unbleached molecules in, the rate of diffusion (or colloquially, the liquidity) of the structure can be inferred.

The ability to label VFs in live cells also facilitates the investigation of VF dynamics by time-lapse microscopy. Because LLPS structures are not membrane-bound unless anchored to another structure, they typically move freely through their medium, with motion primarily driven by collisions with other mobile objects, such as cytoplasmic cargoes. Furthermore, LLPS structures can typically undergo observable fusion and fission events, in which two colliding LLPS

structures fuse into a single larger structure, or a larger structure splits into multiple smaller structures, respectively [139,142,148].

Time-lapse microscopy also permits the analysis of the response to aliphatic diol treatment. Small aliphatic diols are a class of organic molecules that have been repeatedly observed to disrupt and dissolve LLPS structures in both cells and cell-free systems, potentially by perturbing the hydrogen-bonding environment. Because of this effect, response to aliphatic diol treatment is frequently employed as a crude assay for LLPS. Such assays are not without drawbacks however: the most frequently employed diol for this purpose is 1,6-HD, which is toxic to live cells and broadly damages cellular membranes. Alternative diols such as 1,3-Propanediol (1,3-PD) can exhibit reduced toxicity, but diol treatment remains suboptimal when testing condensates in live cells [149].

To study the LLPS phenomenon in the context of IBDV VFs, I developed a flexible system based on the spike-in modality which permitted us to fluorescently label VFs *in-situ* without fixation or permeabilization. To accomplish this, I conjugated the constitutively fluorescent monomeric protein mNG to the N-terminus of VP3, a highly expressed viral protein that is known to substantially partition into VFs during infection. Due to the sensitive nature of the LLPS phenomenon, I screened a variety of candidate reporters during the development of this system and determined that mNG most faithfully reconstituted the behavior of untagged VP3. I determined that simultaneous expression of our mNG::VP3 construct in cells infected with IBDV led to recruitment of mNG::VP3 to VFs, thereby fluorescently labeling them.

We then developed a computer vision-based pipeline to extract and analyze the morphologies of the VFs I visualized, and used a panel of geometric measurements, along with Fourier analysis and calculation of Laplacian energy, to classify and differentiate the observed morphologies. Finally, I demonstrated that our spike-in system for VF labeling could be applied to successfully measure the liquidity of VFs by FRAP. As a basic application of this suite of tools, I measured the morphologies and properties of VFs at eight, sixteen and twenty four hours post infection, and discovered that not only were some significant timepoint dependent morphological changes present, but surprisingly found that IBDV VFs appear to become more liquid at later timepoints, which is the opposite phenomenon as is observed in *Rotavirus*, another dsRNA virus from which properties of the less-studied IBDV have been frequently inferred. These results both validate the suite of tools I have developed for the study of IBDV VFs, but also may represent a notable difference between the physicochemical properties of those VFs and *Rotavirus* viroplasms, to which they are frequently compared.

3.2 Results

3.2.1 IBDV-Infected Cells Develop Punctate Virus Factories (VFs)

As IBDV VFs are enriched in VP3, immunofluorescence microscopy is a commonly used technique for visualizing IBDV infection in cells. Although not commercially available, a mouse monoclonal antibody raised against IBDV VP3 has been produced, and would serve as our primary means to visualize IBDV infection in cells [150]. I generated strain PBG98 IBDV using our in-house reverse genetics system (2.3.5), which was titrated by TCID₅₀ (2.3.6) and used to infect chicken fibroblast (DF-1) cells at an MOI of 1. Infected cells were fixed and stained with

an anti-VP3 monoclonal antibody (2.4.1). Although cells with a variety of phenotypes were observed, cells bearing rounded puncta strongly enriched for VP3 signal were abundant, particularly at 16 hours post-infection (hpi). Cells with puncta were observed as early as 6 hpi and persisted at all time points tested. A timepoint of 18 hpi for visualization was frequently employed, as achieving the highest proportion of punctate cells was not the focus.

3.2.2 mNeonGreen Conjugated VP3 Behaves Similarly to Unlabeled VP3 in Cells

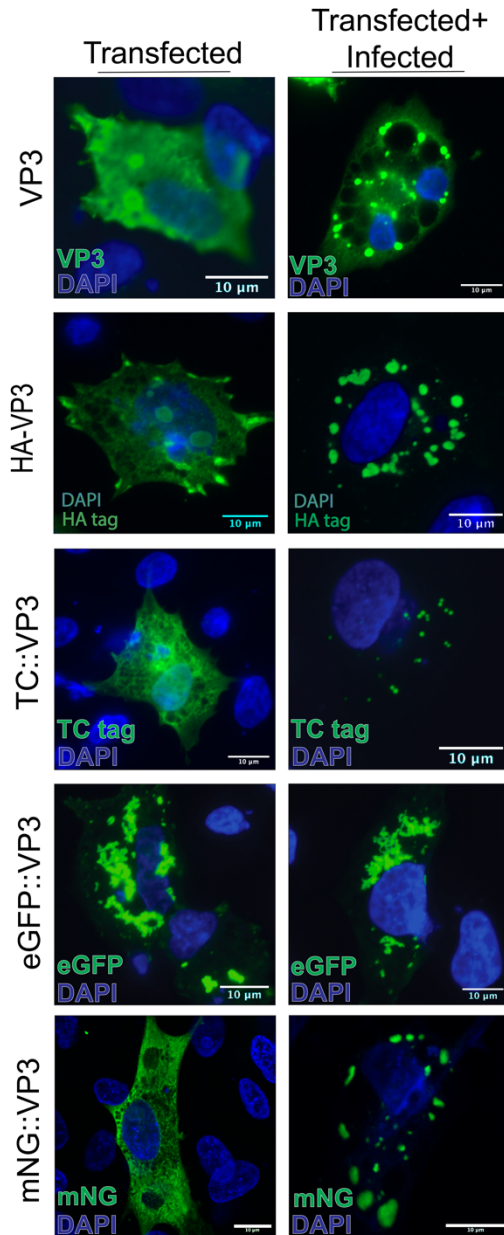


Figure 3.1 mNeonGreen Conjugated VP3 Behaves Similarly to Unlabeled VP3 in Cells. DF-1 cells were transfected with a vector expressing VP3, or reporter-conjugated VP3 constructs (HA::VP3, TC::VP3, eGFP::VP3, and mNG::VP3). Cells were imaged by confocal microscopy at 18 h.p.t., either alone (left column), or in cells simultaneously transfected with reporter::VP3 conjugates and infected with strain PBG98 IBDV (MOI=5). Scale bars, 10μm.

Although immunofluorescence microscopy was effective for visualizing VP3 in infected cells, it was incompatible with live-cell imaging and required a non-commercially available antibody [150]. As such, I modified our VP3 expression plasmid by conjugating a panel of reporters to the protein's N-terminus. The N-terminus was selected due to known replicative defects introduced by C-terminal reporter conjugation and the prior existence of an enhanced GFP (eGFP) N-terminal VP3 conjugate [95]. I generated mNG::VP3, TC::VP3, and HA::VP3, where TC represents the “tetracysteine” motif which can be fluorescently labeled by a reactive substrate, and “HA” is a small epitope of the influenza hemagglutinin (HA) protein against which commercially available antibodies have been raised. mNG::VP3 was generated by DNA fragment synthesis and restriction cloning (2.3.3), whereas TC::VP3 and HA::VP3 were

generated by PCR with mutagenic primers (2.3.2). These constructs were tested alongside eGFP::VP3, and unlabeled VP3 as a control. Cells were transfected with

plasmids expressing the respective reporter constructs under the control of a cytomegalovirus (CMV) promoter using Lipofectamine 2000 (2.3.4), then fixed with paraformaldehyde and stained with DAPI for nuclear visualization. Each plasmid was tested under two conditions: expressed independently and in cells simultaneously transfected with the respective construct and infected with strain PBG98 IBDV. With the exception of eGFP::VP3, all tested reporters approximated the behavior of exogenously expressed VP3: while numerous cells had small, point-like signal enhancements and some exhibited annular structures akin to those observed by Gimenez et al. [98], high-expressing cells exhibited a diffuse, ubiquitous cytoplasmic signal distribution. In the case of eGFP::VP3, I observed many cells with large, extremely bright signal regions. These were morphologically very different from the rounded appearance expected of IBDV VFs or LLPS structures more broadly, and their complex edge geometries led us to hypothesize that they were aggregates.

When co-expressed with IBDV infection, all reporter constructs, except eGFP::VP3, exhibited the behavior expected of unlabeled VP3 to some degree. The eGFP::VP3 again formed aggregates, so I could not determine whether recruitment of eGFP::VP3 to VFs had occurred. In cells expressing TC::VP3, after labeling with FLaSH reagent (a green fluorescent compound that binds the TC motif), fluorescently labeled puncta were noted in infected cells. However, this system requires pretreatment of cells with a quenching agent followed by treatment with FLaSH. This protocol introduces variability and complexity as compared to constitutive fluorescent protein fusion [151]. HA::VP3 and mNG::VP3 behaved the most similarly to wild-type VP3 in our screen: predominantly diffuse when expressed alone, but exhibiting near complete redistribution into morphologically appropriate puncta in the presence of infection (Figure 3.1). I

therefore elected to use mNG::VP3 as our live-cell-compatible reporter-conjugated VP3 and retained HA::VP3 as an immunofluorescence-compatible VP3 construct for situations where direct labeling with our anti-VP3 monoclonal was not optimal.

3.2.3 Computer Vision Facilitates the Extraction of VF Morphologies From Micrographs

Although our spike-in system proved effective for labeling VFs in the context of infection, micrographs alone are not quantitative. Image analysis suites such as Fiji allow the creation of freehand selections, where the outline of a feature, such as a VF, can be traced by dragging the cursor. This relies both on the steadiness of the analyst's hand and a subjective judgement of the object's margin to determine geometry. However, IBDV VFs are micron-scale features, and due to measurement inaccuracies and unconscious biases, measuring them "by hand" is unlikely to yield accurate or consistent data. I therefore aimed to develop a computer vision-based pipeline to quantify and measure labeled VFs in micrographs. Though the pipeline was largely written in Python, the majority of the image operations (and indeed the source code due to shared libraries) are also available in widely-used scientific image analysis platforms such as Fiji/ImageJ [152].

In computer vision, the problem I aimed to solve is termed "segmentation" – broadly, the classification of pixels as either foreground (part of an object) or background (not part of an object). Several algorithms exist for this purpose, and of these I selected the watershed algorithm for segmentation of VFs from micrographs [153]. Our implementation of the

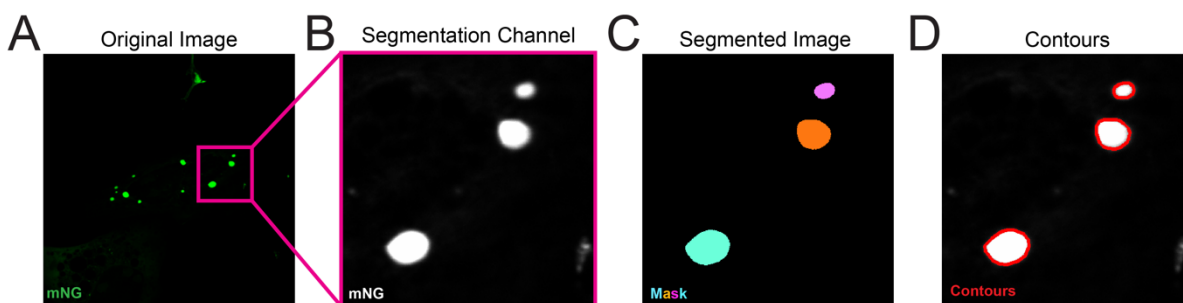


Figure 3.2 Extraction of VF geometries and contours with computer vision. (A) DF-1 cells were simultaneously transfected with mNG::VP3 and infected with strain PBG98 IBDV (MOI=5), and imaged with a Zeiss LSM 980 Airyscan confocal microscope. (B) The channel representing the segmentation target (mNG) was extracted from the micrographs. (C) Watershed segmentation was applied to generate a mask denoting VFs, and the segmented images were labeled to allow individual objects to be analyzed. (D) Contours were detected, isolating VF margin geometry for downstream analysis.

watershed approach was somewhat customized. Candidate VFs were first identified as peak local maxima [154] and high-probability background pixels were identified by percentile-gating intensity. The relative intensity of the image was then used to define “basins” which are “flooded” by the watershed algorithm. For data consistency, segmenting all images for a particular experiment under the same settings is ideal; accordingly, our approach was designed to be as agnostic as possible to variations that typically occur between images within a dataset.

Unconnected blobs of pixels (the identified VFs) were then assigned unique labels (Figure 3.2C). Each labeled VF was also used to define a contour: a multi-segment contiguous line defining the perimeter of the VF (Figure 3.2D). A variety of geometric measurements were then performed on each segmented VF – the area, perimeter, circularity (closeness to a perfect circle), and solidity (degree of convexity) were measured directly from the pixel data. (Figure 3.2C) Because contours encircle each VF, they encode information about the edge morphology of the structures

– as the interface between the heavy and light phases in LLPS, the geometry of this region was of interest. Because contours encircle VFs completely and are by necessity defined by pixel coordinates, I construed each contour as a stepwise periodic function to permit differential geometric analysis. From these functions, I calculated the Fourier spectra and Laplacian energy, and directly measured the curvature parameter κ (2.5.4, 2.5.5) [155].

3.2.4 VFs Across Different Timepoints Form Two Morphological Clusters

Armed with our consistent spike-in labeling system and after developing our pipeline for morphological characterization, I next sought to reassess the question of time-dependent morphological change more rigorously. I selected four timepoints for this study: : 8, 12, 16 and 24 hpi for balanced temporal resolution within the biologically relevant timescale of IBDV infection, as a single cycle of IBDV replication is completed within 24 hours. I simultaneously transfected DF-1 cells on coverslips with mNG::VP3 and infected them with strain PBG98 IBDV at an MOI of 5, and cultured them until their designated timepoint. At each timepoint, cells were fixed with 4% paraformaldehyde, and coverslips were mounted to slides for visualization. Punctate cells were imaged with a 63x oil immersion objective on a Zeiss LSM980 Airyscan scanning confocal microscope, and the images were processed with our computer vision pipeline to extract puncta geometries. All segmentations were manually screened during processing – incidental segmentation of apparent debris was addressed by targeted removal of that individual object from further analysis, and images with failed segmentations were rejected.

For each contour, shown plotted in polar coordinates, I calculated a Fourier spectrum (up to the 255th harmonic), the Laplacian energy, and the curvature (Figure 3.3A, B). As these metrics

encode subtly different but unintuitive aspects of shape variation, I elected for a dimensionality reduction approach [156]. I embedded the shape metrics (Fourier spectral slope, Laplacian energy and curvature variation) in a feature space and used uniform manifold approximation and projection (UMAP) to perform a dimensionality reduction (n_neighbors=50, min_dist=0.1). Although the data clustered visually, I formally identified the clusters using Hierarchical Density-Based Spatial Clustering of Applications with Noise (HDBSCAN), a non-parametric clustering algorithm [157] (Figure 3.3C). I identified two clusters, a major cluster and a minor cluster. I then normalized and

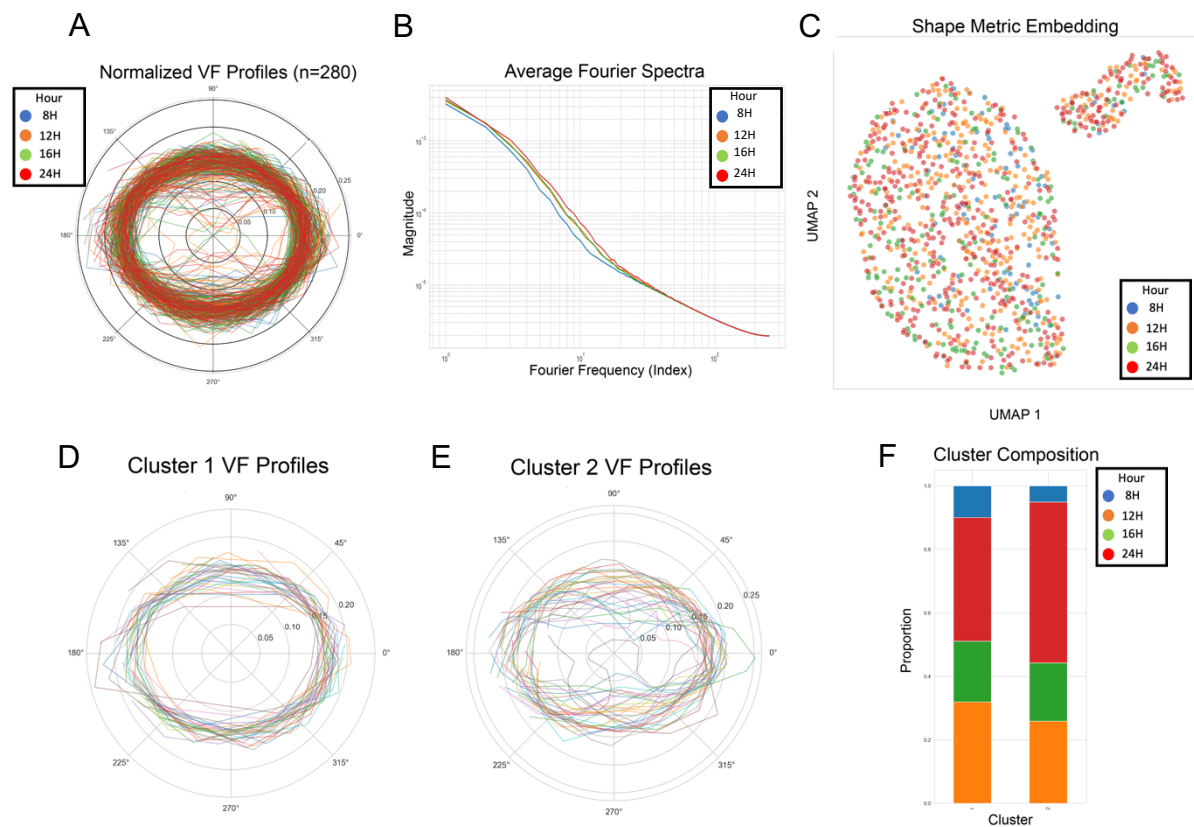


Figure 3.3 VFs Across Different Timepoints Form Two Morphological Clusters. Images of cells simultaneously transfected with mNG::VP3 and infected with IBDV (MOI=5) were captured with a Zeiss LSM 980 Airyscan confocal microscope at 8h.p.i, 12h.p.i, 16h.p.i and 25h.p.i, and processed for Fourier and Laplacian analysis. (A) VFs were segmented from images

and represented as contours, which were normalized and plotted with polar coordinates. (B) Magnitudes of the first 255 Fourier frequencies computed by applying a Fourier transform to the detected contours, grouped by time post infection. (C) Shape metrics (spectral slope, Laplacian energy and curvature variation) were embedded as features and a UMAP plot generated from these data ($n_neighbors = 50$, $min_dist = 1.0$). (D) VF profiles selected randomly from cluster 1 - I have termed cluster 1 the “regular” cluster due to the regularity of its profiles (E) VF profiles selected randomly from cluster 2 - I have termed cluster 2 the “irregular” cluster due to the irregularity of its profiles. (F) Clusters were identified from the original UMAP plot with HDBSCAN and their timepoint composition profiled (proportion of data from each timepoint per cluster plotted).

plotted the contours from each cluster, and noted that cluster two exhibited a greater variation in subjective contour complexity than did cluster one – cluster two contained both very round and very irregular contours (Figure 3.3D, E). I also determined the composition of each cluster – the proportion of contours in the cluster that came from each timepoint. I noted that contours from the 24h timepoint comprised a larger proportion of the cluster two VFs than cluster one, indicating that there is greater morphological variability in properties in the late timepoint VFs (Figure 3.3F).

3.2.5 Some VF Geometric Properties Change Significantly Throughout the Course of Infection, but shape remains largely consistent.

Our analysis of contour morphology is not the only means of analyzing the shapes of detected objects – in fact, most quantitative studies of morphology involve calculating a variety of geometric parameters, including familiar properties such as area and perimeter, as well as less familiar and less intuitive properties such as circularity and solidity. Fortunately, once a segmentation has been determined for an image, calculation of these metrics is straightforward, typically requiring only a single call to a library function [158]. The vast majority of the thirty-

seven measurements per VF thus obtained are not pertinent to the study of VF morphology and are not included herein. I focused our analysis on three “classical” geometric properties of VFs: area, perimeter, and solidity. Area and perimeter accord with common understanding (although area is discretized at the pixel level and perimeter is inherently an approximation of a contour passing through the border pixels), where solidity is defined as the ratio between the area of an object and that object’s convex hull (the smallest convex shape that can contain the object). This is an alternative to the more commonly used circularity, the ratio of the shape’s area to that of a circle with the same perimeter [158]. Although the measure of circularity is useful, LLPS structures in the crowded microenvironment of a cell may attain smooth yet irregular shapes. Where these smooth, irregular shapes would have a low circularity, they would retain a high solidity so long as they are convex. I found that mean VF area and perimeter both exhibit a trend towards greater values at later time points, with inter-time-point differences typically significant ($p < 0.001$) (Figure 3.4A, B). Solidity differed in this respect – although I observed that the lowest observed solidity values trended lower at later timepoints, these extreme data were sparse, therefore no significant difference was observed in solidity across time points (Figure 3.4C).

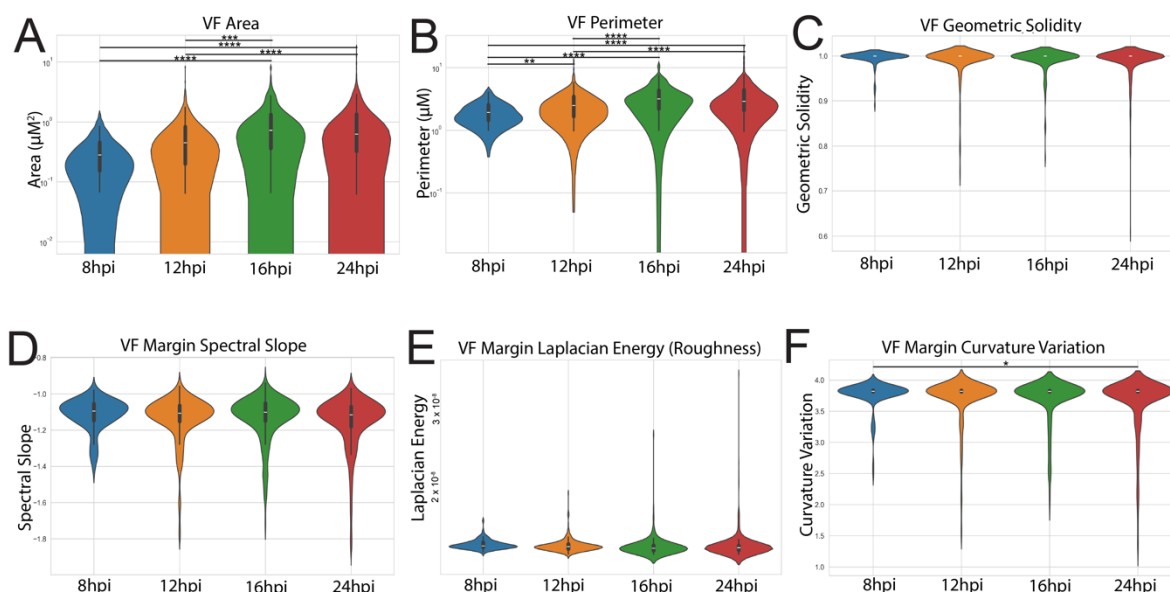


Figure 3.4. Some geometric properties of VFs change significantly over the course of infection, but shape remains largely consistent. VF profiles were grouped by timepoint and analyzed for geometric (A, B, C) and computed margin (D, E, F) shape metrics. For all plots, significant differences indicated with asterisks; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ (A) Mean VF area (B) Mean VF Perimeter (C) Mean VF geometric solidity (D) Mean VF margin spectral slope (E) Mean VF margin Laplacian energy (F) Mean VF margin curvature variation.

We also performed grouped statistical analysis of the metrics calculated from contours. Although trends were visible in the data, no significant difference was noted between time points for the mean Fourier spectral slope or Laplacian energy (Figure 3.4D, E). I did note that VFs at twenty-four h.p.i had a significantly higher curvature variation than VFs at eight h.p.i, though the effect size was small, and no significant differences were noted in curvature variation between any other timepoints.

3.2.6 VFs Show Increased Liquidity at Late Timepoints

As described above, our spike-in system permits the visualization of fluorescently labeled VFs in live cells. I therefore employed FRAP to measure molecular mobility within VFs at different timepoints post-infection. DF-1 cells were cultured in glass-bottom confocal dishes and were

transfected and infected in the same manner as cells used in fixed imaging experiments. Rather than fixing and mounting, however, at the appropriate time points, dishes were transported to the campus imaging core for FRAP analysis. Acquisition and bleaching parameters were kept constant between individual trials, replicates, and conditions (2.4.5). Data were analyzed in Python using a re-implementation of the EasyFRAP-Web method (2.4.5). Data points represent the mean of at least three replicates per condition, lines represent the calculated fit for the data, and error bars represent SEM (Figure 3.5A).

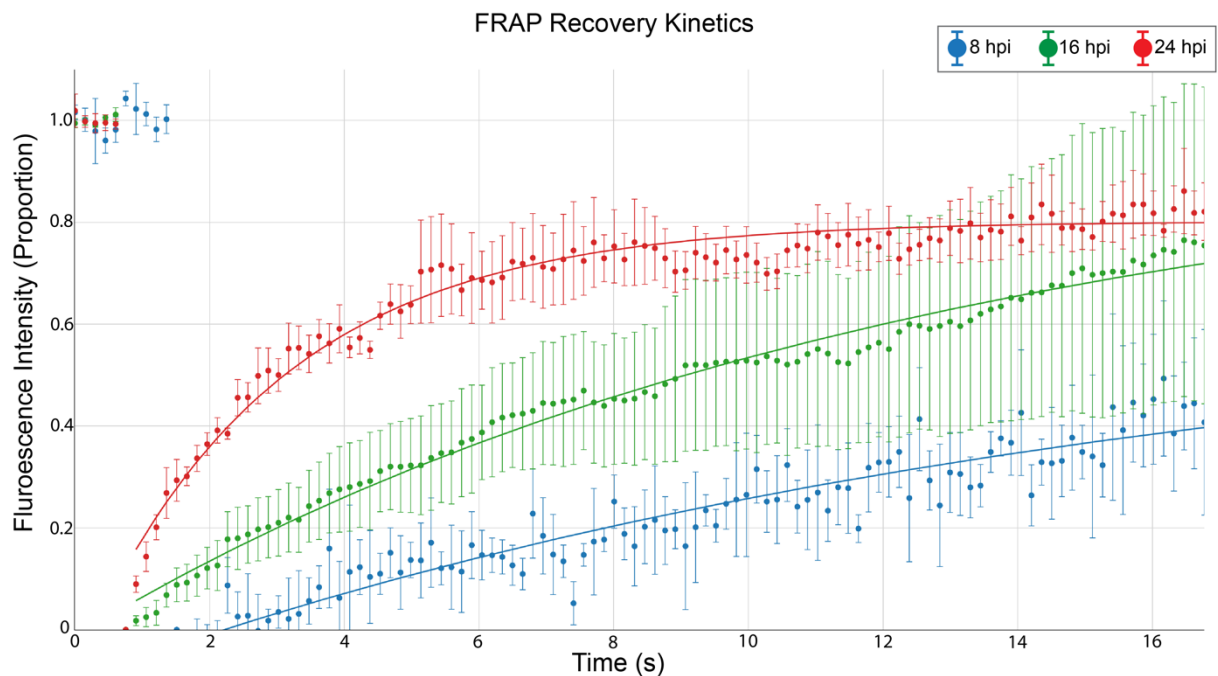


Figure 3.5 VFs Show Increased Liquidity at Late Timepoints.

DF-1 cells were simultaneously transfected with mNG::VP3 and infected with PBG98 IBDV (MOI=5). FRAP experiments were performed at 8, 16, and 24 hours post-infection (h.p.i), and the mean fluorescence of the bleach region was plotted, along with a single-exponential fit curve (solid lines). At 8h.p.i, VFs had a mobile fraction (MF) of 0.646 and a half-recovery time ($t_{1/2}$) of 10.61s. At 16h.p.i, VFs had an MF of 0.972 and $t_{1/2}$ of 8.54s. At 24h.p.i, VFs had an MF of 0.802 and $t_{1/2}$ of 2.02s.

We found that although recovery was observed at all time points, it was substantially more rapid at later time points. At 8 h.p.i, VFs had a mobile fraction (MF) of 0.646 and a half-recovery time ($t_{1/2}$) of 10.61s. At 16h.p.i, VFs had an MF of 0.972 and $t_{1/2}$ of 8.54s. At 24h.p.i, VFs had an MF of 0.802 and $t_{1/2}$ of 2.02s. These data suggest that although VFs are liquid at all timepoints measured, their liquid character increases throughout the course of infection.

3.3 Discussion

Though the rigorous investigation of any cellular structure presents challenges, the mechanics of LLPS formation and the biochemistry that underlie their properties present unique obstacles that necessitate the development of specialized tools. It is also broadly desirable to develop strategies to manipulate and interrogate an LLPS structure – its ephemeral, environment-driven properties necessitate maximizing *in situ* measurements for any investigation. I therefore set out to develop a suite of tools to characterize the LLPS VFs formed by IBDV. Though our primary aim was the development of a broadly applicable suite of tools and techniques, I also sought to expand our understanding of IBDV by applying this suite at different time points post-infection, thereby providing temporal resolution of VF properties.

The simplest means of characterizing VFs during infection is visualization by immunofluorescence microscopy. By their nature, LLPS structures are sites of elevated concentration of biomolecules – this is what defines them. Although not strictly chemically necessary, LLPS structures are overwhelmingly, at least somewhat, proteinaceous [45], making them an attractive target for antibody generation. This is the case for IBDV VFs as well: one of the primary components of VFs, protein VP3, is strongly partitioned into the heavy phase, and I

was able to obtain a monoclonal antibody raised against this viral protein, making the stain both regiospecific and specific to infected cells. However, techniques that rely on immunofluorescent detection of intracellular targets are limited to fixed and permeabilized cells, permitting static analysis but precluding dynamic study.

Although this limitation applies to immunofluorescent detection of any intracellular target, it is of particular concern in the study of LLPS. LLPS structures are dynamic – they diffuse internally, are generally mobile unless tethered or constrained, undergo elastic deformation, and may even exchange molecules with their surroundings when those molecules are chemically compatible [159]. These properties are central to the behavior of LLPS structures – they dictate how the structures move, interact, and what chemistry may occur within them. As such, interrogation methods constrained to fixed cells inherently cannot probe fundamental aspects of LLPS structural properties.

We therefore leveraged the “spike-in” modality to fluorescently label IBDV VFs in live cells during infection. By simultaneously expressing reporter-conjugated VP3 in cells during IBDV infection, reporter VP3 is recruited to the VFs formed by the infection and, upon intermixing with the unlabeled VP3 encoded by the virus, labels the entire structure. Spike-in is attractive for several reasons, including the ability to sidestep the need to edit the virus to encode a fluorophore. Although reporter IBDV has been generated in the past, using both the TC-tag system and the split-GFP system, these reporter viruses were attenuated, and the inserted tags were unstable, and were evicted from the viral genome within several passages [94]. Spike-in, by

contrast, does not require an edited virus and requires only simple transfection of cells with an expression plasmid. However, the technique is not without limitations.

The first is somewhat unique to the technique: exclusion of simultaneous infection. Cells respond to both lipid nanoparticle transfection and viral infection, and a cell already engaged in such a response is hostile to infection and transfection. This necessitates the virus and transfection reagent reaching the target cells at approximately the same time – prior transfection or infection leads to drastic reductions in the proportion of cells that are both transfected and infected – only untransfected cells tend to be infected, and vice versa. This mutual inhibition effect can be mitigated in some viruses: in the case of ReoV, infection with infectious subviral particles (ISVPs) as opposed to virions overcomes much of the exclusionary effect. However, no such option exists in the case of IBDV. In our testing, I found that simultaneous transfection and infection were optimal, along with a relatively high MOI of 5. Even then, the proportion of simultaneously transfected and infected cells was not large, necessitating the preparation of many cells in order to collect sufficient data.

The second limitation is not unique to spike-in, and is rather a consideration that must be made when investigating LLPS structures by any means that alter their composition: LLPS is a sensitive phenomenon. As was exhibited by Barkley *et. al.* and others, even small, typically “benign” reporters such as epitope tags can substantially modulate the physical properties of LLPS structures as compared to their unlabeled counterparts [160]. This sensitivity is a consequence of the biology of LLPS formation – it cannot be avoided, and instead must be mitigated where possible, and must inform experimental design and analysis. Mitigation is

achieved by tag selection. As each LLPS system differs, there is no panacea reporter that minimally affects all LLPS – candidate reporters must be selected and screened, and our data lends credence to this principle. eGFP was long considered (and is still sometimes labeled) a “monomeric” fluorescent protein. While this is true in comparison to the truly multimeric GFP, eGFP retains some self-affinity [161]. In most cases, this does not present a problem. However, in a sensitive system like a protein with the propensity to phase separate, conjugation with eGFP can lead to the formation of aggregates as I observed, or even spurious phase separation. Further, even labeling with a small, reactive tag such as TC led to the formation of morphologically distinct VFs. It was only by generating and screening a panel of reporters that I was able to select HA::VP3 and mNG::VP3 as appropriate candidates for our reporter.

However, even when candidate reporters exhibit appropriate behaviors, they may still influence the properties of VFs. Unfortunately, without the means to measure the physical properties of unlabeled VFs, it is challenging to quantify, or even detect, such perturbations. This limitation must be addressed by experimental design. Any modified LLPS structure, either created from modified components (as in reporter viruses) or modified *post-hoc* by the spiking in of a reporter must be treated as a distinct species – the measured properties cannot be compared to those of unlabeled structures. Our data therefore is entirely comparative – spike-in labeled VFs are always compared to other spike-in labeled VFs.

With a system permitting the labeling of VFs in live cells developed, I turned our attention toward extracting maximum utility from static analysis. VFs, as biomolecular condensates, are not assembled into a particular geometry, rather their geometry is an emergent property of their

underlying biophysics. I therefore hypothesized that VF morphology might act as a proxy for fundamental biochemical changes occurring throughout the course of infection and set out to measure those morphological properties. To that end, I developed a computer vision pipeline for the automated segmentation and analysis of VFs in micrographs. Computer vision is of particular utility in this case because it can exhibit two properties advantageous for the detection of subtle changes: consistency and parallelism. Manual demarcation of VFs for measurement is not tenable at scale, human fatigue and imprecision will introduce unacceptable “noise” into a dataset where any “signal” may be weak. The adoption of a computer vision pipeline also enabled us to process hundreds of VFs quickly – for the detection of weak phenomena, extraction of large datasets of this nature is invaluable. Because LLPS structures frequently exhibit rounded, hard-edged morphologies similar to IBDV VFs, these tools are broadly applicable to the study of other viral condensates as well as LLPS structures more broadly.

We applied several mathematical techniques to extract meaningful information from segmented VFs. By conceptualizing the contour (border shape) of each VF as a periodic function, I was able to calculate metrics such as Fourier spectra, Laplacian energy, and curvature from each VF. The Fourier spectrum characterizes how structural variation in a system is distributed across spatial scales, revealing whether complexity arises from large-scale organization, fine-scale features, or a combination of both [162]. This metric captures the distribution of “scales” in the data – whether VF borders defined by low-frequency variation such as large lobes, or high frequency variation such as numerous small bumps. Laplacian energy quantifies the total degree of local spatial irregularity in a system by measuring how strongly each element deviates from its immediate neighborhood, providing a global, coordinate-independent measure of structural

complexity [163]. Essentially, Laplacian energy collapses “shape complexity” to a single value. Curvature, though related, is distinct in that it quantifies the local bending of a structure. Though not often applied to the analysis of micrographs, these metrics are standard tools of geometric analysis, and are well suited to detection of small variations between similar structures.

Although our data did reveal clustering of VFs into “broadly regular” and “irregular” groups, the latter of which was overrepresented in the latest timepoint, I did not observe dramatic shifts in shape metrics over the course of infection. Indeed, of the measurements I made (including the more typical geometric measurements of area, perimeter, and solidity), only area and perimeter showed consistently significant differences across timepoints, both tending to increase at later timepoints. Although I did discover that the mean variation of curvature for VFs at twenty-four hours post infection was significantly lower than that of VFs at eight hours post infection, the effect size was small and only existed between these two timepoints. I did note generally increasing presence of extreme values for spectral slope and Laplacian energy at late timepoints, but these were rare and did not significantly alter the distributions at each timepoint. I have therefore somewhat unsensationally determined that IBDV VFs grow larger throughout the course of infection, but their shapes do not significantly change.

Although the morphological properties (at least those measurable within the constraints of our system) generally did not significantly vary across timepoints, the same cannot be said for the dynamic properties of VFs. Leveraging the compatibility of our spike-in labeling modality with live-cell imaging, I used FRAP to measure diffusion within VFs at different timepoints post infection. I found that at all measured timepoints, VFs exhibit liquid-like characteristics: they

recover to fairly high levels on relatively short timescales – but recovery was substantially faster and more complete at later timepoints. Mobile fraction, a measure of what proportion of molecules are free to diffuse within the structure, increased from 0.646 at eight hours post infection to 0.972 at sixteen hours post infection, and was reduced slightly to 0.802 at twenty-four hours post infection. For the same time points, half recovery time decreased from 10.61s to 8.54s, and finally to 2.02s at twenty-four hours post infection.

This is notable in isolation, and more so in the context of the dsRNA virus field. Although it is possible that the change in liquidity is incidental to some other process, it is also possible that IBDV leverages this temporal liquidity gradient for a replicative advantage – perhaps at early timepoints, stricter exclusion of host surveillance factors is a worthy trade-off for slower internal diffusion and mixing. In the context of dsRNA viruses, the finding is particularly notable because it represents the opposite effect observed in rotavirus whose LLPS replicative structures, termed viroplasms, become “harder” (less liquid) at later timepoints. Geiger *et. al.* noted reduced responsiveness of viroplasms to dissolution with propylene glycol, another aliphatic diol with condensate disrupting properties akin to 1,6-HD or 1,3-PD, at 12 h.p.i as compared to 4 h.p.i. They also noted reduced FRAP recovery of viroplasms at the 12 hour time point, which coincided with measured hyperphosphorylation of NSP5, suggesting a chemical mechanism for the changing material properties of viroplasms [149].

Compared to *Rotavirus*, IBDV is understudied, particularly at the biochemical level. As such, where information about IBDV is not known, it is frequently assumed that mechanisms

analogous to rotavirus or ReoV exist for IBDV. Our findings challenge that assumption and highlight the importance of direct investigation where possible.

3.4 Conclusions

In this study, I have developed and demonstrated a system for fluorescent detection of IBDV VFs in both fixed and live cells, that is compatible with dynamic analysis modalities such as FRAP. To achieve this, I leveraged a “spike-in” approach, where exogenously expressed reporter conjugated VP3 is “recruited” to VFs during simultaneous infection, causing the reporter to partition into the VF along with the VP3 carrier. I screened multiple reporters from different classes and selected mNG as the optimal reporter based on its performance in our screen and compatibility with fixed and live imaging techniques. I also developed a computer vision pipeline for the detection and measurement of VFs in micrographs, one which incorporates both traditional geometric measurements and more advanced differential geometry for sensitivity and accuracy. Because these tools were designed for geometric and liquidity analysis, they are in principle transferrable to other systems that form morphologically similar bodies including other viral condensates, cellular condensates, or visually hard-edged structures in micrographs generally. As an initial investigation with this suite of tools, I measured the morphology and liquidity of IBDV VFs at multiple timepoints post infection. Our data confirmed previous findings that IBDV VFs grow larger throughout the course of infection, and newly revealed that the morphology of VFs remains relatively consistent throughout infection. Surprisingly, I found that IBDV VFs exhibit increased liquidity at later timepoints, a result opposite to the behavior of analogous LLPS replicative bodies formed by the dsRNA *Rotavirus*, a member of the *Reoviridae* family. Our results offer insights into the temporal dynamics of VF morphology and physical properties through the course of infection, highlight a newly discovered difference between

members of the *Birnaviridae* and the *Reoviridae*, and provide a platform of tools and technologies that can be leveraged to explore the determinants of IBDV VF properties.

Chapter 4: Defining the minimal components required for the formation of IBDV VFs and for driving the LLPS process

4.1 Introduction.

4.1.1 Modes of phase separation

As discussed above, although the demixing phenomenon that creates the heavy and light phases in LLPS is generally spontaneous above C_{Sat} , not all LLPS is driven by a single biomolecule, nor does it always occur in isolation. Among the dsRNA viruses described herein, rotavirus and ReoV provide useful examples of two distinct “modes” of phase separation, differing in both the number of scaffold biomolecules and the cellular microenvironment in which phase separation occurs. The formation of viroplasms in rotavirus infection is associated tightly with LDs. Viroplasm scaffold proteins NSP2 and NSP5 associate closely with LD resident proteins perilipin (PLIN1) and ADRP [101], an association which has functional significance. If LD biogenesis is disturbed by pharmacological inhibition of fatty acid (FA) synthesis, viroplasm growth and function is interrupted [101]. This suggests that LDs may help to organize the assembly or maturation of viroplasms. By contrast, ReoV μ NS appears to nucleate biomolecular condensates completely independently, with no apparent reliance on LDs or membranes for organization or maturation.

The work performed by Gimenez *et. al.* and Zanetti *et. al.* indicates that IBDV VFs nucleate more similarly to rotavirus viroplasms than μ NS condensates [98,99]. IBDV VF-resident VP3

associates closely with PI3P-enriched EEs both in early infection and when exogenously expressed in cells, This suggests that EEs act as “nucleation sites” for VF biogenesis. This hypothesis is biophysically reasonable – as scaffold molecules must reach C_{Sat} for phase separation to occur, when scaffold biomolecules associate with a cellular component or compartment, it is likely that C_{Sat} will be reached locally in proximity to these sites, and thus phase separation will first occur there.

Although the utilization of a cellular “platform” for the nucleation or maturation of VFs in IBDV appears more similar to rotavirus viroplasms than the spontaneous cytoplasmic nucleation ReoV VFs, the scaffold composition of IBDV VFs has, as of yet, not been determined. ReoV μ NS exhibits phase separation independently – even at low concentrations, μ NS phase separates with no apparent reliance on other biomolecules, cellular or viral [44]. Rotavirus viroplasms, by contrast, are a prototypical example of a bipartite scaffold, as neither NSP2 nor NSP5 phase separates independently in cellular conditions, but phase separate readily when mixed in almost any ratio [149].

As discussed above, I hypothesized that VP3 acted as a proteinaceous scaffold for the formation of IBDV VFs. I therefore aimed to characterize the scaffolding behavior of VP3 and to determine whether, like ReoV μ NS, VP3 phase-separated independently, or whether, like rotavirus NSP2 and NSP5, multiple biomolecules were necessary for phase separation to occur.

4.2 Results

4.2.1 VP3 Is Necessary but Not Sufficient to Drive the formation of cytoplasmic puncta

Previously, I determined that co-transfecting cells with reverse genetics plasmids encoding segments A and B results in the rescue of infectious IBDV [96][94]. To determine the necessity of VP3 in the formation of VFs, here I generated a truncated variant of segment A lacking the VP3 coding sequence (CDS) (SegAΔVP3) (Figure 4.1).

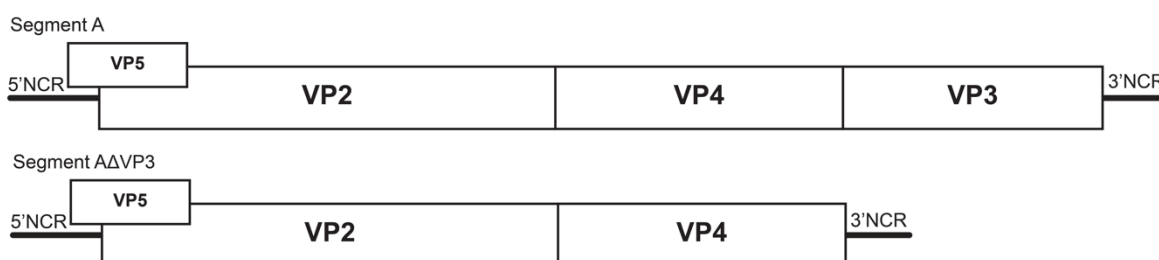


Figure 4.1 Segment AΔVP3 Schematic. Schematic diagram of wild type (wt) Segment A (A) compared to Segment A ΔVP3 (B).

When DF-1 cells were transfected individually with wild-type segment A (SegA) or a wild-type segment B containing an HA tag fused to the N-terminus (HA::SegB), punctate structures were not observed, but when cells were co-transfected with SegA and HA::SegB together, puncta were observed, as expected. In contrast, no puncta were observed in cells that were co-transfected with SegAΔVP3 and HA::SegB together, although it was not possible to image SegAΔVP3, as the antibody I used was raised against VP3.

Furthermore, when cells were co-transfected with SegAΔVP3, HA::SegB, and a wt VP3 under the control of a CMV promoter (VP3), the punctate phenotype was recovered (Figure 4.2).

Taken together, these data indicated that VP3 was necessary but not sufficient for the formation of cytoplasmic puncta.

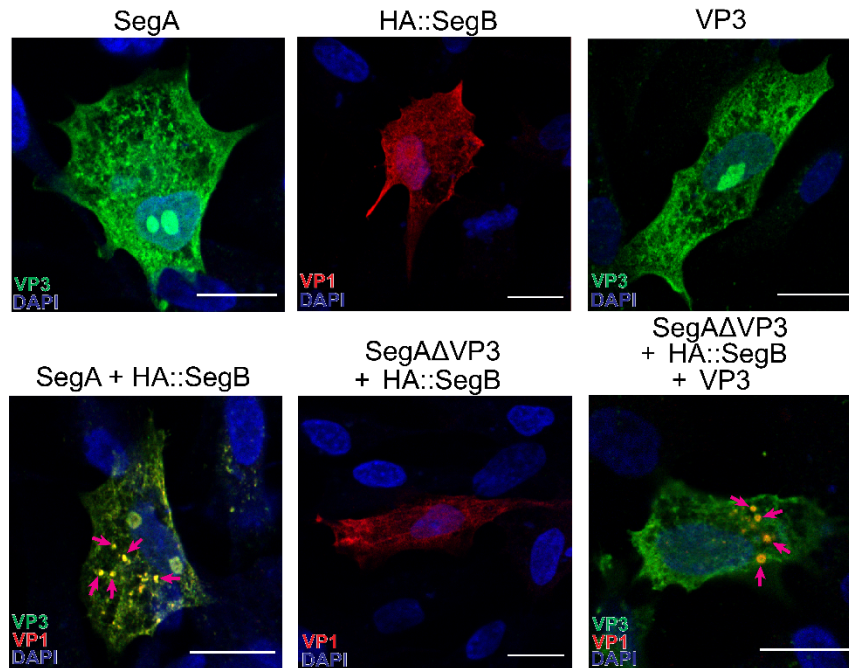


Figure 4.2 VP3 Is Necessary but Not Sufficient to Drive VF Phase Separation. DF-1 cells were transfected with Segment A (SegA), HA::Segment B (HA::SegB), VP3 alone, or SegA + HA::SegB, SegAΔVP3 + HA::SegB, or SegAΔVP3 + HA::SegB + VP3 (A). Cells were fixed at 34 hours post transfection (h.p.t.), and stained with DAPI (blue), and antibodies against VP3 (green), or HA (red). Scale bars, 50μm.

4.2.2 VP3, VP1, and RNA Are the Minimal Components Necessary for the Formation of Phase Separated Biomolecular Condensates

Next, I sought to determine the minimal components of IBDV phase-separated biomolecular condensates. As SegA did not lead to the formation of cytoplasmic puncta, and encodes VP2, 3, 4, and 5, I concluded that VP3 did not form condensates in the presence of these other proteins. As SegB did not lead to the formation of cytoplasmic puncta, and encodes VP1 which synthesizes vRNA, I concluded that VP1 and vRNA did not lead to the formation of condensates in the absence of other factors. As co-transfection of SegA and SegB did form cytoplasmic puncta, I hypothesized that VP3 and VP1 either in the presence or absence of vRNA would be necessary and sufficient for the formation of biomolecular condensates. To test this hypothesis, DF-1 cells were transfected with plasmids encoding VP3, VP1 fused to FLAG at the C terminus

(VP1::FLAG), or HA::SegB. No cytoplasmic puncta were observed in cells expressing VP3, VP1::FLAG, or HA::SegB alone. In contrast, cytoplasmic puncta were observed in cells co-expressing VP3 and HA::SegB, demonstrating that puncta were formed in the presence of VP3, VP1, and vRNA (Figure 4.3). It was not possible to image cells co-expressing VP3 and VP1::FLAG as the antibodies against VP3 and FLAG were the same isotype. Therefore, I imaged cells co-expressing mNG::VP3 and VP1::FLAG and also observed some puncta (Figure 4.3).

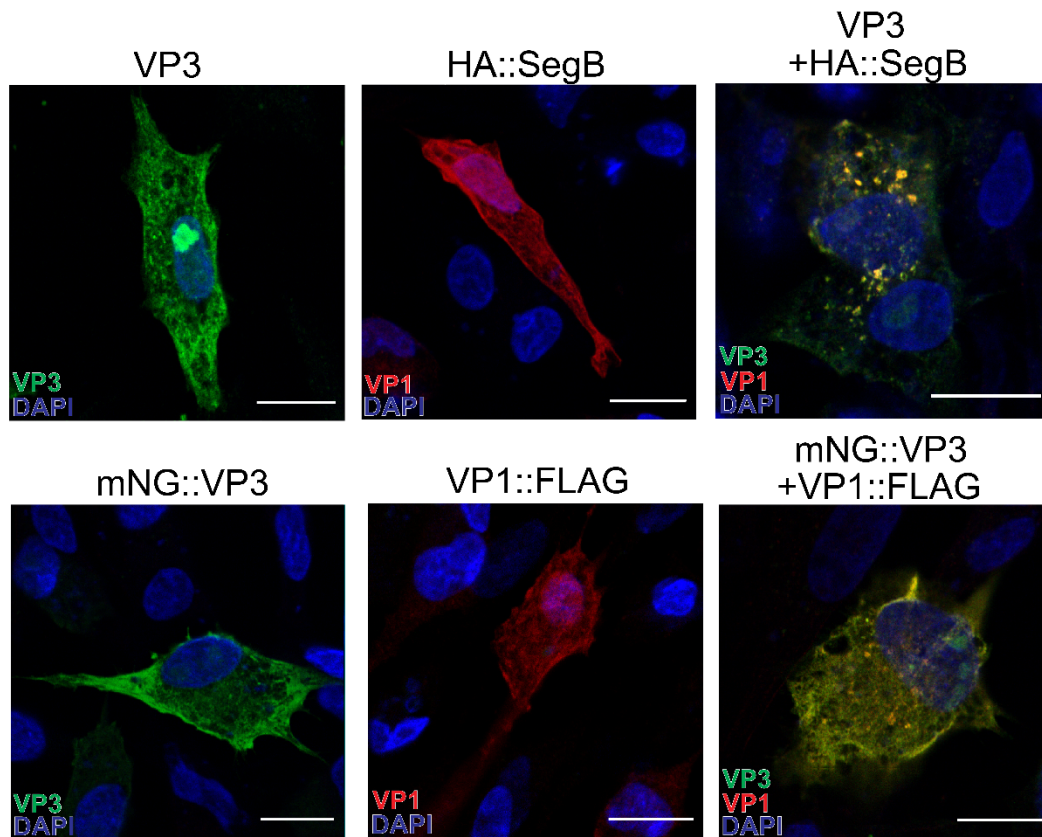


Figure 4.3 The Minimal Components of IBDV VF_s Are VP3 and VP1. DF-1 cells were transfected with mNG::VP3, VP1::FLAG, VP3, HA::Segment B (HA::SegB) or a mixture of VP3 + HA::SegB (A). Cells were fixed at 34h.p.t., and the nuclei were stained with DAPI (blue). mNG shown in green, Segment B (visualized by staining with anti-HA monoclonal antibody) and VP1 (visualized by staining with anti-FLAG monoclonal antibody) are shown in red. Scale bars, 50 μ m.

As the presence of cytoplasmic puncta does not necessarily equate to phase separated biomolecular condensates, I next imaged cells co-expressing mNG::VP3 and VP1, or mNG::VP3 and SegB by live-cell confocal microscopy. At 34 hours post transfection, the puncta in cells expressing mNG::VP3 and VP1 showed limited recovery by FRAP, with a mobile fraction of 0.465. In contrast, the puncta in cells expressing mNG::VP3 and SegB showed much more robust FRAP recovery with a mobile fraction of 0.911, indicative of true liquid-like characteristics (Figure 4.4). While the plasmid encoding VP1 under the control of a CMV promoter only expresses the VP1 protein, the plasmid encoding SegB expresses both VP1 and viral (v)RNA, as Seg B is part of the reverse genetics system which contains self-cleaving ribozymes upstream and downstream of the VP1 coding sequence, which makes non-capped RNA which can be utilized by the polymerase to synthesize vRNA. Taken together, these data demonstrate that the minimal components required for IBDV phase-separated biomolecular condensates to form were VP3, VP1, and vRNA.

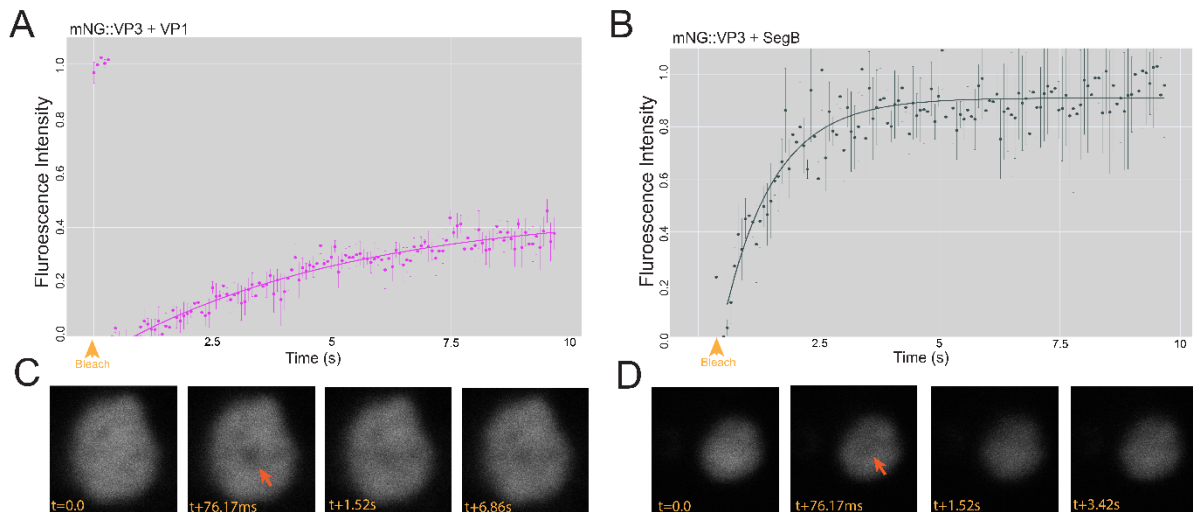


Figure 4.4 The Minimal Components of IBDV Phase Separated Biomolecular Condensates Are VP3, VP1, and RNA. DF-1 cells were co- transfected with a mixture of mNG::VP3 and VP1::FLAG and a time lapse series was captured by live confocal microscopy at 34 h.p.t. with a single iteration of point bleaching on the 5th frame (yellow arrowhead). Intensity data was extracted and used to calculate a FRAP recovery curve. Results are from 3 replicates; error bars

= SEM (A). DF-1 cells were co- transfected with a mixture of mNG::VP3 and SegB and a FRAP recovery curve generated in the same way (B). Representative recovery images for a photobleached puncta formed in the presence of VP1::FLAG with the time post bleach (t) shown in milliseconds (ms) (C). Representative recovery images for a photobleached puncta formed in the presence of SegB with the t post bleach shown in ms (D).

4.3 Discussion

In previous work, the Broadbent Lab determined that the VFs of birnaviruses such as IBDV were formed through the process of LLPS [94,95], but the molecular mechanism of how birnaviruses drive LLPS to form their VFs remained unknown. As a major component of IBDV VFs is the VP3 protein [95,96], I sought to investigate its role in forming VFs. When I expressed untagged VP3 independently in cells, I did not observe puncta characteristic of VFs, suggesting that VP3 alone was insufficient to drive phase separation. Furthermore, I removed the VP3 gene from our reverse genetics system and demonstrated the absence of cytoplasmic puncta even when all other viral proteins and vRNA were expressed. Moreover, when exogenous VP3 was added, the punctate phenotype was recovered. Taken together, these data demonstrate that VP3 is necessary, but not sufficient for the formation of IBDV VFs.

Previously, the Broadbent Lab had expressed eGFP::VP3 in cells and observed cytoplasmic puncta [95]. However, as described in Chapter 3, Section 3.2, the nature of the tag fused to VP3 is important for determining VP3's cellular distribution. It is likely that in the previous study, the eGFP tag caused the formation of puncta in cells transfected with eGFP::VP3 alone, which were artifacts. Therefore, based on the more extensive study I have conducted, I conclude that VP3 does not form puncta in cells when expressed alone, and the nature of the tag used is important in determining the cellular distribution of VP3. This is in contrast to the scaffold protein of ReoV

(μ NS), which spontaneously phase separates in transfected cells in the absence of other viral components [44], but is more similar to Rotavirus, where nucleation of replicative biomolecular condensates is driven by two viral proteins, NSP2 and NSP5 [42]. Neither NSP2 nor NSP5 phase-separate when expressed independently; only upon co-expression do phase separated condensates appear. However, because the biochemical definition of an LLPS scaffold requires only that the component be necessary for phase separation, our data support this classification of VP3 in the context of IBDV VF phase separation.

Interestingly, VP3 has been reported to form small foci in QM-7 cells stably expressing the protein alone [98,99]. However, these foci did not resemble the smooth or round structures that are characteristic of LLPS, and the authors did not assess their physical properties. In the present study, I also observed some small foci in DF-1 cells expressing VP3 alone. These formed what I would describe as a granular appearance and were particularly notable after fixation with paraformaldehyde (for example, see Figure 4.3, VP3). However, these foci did not have the same appearance as *Bona fide* VFs and were too small to meaningfully assess liquid-like recovery by FRAP. It is known that VP3 interacts with the cytoplasmic leaflet of endosomal membranes [92,98], and it is possible that accumulation of VP3 along these membranes is responsible for the granular appearance I observed in DF-1 cells, and the foci that have previously been reported in QM-7 cells. Moreover, in some cells, I also observed a ring-like “annular” phenotype when VP3 was expressed alone, which might be consistent with VP3 interacting with intracellular membranes.

Next, I sought to define the minimal component set for IBDV phase separation. Although I was able to detect some punctate structures in cells simultaneously expressing VP3 and VP1, these showed limited recovery by FRAP. It was only when I simultaneously expressed VP3 with IBDV Segment B (capable of generating both VP1 protein and vRNA) that I observed robust, liquid-like FRAP recovery, indicating that the minimal component set for phase separation consisted of VP3, VP1, and vRNA.

An interesting dynamic exists between these core components: VP1 acts as the RdRp, and one of VP3's various functions is to promote VP1's RNA replication activity [164]. When in complex with the 16 C-terminal residues of VP3, the catalytic domain of VP1 undergoes a notable remodeling from a "closed" to an "open" state by virtue of an 8Å movement of a VP1 domain termed the B-loop. This interaction results in a marked upregulation of VP1's catalytic activity *in-vitro*. Furthermore, the (+) sense vRNA produced by VP1 also serves as a template for protein synthesis, and both VP1 and VP3 are encoded by vRNA. Succinctly, VP3 activates VP1, which produces vRNA, thereby facilitating the synthesis of additional VP3 and VP1. Since these three components also form the minimal component set for IBDV VF phase separation, the phase-separating components and the core replicative components are one and the same. Furthermore, as a positive-feedback mechanism potentially exists, this may in part help explain our observations from Chapter 3, where liquidity of the IBDV VFs increased over time.

One limitation of the data in this chapter is that it relies on the transfection of cells with plasmids and does not study VP3 in the context of infection. Our data may therefore be an oversimplification. During infection, VP3 is not synthesized from a monocistronic vRNA, and is

rather derived from the cleavage of a VP2-VP4-VP3 polyprotein encoded by the large ORF of Segment A. The phase-separating component set, therefore, does not form an independent catalytic cycle, as VP2 and VP4 production are inherent byproducts of VP3 synthesis during infection. Rather, the interrelated nature of the minimal phase separation set with the core replicative machinery highlights the potential for the sensitive dependence of VF formation and properties on the behaviors of individual components.

Interestingly, as many cellular condensates, such as P-bodies, SGs, and the nucleolus, are driven by complex, multi-component mixtures including RNA, they are more akin to the minimal component set I have determined for IBDV VFs than the condensates formed by the *Reoviridae*. The simpler condensate-formation dynamics exhibited by ReoV may be advantageous to the virus, as, rather than requiring a collection of biomolecules that must all be synthesized and reach a concentration ratio conducive to phase separation, ReoV μ NS need only reach its own saturation concentration, C_{sat} , and phase separation proceeds spontaneously. The more complex formation dynamics of multi-scaffold condensates also raise the possibility of greater complexity in property determination, as their physical properties are determined by the individual properties, interactions, and relative quantities of their components. Our determination of the minimal composition of IBDV VFs, therefore, raised questions as to how the physical properties of the structures themselves are determined and modulated.

Chapter 5: The Structure and Function of the VP3 C Terminus

5.1 Introduction.

As previously discussed, LLPS as a phenomenon is broadly driven by a network of weak homo and heterotypic intermolecular interactions. In ReoV, the non-structural protein μ NS is a scaffold protein, which self-assembles into phase-separated structures in the absence of other viral components [44], yet only the extreme N- and C-terminal sequences of μ NS are likely to be disordered, and within the minimal region responsible for the formation of viral factory-like structures, there are two predicted alpha helices that have been proposed as principal interacting elements for μ NS self-assembly [160]. By contrast, in rotavirus, both NSP2 and NSP5 are required to drive LLPS. NSP5 is an IDP that can assemble into several higher-ordered oligomers [165] and the Carboxy (C-) terminal region of NSP2 is flexible, allowing it to participate in domain-swapping interactions that may be important in viroplasm formation [40].

Previous work demonstrated that VP3, a dsRNA-binding viral protein, was robustly expressed during infection and strongly partitioned into VFs [92,95]. Moreover, I previously demonstrated that VP3 was necessary for the formation of IBDV VFs (Chapter 4). Here, I hypothesized that VP3 might act as a driver for the LLPS phenomenon that underlies the formation of the factories. Previous work from our lab also demonstrated that, when tagged to eGFP, VP3 formed cytoplasmic inclusions morphologically similar to VFs, which, coupled with the findings of Gimenez *et. Al.*, suggested that VP3 might have a function analogous to ReoV μ NS [98]. Additionally, I found that the complete structure of VP3 had not yet been solved. As such, I set out to predict the structure of VP3, evaluate whether it contained an IDR, and determine if a

structure-functional relationship existed between VP3 and the phase-separating behavior of IBDV VFs.

5.2 Results

5.2.1 The C-terminal domain of VP3 lacks predicted secondary structure.

Given that VP3 is a major component of the IBDV VF structures, I hypothesized that VP3 would act as a scaffold protein and show structural features conducive to driving LLPS. Only the structure of the VP3 “core” domains (residues 82-220) had previously been solved by X-ray crystallography [166], so I used AlphaFold2 to model the structure of the full-length IBDV VP3 monomer from IBDV strain PBG98 (Figure 5.1D). The prediction was in good agreement with the crystal structure of the core domains, but notably lacked secondary structure in the C-terminal 36 residues (residues 221 – 257), hereafter referred to as the C-terminus. This correlated with a large predicted aligned error (PAE) in residue pairs in the C-terminus (Figure 5.1A), suggesting a low confidence in the position of these residues relative to the rest of the structure. Moreover, these data were in agreement with the results of a disorder prediction algorithm, which indicated an increased propensity for disorder in the C-terminus as well as the N-terminus, and within two predicted “hinge” regions between structured portions of the core (Figure 5.1B) [137]. This result inversely correlated with the Shannon entropy of the sequence [138] (Figure 5.1B). Taken together, the residues of the C-terminus lacked predicted secondary structure, showed high IUPred3 disorder scores, and reduced sequence entropy, which are typical of a low-complexity IDR when measured by classical methods [167].

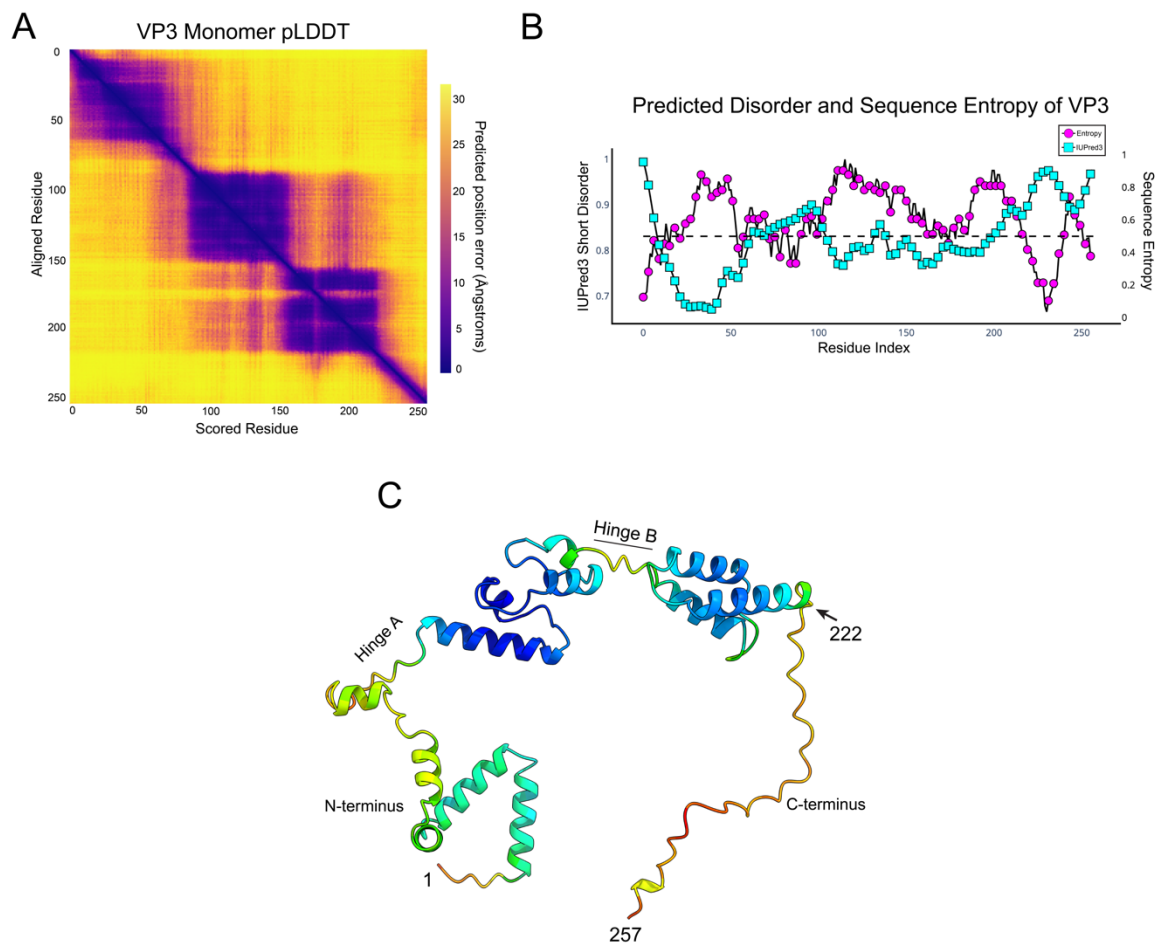


Figure 5.1 VP3 encodes a C-terminal domain with a predicted lack of secondary structure. A heatmap of the PAE values for the AlphaFold2 prediction of the VP3 monomer. Values represent AlphaFold's predicted error in the relative position of each pair of residues, measured in Angstroms and plotted on the x and y axes (yellow depicting high error and purple depicting low error) (A). Sequence analysis of VP3 Shannon Entropy (magenta) as measured for a dynamic 20-residue window centered on each residue, compared with the IUPred3 Short Disorder plot (blue). The horizontal dashed line represents an IUPred3 Short Disorder of 0.5 (B). The AlphaFold2 predicted structure of monomeric VP3. Residues are colored by pLDDT; termini and hinges labeled (C).

5.2.2 The VP3 C-terminal domain is predicted to be a highly dynamic IDR.

MD simulations of the VP3 monomer were employed to explore the predicted behavior of the C-terminus compared to VP3 as a whole. Each simulation was 20 nanoseconds in duration and the alpha carbons of VP3 were visualized as animated three-dimensional scatter plots of atomic coordinates (Movie S1, Figure 5.2A).

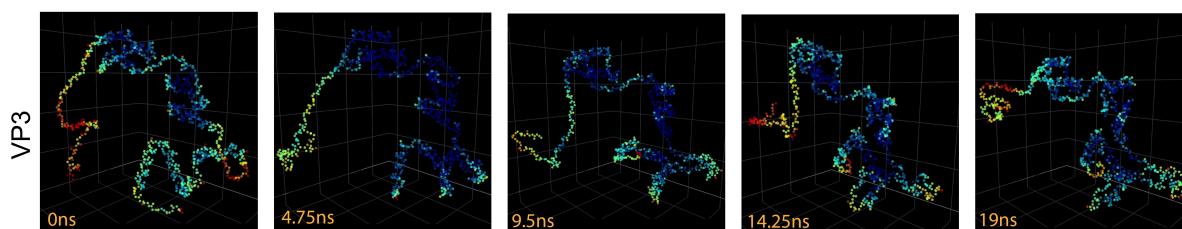


Figure 5.2 Monomeric VP3 molecular dynamics simulation. Evenly spaced still frames from one 20ns MD simulation of monomeric VP3. Points indicate the location of backbone atoms. VP3 molecule is oriented with the C-terminus on the left and the N-terminus on the right. Colors indicate relative atomic velocity (red is highest, blue is lowest) (A).

These data demonstrated that the C terminus has a greater breadth of the conformation space than other domains of the VP3 monomer. To quantify the relative mobility of each residue, six independent simulations were run, and the B factor, a metric associated with thermal motion, was calculated for each alpha carbon in each simulation. The average per-atom B factor values were plotted, revealing an elevated B factor at the C-terminus (Figure 5.3A), indicating increased predicted mobility. However, the B factor is an imperfect metric for distinguishing order from disorder, as ordered regions of proteins can undergo substantial thermal motion in MD simulations if they are adjacent to flexible “hinge” or “loop” regions [168]. Therefore, to determine whether the behavior of the C-terminus differed from that of other regions of the protein, I calculated pairwise correlation coefficients for atomic motion across the VP3 monomer (atomic motion correlation analysis) and plotted the results as a heatmap (Figure 5.3B). This

revealed that the movement of the C-terminus was poorly correlated with other regions of the protein (Figure 5.3B, bottom-right corner), suggesting it moves independently of the rest of the protein. As the C terminus is 36 amino acids in length, I next determined the atomic motion correlation of equally-sized sections of 36 amino acids in length across the whole VP3 protein and compared these data to the C terminus, to determine whether a given region was significantly more ordered than the C-terminus or not (Figure 5.3C). If a given region of the protein was significantly more ordered than the C-terminus, it had a p value below the 0.05 cut-off (red line), whereas if a particular region was not significantly more ordered than the C-terminus, then the p value was higher and hence gave a peak (Figure 5.3C).

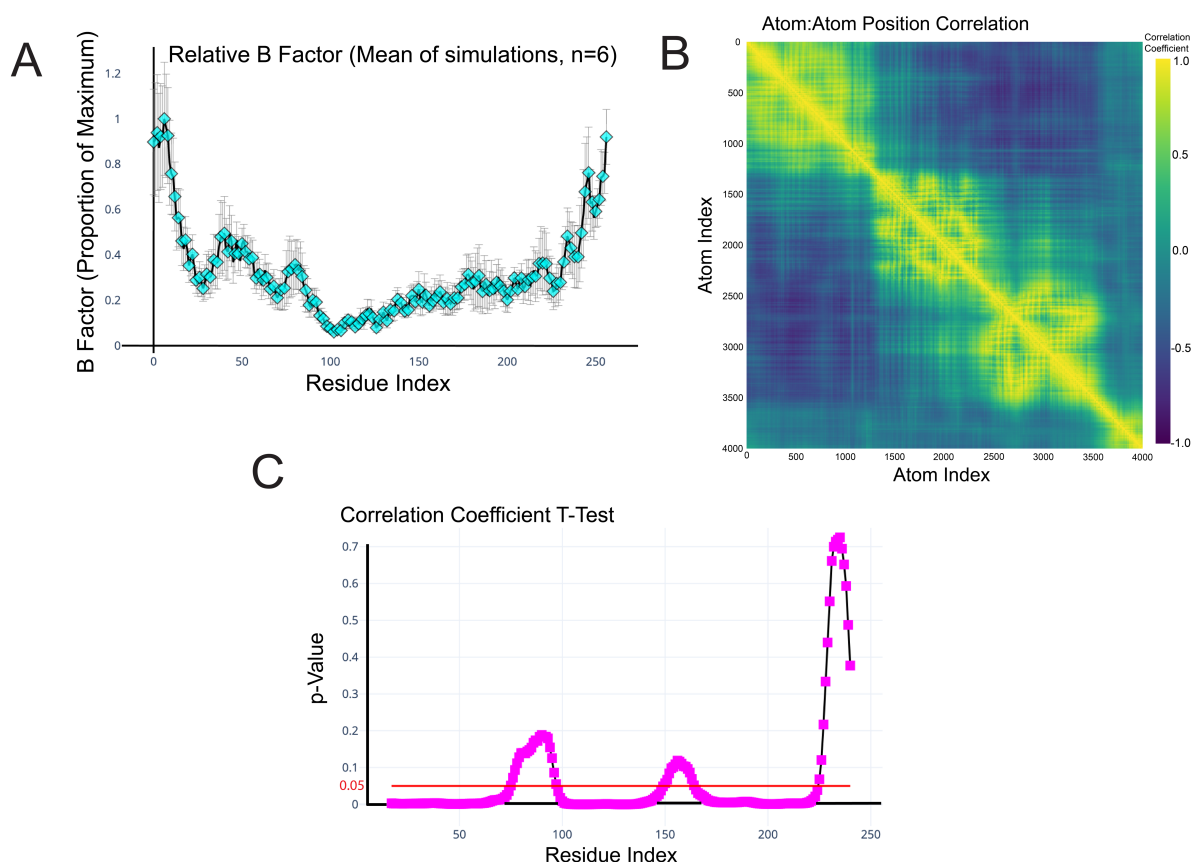


Figure 5.3 The VP3 C-terminal domain is predicted to be a highly dynamic IDR. The mean relative B-factor, calculated from MD simulations of the VP3 monomer (6 independent 20-nanosecond simulations), was plotted on the y-axis against VP3 amino acid residue number on

the x-axis. Error bars represent the standard error of the mean (SEM) (A). Pearson's product-moment correlation coefficient between the trajectories of each atom pair was calculated and plotted as a heatmap. Yellow represents perfect positive correlation, purple represents perfect negative correlation, blue/teal suggests a lack of correlation (B). The movement of the atoms (average positional correlation coefficient) within the 36 amino acids of the C terminus was compared to equally-sized windows of 36 amino acids in length spanning the whole length of the VP3 protein sequence, and the p-values were plotted on the y axis against the VP3 amino acid residue number on the x axis. Regions of the VP3 region that were significantly more ordered than the C-terminus had a p value below the 0.05 cut-off (red line) (C).

The VP3 protein was significantly more ordered than the C-terminus at all regions except the C-terminus itself, and amino acids 74-96 and 149-163, both of which correspond to the predicted unstructured hinge regions of the protein, and the latter of which corresponds to a "loop" region in the VP3 core structure solved by X-Ray diffraction [166]. Taken together with the calculated B-factor values and the broad conformational landscape explored by the C-terminus, the results of MD simulations demonstrated that the VP3 C-terminus is predicted to be a highly dynamic IDR. Moreover, when I performed MD simulations on a panel of VP3s from diverse IBVD strains, all exhibited the same elevated B factor at the C terminus, and there was no statistically significant difference among them (Figure 5.4B), demonstrating that the VP3 C-terminal IDR is conserved across multiple IBVD strains. This result is unsurprising given the relatively well-conserved nature of the VP3 C-terminus sequence across even diverse strains (Figure 5.4A).

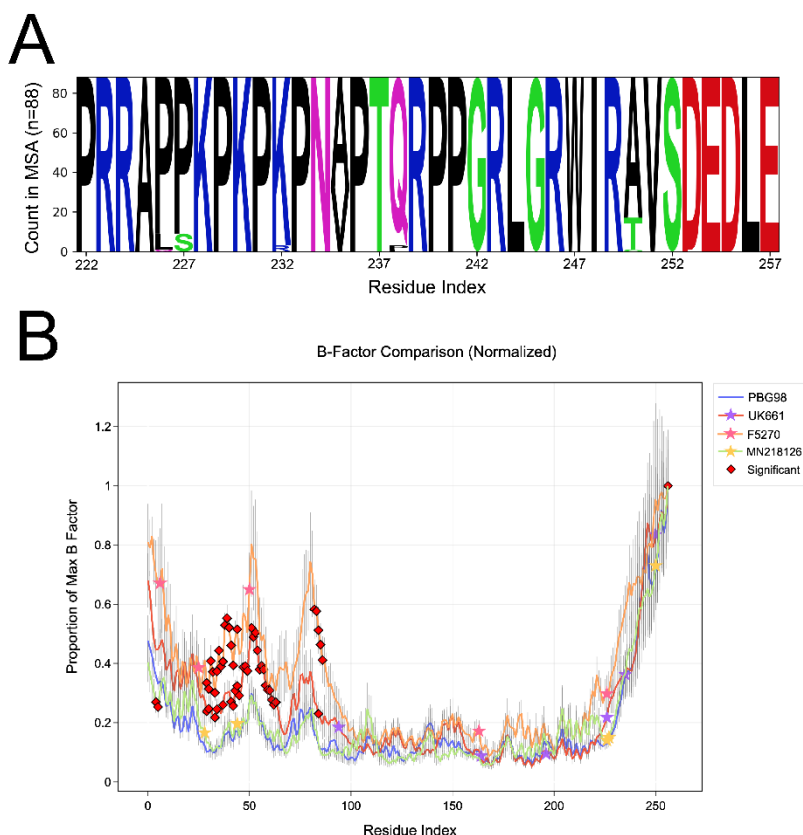


Figure 5.4 The sequence and flexibility of the VP3 C-terminal domain

are conserved between strains of IBDV. 88 sequences of IBDV VP3 were obtained from GenBank and aligned using SnapGene. The 36-residue C-terminal region from each sequence was isolated and processed into a “sequence logo” using the Logomaker python package (A). The structures of VP3 from four IBDV strains (PBG98, UK661, F5270, and MN218126) were predicted using AlphaFold2, and monomeric MD simulations were performed. Mean relative B-factor, calculated from MD simulations of the VP3 monomer (6 independent 20-nanosecond simulations), was plotted on the y axis against VP3 amino acid residue number on the x axis. Error bars represent the standard error of the mean (SEM). Amino acid positions where each strain differs from PBG98 are indicated with a colored star, and positions where the B factor differs significantly ($p < 0.05$) from PBG98 are indicated with a red diamond (B).

5.2.3 IBDV VP3 can exist as a homodimer with two C-terminal dynamic IDRs extending from the core.

In infected cells, VP3 can exist as a homodimer with the dimerization domain of VP3 predicted to lie within the N-terminus of the protein [169]. I therefore generated a predicted structure for

the VP3 homodimer using AlphaFold3. The VP3 dimer model featured interactions between the N-termini of the monomers, but, owing to the flexible hinge regions predicted in the monomer, the “core” regions wrapped around the N-termini, resulting in an overall compact conformation. The C-termini of both subunits extended from the compact cores and exhibited the same low confidence as in the monomer model (Fig. 5.6A and B). I performed MD simulations using the VP3 homodimer. As with monomeric VP3, each simulation was 20 nanoseconds in duration, and the alpha carbons of VP3 were visualized as animated three-dimensional scatter plots of atomic co-ordinates (Movie S2, Figure 5.5A).

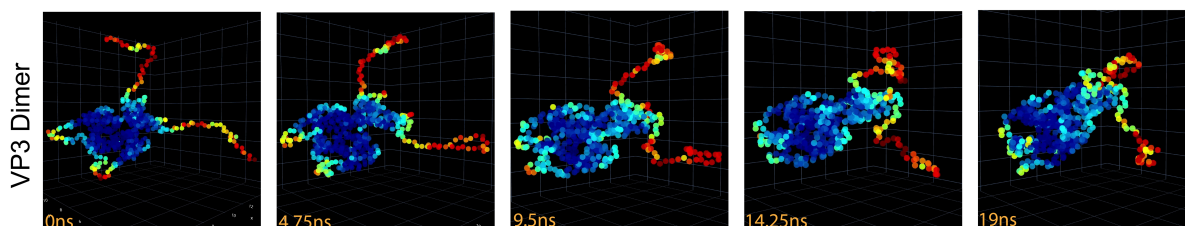


Figure 5.5 Dimeric VP3 molecular dynamics simulation. Evenly spaced still frames from one 20ns MD simulation of monomeric VP3. Points indicate the location of alpha carbon atoms. VP3 molecule is oriented with the C-terminus on the left and the N-terminus on the right. Colors indicate relative atomic velocity (red is highest, blue is lowest) (A).

The resulting trajectories of the alpha carbons in the VP3 homodimer were visualized as an ensemble of 10 evenly spaced time points from a single 20-nanosecond MD simulation (Figure 5.6C), demonstrating the breadth of the conformational space explored by the C termini. B-factors were calculated for the alpha carbons in the homodimer, and these data indicated that the elevated C-terminal B-factors observed in the monomer simulations were also observed for both subunits of the dimer (Figure 5.6D).

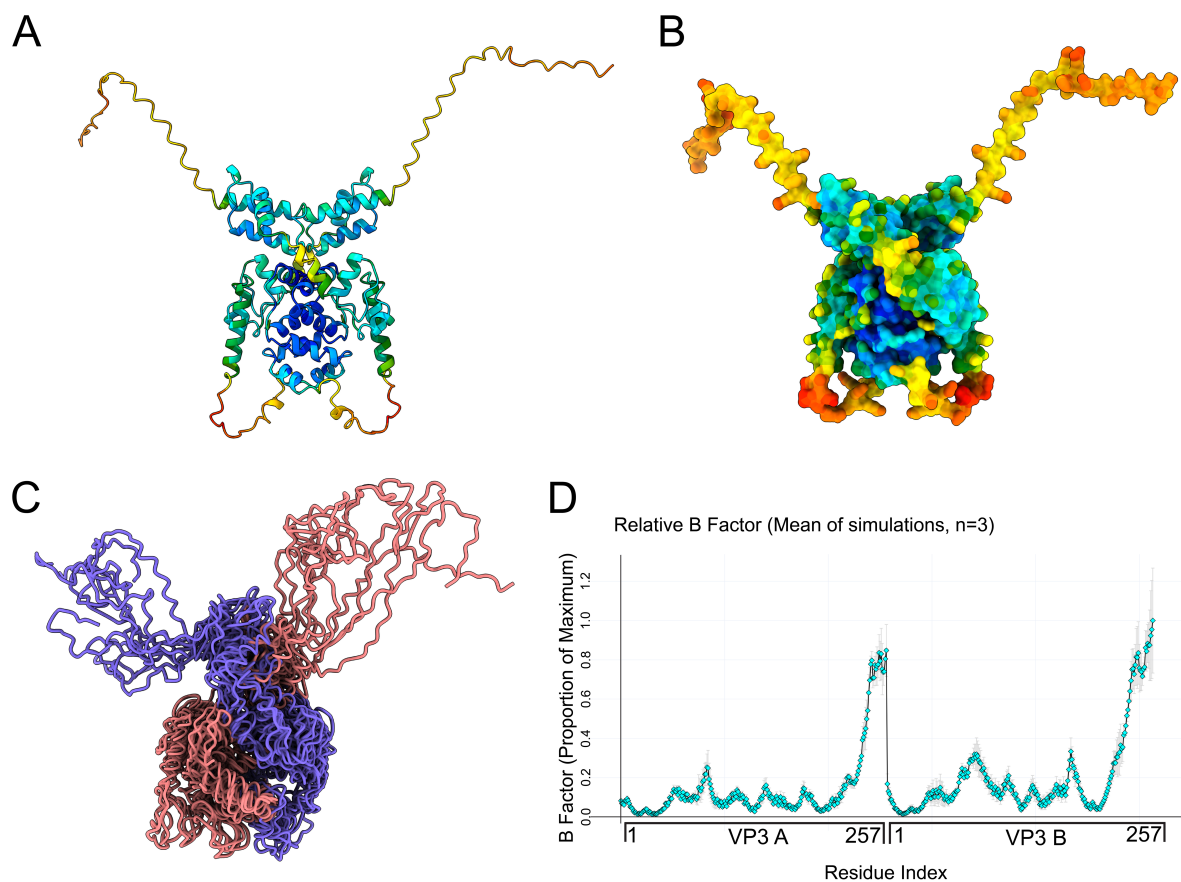


Figure 5.6 IBDV VP3 can exist as a homodimer with two C-terminal dynamic IDRs extending from the core. A cartoon rendering of the VP3 homodimer as predicted by AlphaFold3, visualized in UCSF ChimeraX, and colored by positional confidence (red indicates lower confidence; blue indicates higher confidence) (A). A solvent-accessible surface of the VP3 homodimer, visualized by UCSF ChimeraX, and colored by positional confidence (red indicates lower confidence; blue indicates higher confidence) (B). An ensemble of structures representing ten evenly spaced timepoints of a single 20-nanosecond MD simulation. Structures were extracted from the trajectory and rendered as an ensemble in UCSF ChimeraX without alignment, colored by monomer (one monomer in purple, the other in pink) (C). The mean relative B-factor was calculated from the MD simulations of the VP3 homodimer (6 independent 20-nanosecond simulations) and plotted on the y-axis against the amino acid residue number of two VP3 monomers (A and B) concatenated on the x-axis. Error bars represent SEM (D).

5.2.4 The C-terminal IDR is not required for the recruitment of IBDV VP3 into VFs.

We next aimed to compare the behavior of the full-length VP3 with a VP3 molecule that lacked the 36-residue C-terminus. I therefore truncated the mNG::VP3 construct after residue 221 of VP3, to form mNG::VP3 Δ C. An alignment of the predicted structures of VP3 and VP3 Δ C using AlphaFold3 and PyMOL revealed identical predicted secondary structure configurations, with differences between the structures arising only from the flexible linker regions, demonstrating that there were no predicted folding defects in the remaining protein (Figure 5.7A, B, C).

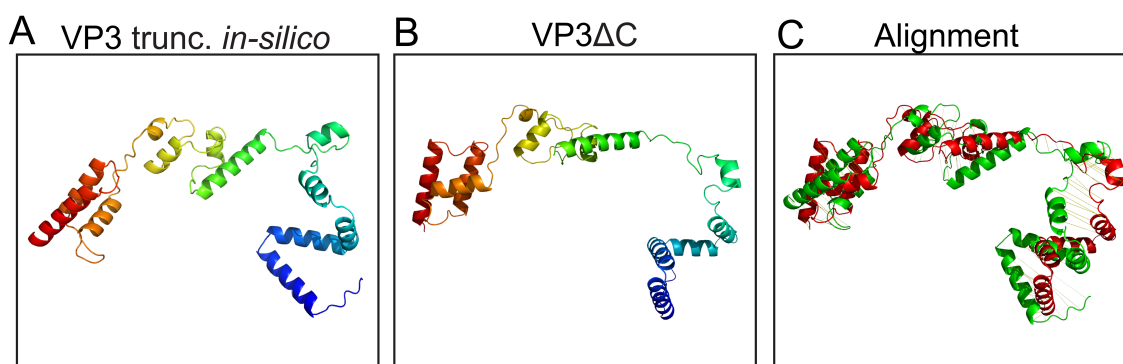


Figure 5.7 Predicted folding of VP3 Δ C matches the predicted folding of residues 1-221 in full-length VP3. The model of full-length VP3 generated by AlphaFold was truncated *in silico* by the removal of all atoms downstream of residue 221 (A). The structure of VP3 Δ C was predicted *de novo* by AlphaFold from the truncated amino acid sequence (B). The structures of residues 1-221 from the full-length model and the VP3 Δ C model were aligned in PyMOL, revealing agreement between the secondary structural elements (C).

We then transfected DF-1 cells with the mNG::VP3 Δ C construct, and stained the cells with α -VP3, revealing a complete overlap of the mNG and VP3 signals, and a cytoplasmic distribution matching that of full-length VP3. Moreover, colocalization analysis of the mNG::VP3 Δ C signal and the anti-VP3 signal revealed a Manders' coefficient of 0.903 and Spearman's rank correlation of 0.793 (Figure 5.8A), demonstrating that truncating the C terminus did not substantially affect the intracellular localization of the protein in transfected cells, nor the binding of the anti-VP3 antibody.

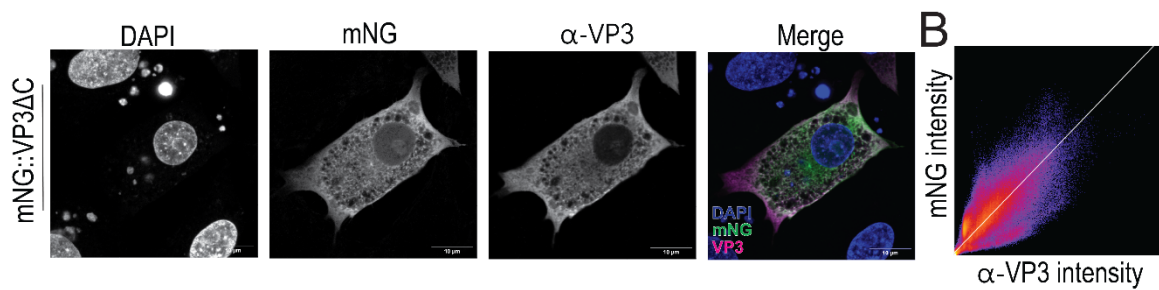


Figure 5.8 VP3ΔC exhibits diffuse expression and does not show evidence of gross folding defects. DF-1 cells were transfected with mNG::VP3ΔC, fixed 18h.p.t., and immunostained with an antibody raised against full-length VP3 (nuclei stained with DAPI). The signal from individual channels is shown in black and white, with the merged image in color) (A). Colocalization analysis of the mNG::VP3ΔC signal and the anti-VP3 signal (B).

To determine if deletion of the C-terminus altered the recruitment of exogenous VP3 to VFs, DF-1 cells were transfected with either mNG, mNG::VP3, mNG::VP3ΔC, or a construct where the 36 C terminal region of VP3 was fused to mNG (mNG::Cterm) and simultaneously infected with IBDV strain PBG98 (Figure 5.9 B, C). The mNG signal was observed to be redistributed from diffusely cytoplasmic to punctate in infected cells transfected with mNG::VP3, mNG::VP3ΔC, or mNG::Cterm, indicating that deletion of the C-terminus did not prevent the recruitment of exogenous VP3 to IBDV VFs, and indicating that the C-terminus itself was also recruited to the VFs. As VP3ΔC contains the core region which is known to interact with RNA [121], and the C terminus is known to interact with VP1 [170], I conclude that the VP3ΔC is recruited to the VF through interaction with RNA, and that the C term is recruited to the VF through interaction with VP1.

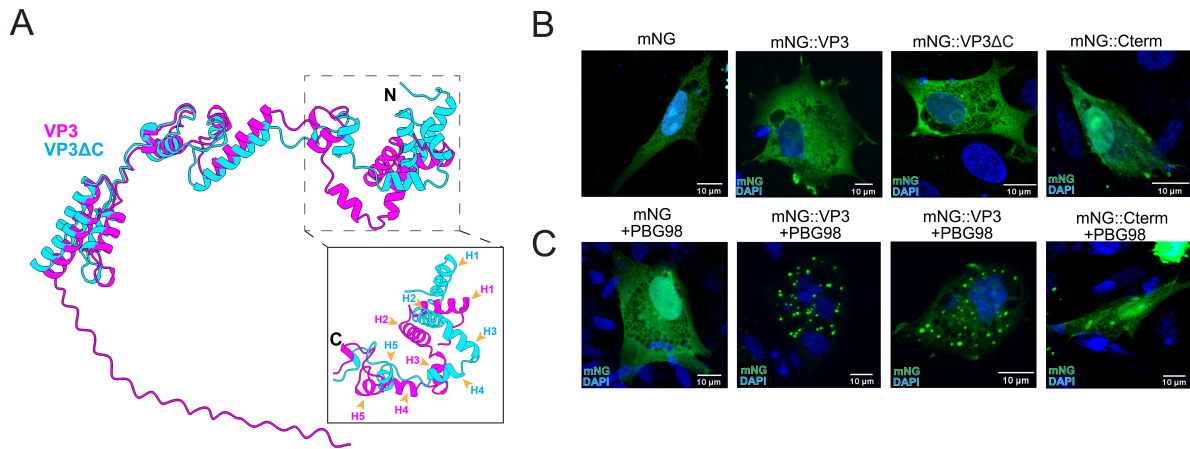


Figure 5.9 The C terminal IDR is not required for the recruitment of IBDV VP3 into VFs. AlphaFold3 predictions for the wt VP3 (magenta) and the VP3ΔC (cyan) were aligned with ChimeraX and overlaid (N terminal helices numbered H1-5) (A). DF-1 cells were transfected with mNG, mNG::VP3, mNG::VP3ΔC, or mNG::Cterm (B), or were co-transfected with the plasmids and simultaneously infected with IBDV strain PBG98 (MOI 5) (C). Cells were fixed at 12h.p.i, and the nuclei were stained with DAPI (blue) and the mNG signal is shown in green. Scale bars, 10μm.

5.2.5 The presence of VP3ΔC does not significantly impede IBDV replication.

Since I had identified that truncation of the VP3 C-terminus did not preclude VP3 recruitment to VFs formed during infection, I sought to determine whether rescue of viable virus lacking the C-terminus was possible. I used our in-house reverse genetics system that involves two plasmids, one encoding segment A, and one encoding segment B [171]. As VP3 is located at the C terminus of the polyprotein that is translated from segment A (Figure 4.1A), it was possible to remove the C terminus of VP3 by truncating the segment A coding region by 36 amino acids, to form SegAΔC. DF-1 cells simultaneously transfected with SegAΔC and SegB failed to exhibit any detectable cytopathic effect (CPE) after blind passage, indicating that the absence of the C-terminus prevented the generation of productive virus. I further attempted a rescue *in-trans*, by simultaneously transfecting DF-1 cells with SegAΔC, SegB, and VP3, and blind passage was attempted in cells also transiently expressing VP3. However, no CPE was detectable. To

determine if the presence of VP3ΔC acted in a “dominant negative” manner and impeded viral replication, I simultaneously infected and transfected cells with strain PBG98 IBDV alongside mNG, mNG::VP3, mNG::VP3ΔC, and mNG::Cterm, and I measured viral replication by RT-qPCR and TCID₅₀ at 7, 16 and 24 hours post infection (h.p.i). I found no significant difference in viral replication between infected and co-transfected cells at any timepoint (Figure 5.10A, B).

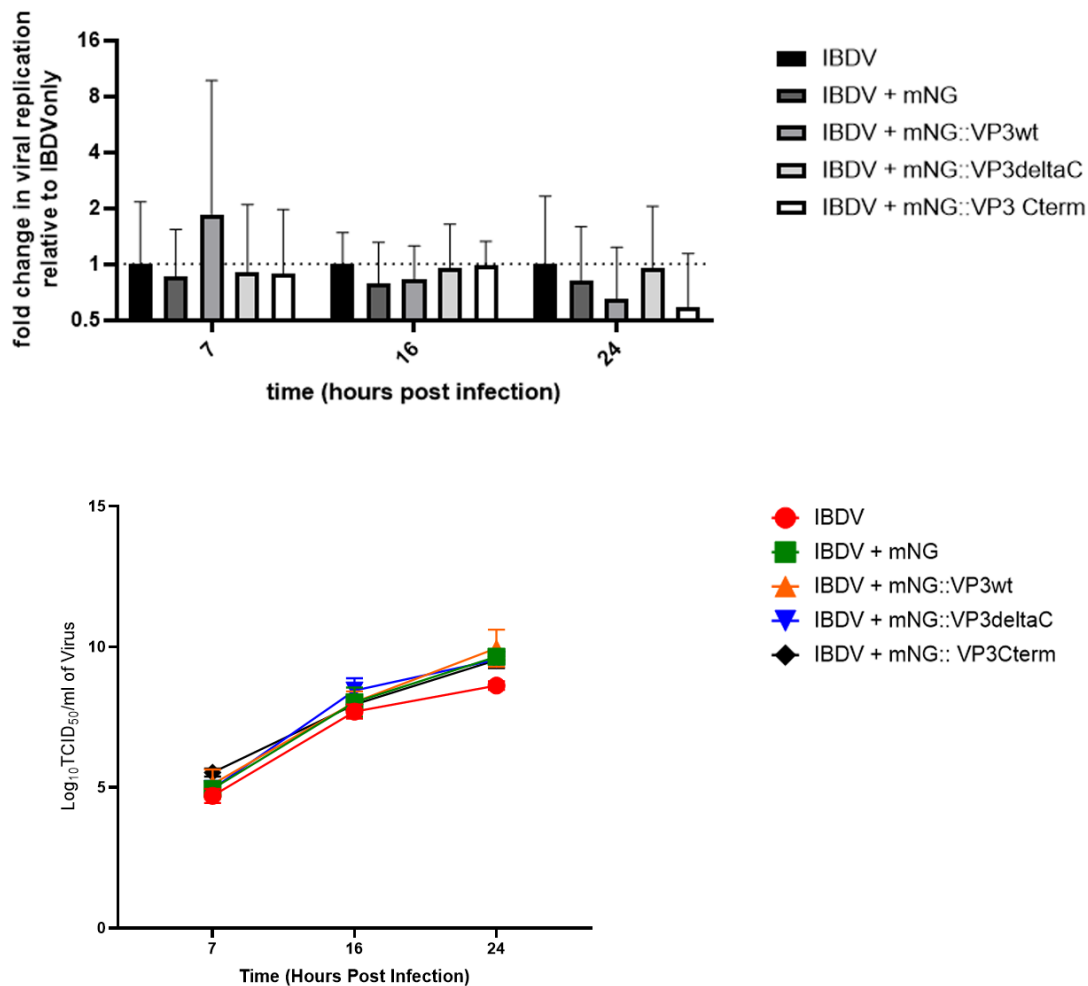


Figure 5.10 Exogenous expression of VP3 and VP3 variants does not significantly impact IBDV replication kinetics. DF-1 cells were infected with IBDV strain PBG98 alone or infected with IBDV strain PBG98 and simultaneously transfected with plasmids expressing mNG, mNG::VP3, mNG::VP3ΔC or mNG::Cterm in triplicate. Cells were lysed, RNA extracted at 7, 16, and 24 h.p.i., and viral replication was quantified by RT-qPCR and plotted as fold change normalized to IBDV infection alone (A). The titer of virus in the supernatant from the same samples was also assessed by TCID₅₀, and the resultant growth curve was plotted (B).

Taken together, these data indicate that although a virus encoding VP3 Δ C cannot yet be rescued, the presence of VP3 Δ C does not significantly impede the replication of wild-type virus, despite evidence that it is recruited to IBDV VFs. Although it is possible that truncated SegA RNA might be the reason for the inability to rescue the virus (potentially packaging signals might extend into the SegA C terminus), it might still be possible to rescue the virus in a cell line that stably expresses VP3, but this was beyond the scope of the present study.

5.2.6 The IBDV VP3 C terminal IDR is not required for the formation of cytoplasmic puncta.

Although our data indicated that VP3 recruitment to IBDV VFs was independent of the C-terminus, the VFs formed by co-infection and transfection contained a mixture of exogenously expressed VP3 Δ C and full-length VP3 derived from the infecting virus, which prevented an analysis of how the VP3 C terminus affected the formation and properties of the VFs. To address this, I transfected DF-1 cells with SegA Δ C and HA::Seg B. Similarly to the wt plasmids, cells expressing SegA Δ C and HA::SegB alone exhibited a diffuse cytoplasmic staining pattern, but when cells co-expressed both, cytoplasmic puncta were observed (Figure 5.11A, B), demonstrating that the VP3 C-terminal IDR was not an absolute requirement for the appearance of cytoplasmic puncta.

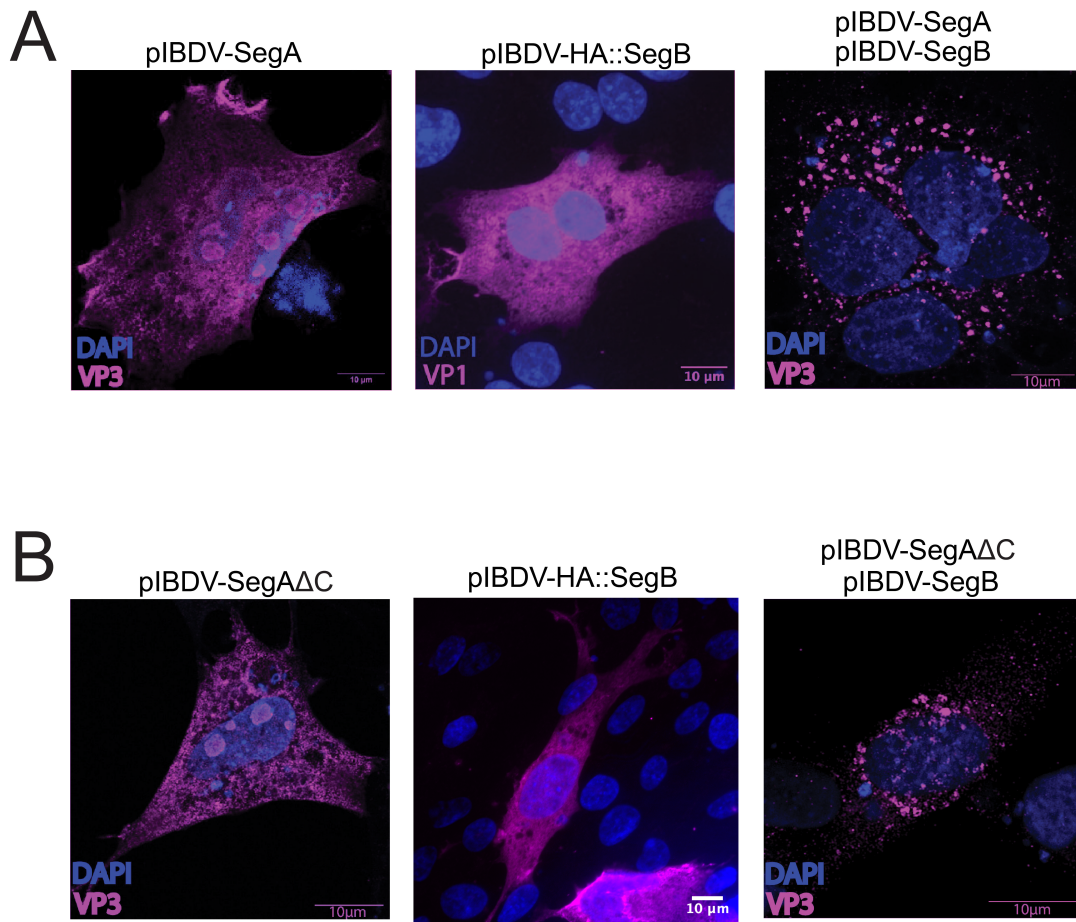


Figure 5.11 The IBDV VP3 C-terminal IDR is not required for the formation of cytoplasmic puncta. DF-1 cells were either transfected with SegA or HA::SegB, and fixed and stained with anti-VP3 or anti-HA antibodies at 18 h.p.t., or DF-1 cells were simultaneously transfected with both SegA and SegB, and fixed and stained with an anti-VP3 antibody at 18 h.p.t. (A). DF-1 cells were either transfected with SegAΔC or HA::SegB, and were fixed and stained with anti-VP3 or anti-HA antibodies at 18 h.p.t., or DF-1 cells were simultaneously transfected with both SegAΔC and SegB, and fixed and stained with an anti-VP3 antibody at 18 h.p.t. The nuclei were stained with DAPI (blue) and the VP3 or HA-VP1 signals are shown in magenta. Scale bars, 10μm (B).

5.2.7 The IBDV VP3 C-terminal IDR promotes the formation of cytoplasmic puncta and influences their size and shape.

To evaluate how the VP3 IDR affected the physical properties of the puncta, I modified our SegB plasmid by adding mNG to the C-terminus of the VP1 coding sequence (SegB::mNG) (Figure 5.12A). I then transfected cells with either a mixture of SegA and SegB::mNG, or a mixture of SegA Δ C and SegB::mNG, and I detected the presence of mNG-positive cytoplasmic puncta that colocalized with the VP3 signal (Figure 5.12B).

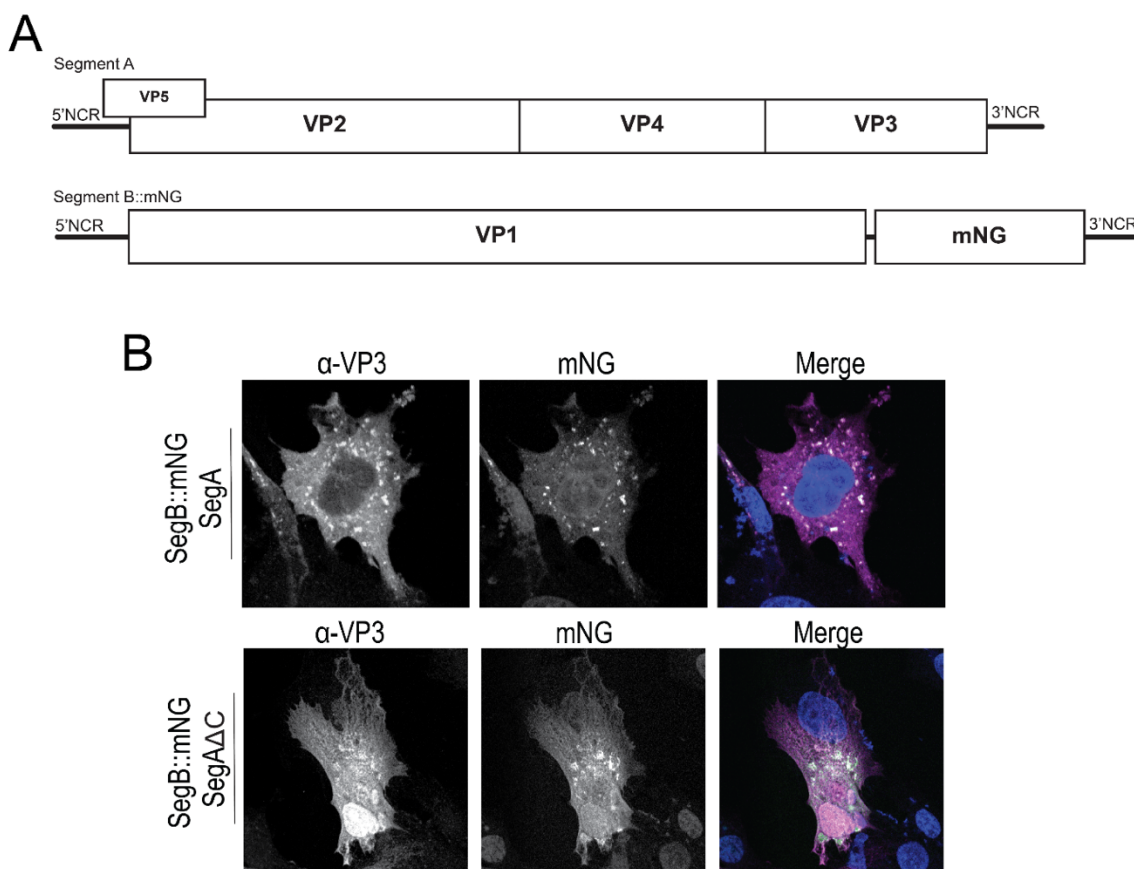


Figure 5.12 A modified reverse genetics system permits imaging of VFs in live cells.

Schematics for the modified IBDV reverse genetics system, with SegA displayed on the top, and SegB::mNG displayed on the bottom (A). DF-1 cells were co-transfected with a mixture of pIBDV-SegB::mNG and either pIBDV-SegA or pIBDV-SegA Δ C, fixed at 34h.p.t., and immunostained with anti- VP3 (nuclei stained with DAPI). The signal from individual channels shown in black and white, with the merged image in color (B).

Next, fifty randomly selected cells from each of three independent replicates of cells transfected with either SegA and SegB::mNG, or SegA Δ C and SegB::mNG were imaged, and the puncta were counted. Although cells containing puncta were observed in the absence of the C-terminus, they were detected at a significantly reduced rate ($p < 0.0001$) (Figure 5.13A). Furthermore, among the cells that did contain puncta, the mean number of puncta per cell was significantly reduced in the Δ C system compared to wt ($p < 0.0001$) (Figure 5.13B). Taken together, these data demonstrate that although the IBDV VP3 C-terminal IDR was not required for the formation of cytoplasmic puncta, it did promote their formation.

Six morphological properties of the puncta were then measured: area, Crofton perimeter, single-puncta mean fluorescence intensity, single-puncta intensity standard deviation, eccentricity, and geometric solidity. Puncta that formed in the presence of wt VP3 had a significantly larger average area and perimeter than puncta formed in the presence of VP3 Δ C ($p < 0.0001$) (Figure 10.13C, D). Moreover wt VP3 puncta had a significantly greater mean fluorescence intensity, and a greater single-puncta intensity variability than VP3 Δ C puncta ($p < 0.0001$) (Figure 5.13E, F), consistent with the VP3 C-terminal IDR contributing to the size and intensity of the puncta. Finally, wt VP3 puncta had a significantly higher mean eccentricity than VP3 Δ C puncta ($p < 0.05$), and exhibited significantly higher mean geometric solidity ($p < 0.0001$), indicating that the VP3 C-terminal IDR contributed to the “roundness” or “smoothness” of the puncta. Taken together, these data demonstrate that the VP3 C-terminal IDR influenced the size and shape of the cytoplasmic puncta.

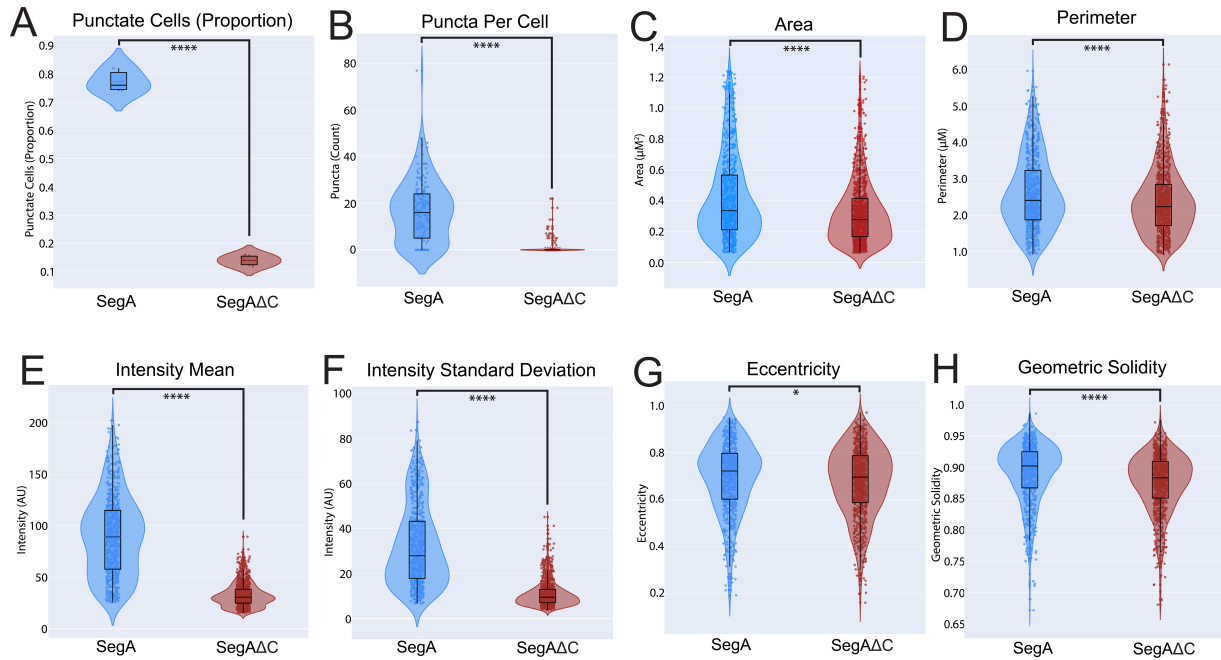


Figure 5.13 The IBDV VP3 C-terminal IDR promotes the formation of cytoplasmic puncta and influences their size and morphology. DF-1 cells were co-transfected with either SegA and SegB::mNG, or SegA Δ C and SegB::mNG. From each of 3 replicates, 50 cells were randomly selected at 34h.p.t., and their phenotype (punctate or non-punctate) was classified (A), and the puncta per cell were counted (B). Fifty cells prepared in the same manner were then imaged individually by confocal microscopy, and puncta were segmented by watershed segmentation and the area, perimeter, mean fluorescence intensity, intensity standard deviation, eccentricity, and geometric solidity (a measure of morphological “roundness” or “smoothness”) were measured with the Scikit-image python module (C – H).

5.2.8 The VP3 C-terminus affects the physical properties of VFs.

Finally, I aimed to characterize differences in the physicochemical properties of puncta formed in the presence of the wt VP3 or the VP3 Δ C by performing FRAP. Briefly, I transfected DF-1 cells with either a mixture of SegA and SegB::mNG, or a mixture of SegA Δ C and SegB::mNG, and I captured time-lapse series by live confocal microscopy at 14 h.p.t., with a single iteration of point bleaching on the 5th frame of each series. I observed a notable difference between the recovery behavior of puncta formed in the presence of wt VP3 and the VP3 Δ C (Figure 5.14A, B). Puncta in cells expressing VP3 Δ C exhibited a partial recovery in fluorescence after

photobleaching, but the mobile fraction was calculated as only 0.291, indicating that only 29.1% of the molecules exhibited any diffusion. This was in sharp contrast to the behavior of puncta formed in cells expressing the wt VP3, where although the recovery half-time was longer at 3.517 seconds (compared to 0.341 seconds in the VP3 Δ C puncta), the calculated mobile fraction was 0.704, indicating that 70.4% of the molecules exhibited diffusion, a significantly greater proportion compared to the VP3 Δ C puncta ($p < 0.01$).

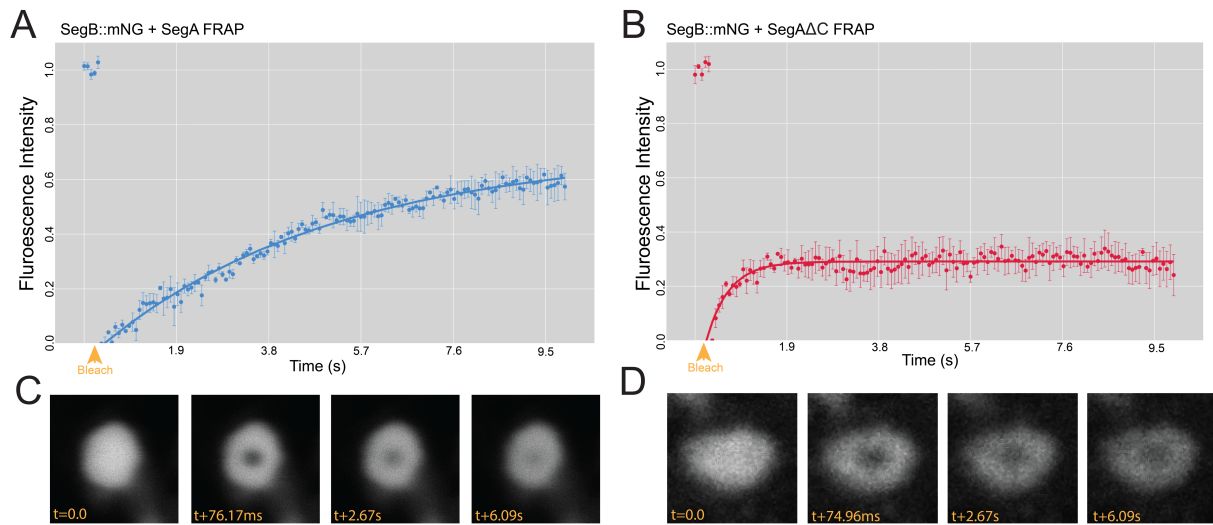


Figure 5.14 The VP3 C-terminal IDR significantly contributes to the mobile fraction of the molecules within the cytoplasmic puncta. DF-1 cells were co- transfected with a mixture of SegA and SegB::mNG and a time lapse series was captured by live confocal microscopy at 14 h.p.t. with a single iteration of point bleaching on the 5th frame (yellow arrowhead). Intensity data was extracted and used to calculate a FRAP recovery curve. Results are from 3 replicates; error bars = SEM (A). DF-1 cells were co- transfected with a mixture of SegA Δ C and SegB::mNG and a FRAP recovery curve generated in the same way (B). Representative recovery images for a photobleached puncta formed in the presence of SegA with the time post bleach (t) shown in milliseconds (ms) (C). Representative recovery images for a photobleached puncta formed in the presence of SegA Δ C with the t post bleach shown in ms (D).

These data demonstrate that the VP3 C-terminal IDR affected the physical properties of the VFs. Moreover, I also evaluated the liquidity of the puncta formed in the chicken macrophage cell-line HD11 and found similar results: the mobile fraction of the puncta formed in the presence of wt

VP3 was larger than the puncta formed in the presence of VP3 Δ C (Figure 5.15A, B), demonstrating this phenomenon was not cell-type specific.

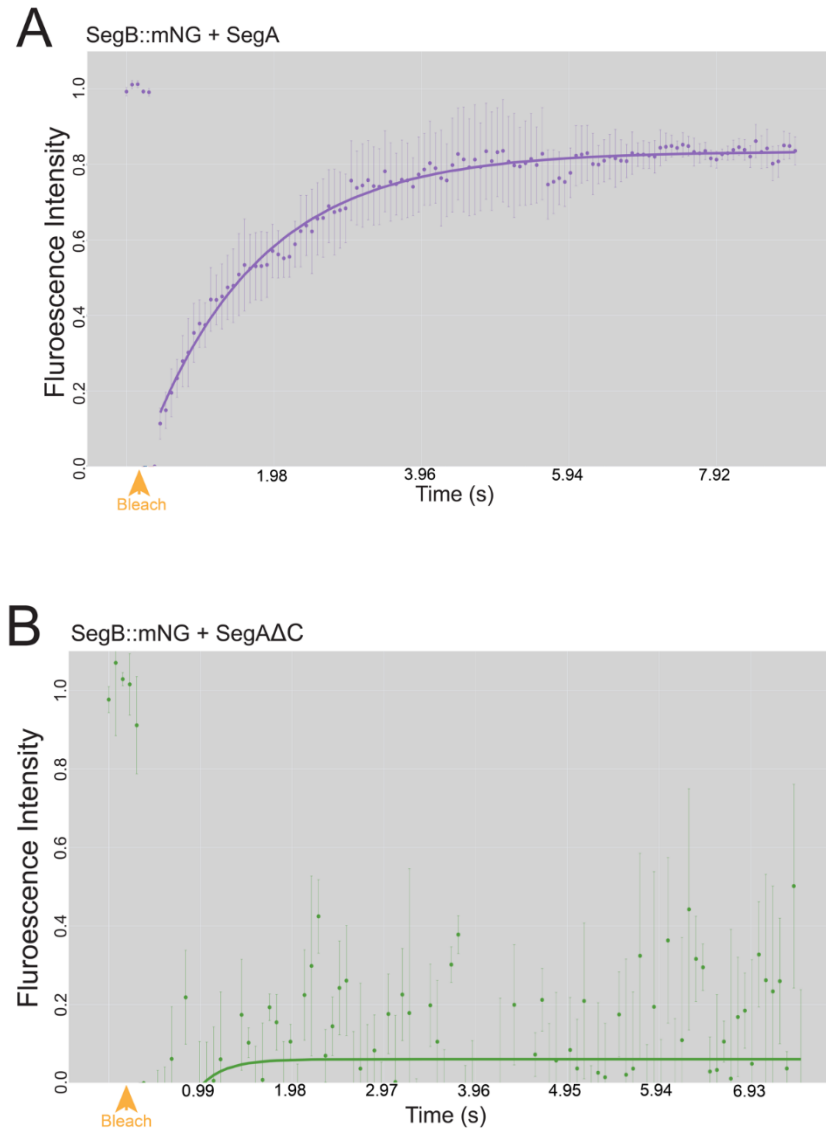


Figure 5.15 The VP3 C terminal IDR significantly contributes to the mobile fraction of the molecules within the cytoplasmic puncta in a macrophage cell line (HD11). HD11 cells were co- transfected with a mixture of SegA and SegB::mNG, and a time lapse series was captured by live confocal microscopy at 34 h.p.t. with a single iteration of point bleaching on the 5th frame. Intensity data was extracted and used to calculate a FRAP recovery curve. Results are from 3 replicates; error bars = SEM (A). HD11 cells were co- transfected with a mixture of SegA Δ C and SegB::mNG and a FRAP recovery curve generated in the same way (B).

To assess other behaviors associated with LLPS, I transfected DF-1 cells with either a mixture of SegA and SegB::mNG or a mixture of SegA Δ C and SegB::mNG, and observed the cells by live-cell confocal microscopy, capturing images every 5 frames to make a time lapse. Interestingly, I observed that both the cytoplasmic puncta made in the presence of either wt VP3 or VP3 Δ C were mobile, moving throughout the cytoplasm on similar, rapid timescales, and underwent fusion events (Figure 5.16A, B), indicating that these key liquid-like properties were retained in the absence of the VP3 C-terminus.

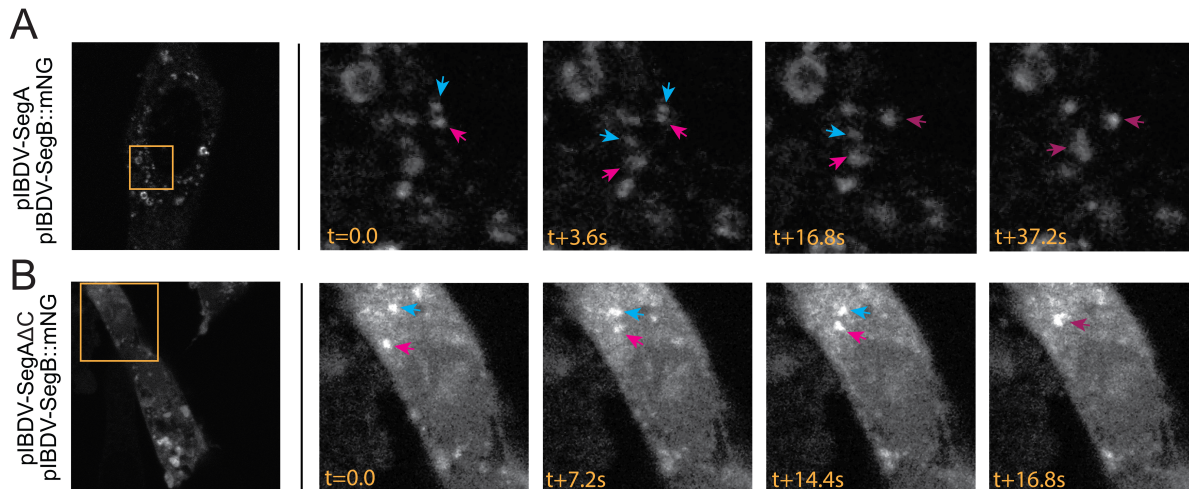


Figure 5.16 Puncta formed in the presence or absence of the VP3 C-terminal IDR are dynamic. DF-1 cells were transfected with a mixture of SegA and SegB::mNG (A), or DF-1 cells were transfected with a mixture of SegA Δ C and SegB::mNG (B). At 34h.p.t., cells were imaged by live-cell confocal microscopy, capturing images every 5 frames to make a time lapse. Still frames from the timelapses are shown. The leftmost images show whole-cell images (mNG signal shown in white), with stills from the inset regions (orange boxed regions) shown thereafter. The timestamp (t) of imaging is shown in seconds (s). Arrows show individual puncta that undergo fusion.

Furthermore, I characterized the puncta's response to treatment with small aliphatic diols, which have been shown to disrupt LLPS structures [139]. Briefly, I transfected DF-1 cells with either a mixture of SegA and SegB::mNG, or a mixture of SegA Δ C and SegB::mNG, and treated the

transfected cells with 1,3-PD, an aliphatic diol that exhibits lower toxicity than the more commonly used 1,6-HD, while capturing time lapse images. Although the toxicity of 1,3-PD was reduced as compared to 1,6-HD, eventually the cells did succumb to the treatment, meaning our observations were limited to approximately ten minutes post-treatment where I could reliably image the puncta. Over the course of the time lapse, 1,3-PD caused a moderate degree of disruption to puncta made with both wt VP3 or VP3 Δ C (Figure 5.17A, B). The margins of most puncta exhibited a loss of definition, although complete dissolution was only observed for a subset of smaller puncta. Both puncta formed in the presence of wt VP3 and in the presence of VP3 Δ C were sensitive to diol treatment, but the responses varied too widely between cells and between puncta within a single cell to quantitatively distinguish differences in the behavior of the puncta made with either wt VP3 or VP3 Δ C.

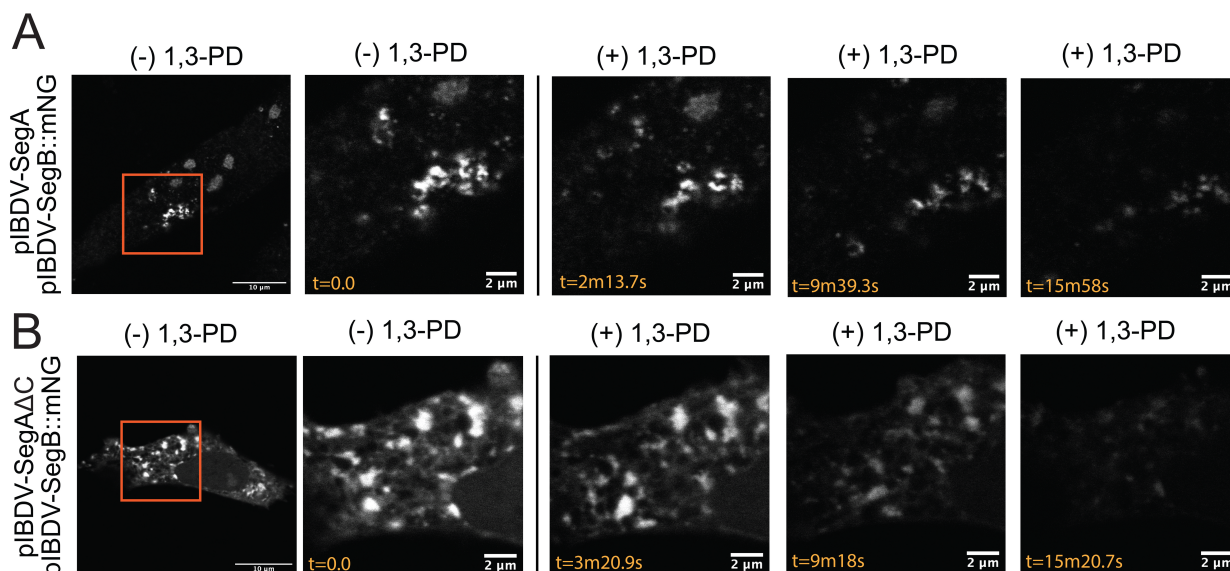


Figure 5.17 Puncta formed in the presence or absence of the VP3 C-terminal IDR are sensitive to aliphatic diol treatment. DF-1 cells were transfected with a mixture of SegA and SegB::mNG (A), or DF-1 cells were transfected with a mixture of SegA Δ C and SegB::mNG (B). Transfected cells were treated with 1,3-PD, while capturing time lapse images. Briefly, cells were initially maintained in media for acquisition, and at time (t)=0 seconds (s), 1,3-PD was added to achieve a final concentration of 4% (v/v). The leftmost images show whole cells (mNG signal shown in white), with stills from the inset regions (orange boxed regions) shown thereafter. The timestamp (t) of imaging is shown in seconds (s).

5.3 Discussion

Having identified the critical role of VP3 in the formation of IBDV VFs, I used AlphaFold modeling and MD simulations to robustly characterize the behavior of the predicted structure of the IBDV VP3 monomer and homodimer. Using AlphaFold2 and AlphaFold3, I predicted the structures of full-length monomeric and dimeric VP3. In both cases, the C-termini of the VP3 protein was predicted to lack secondary structural elements (alpha helices, beta strands), and the atoms in this region had high pLDDT values (a metric of AlphaFold's "confidence" in the position of that atom). Due to the remarkable effectiveness of AlphaFold, lack of predicted

secondary structure and elevated pLDDT is reasonably predictive of genuine structural disorder. To further investigate this possibility, I analyzed the amino acid sequence of VP3 using IUPred3 Short Disorder and measured the Shannon Entropy of the sequence at each position and found that IUPred3 scores were elevated (>0.5) in the C-terminus, while Shannon entropy values were reduced for the same residues. IUPred3 is an algorithm designed to predict the disorder propensity of amino acid sequences, and Shannon Entropy detects reduced sequence complexity in parts of an amino acid sequence, both of which are properties frequently associated with *bona fide* IDRs.

However, to characterize the behavior of the C-terminus (and VP3 as a whole) more definitively, I aimed to robustly characterize the behavior of the protein in solution with MD simulations. Using GROMACS, I performed 6 unperturbed 20 nanosecond simulations of the VP3 monomer and dimer in charge-neutralized aqueous solution and used a custom tool to correct for periodic boundary conditions and output final trajectories for each atom. The relatively rapid motion and conformation space exploration exhibited by the C-terminus were apparent when the trajectories were visualized. To quantify this behavior, I calculated B-factor values for each alpha carbon in the VP3 chain. In the monomer, both the N and C termini exhibited markedly elevated B factor values, whereas in the dimer, only the C-termini exhibited elevated B factor values, likely due to mutual stabilization of the N-termini atoms which lie at the dimer interface.

B factor is an imperfect metric for disorder - despite the elevated B factor observed at the N-terminus, this region was predicted to be largely structured, consisting of a bundle of alpha helices. This region, however, was connected to the core by a short, unstructured “hinge” region.

I therefore hypothesized that the elevated B factor values for the N-terminus were the result of motion in this hinge region causing the atoms to move as a group. To distinguish between the behavior of the N and C-termini, I calculated Pearson product-moment correlation coefficients for every pair of atoms in the simulation, in effect measuring how well the motions of any atom correlated with the motions of any other atom in the structure. Atoms that are part of an overall stable globular structure, and particularly those that are part of the same secondary structural element, would be expected to exhibit strong correlation by this metric, whereas the motions of atoms in a disordered region would be expected to correlate poorly with each other. To determine if motions in the C-terminus were significantly less correlated than those of atoms elsewhere in the protein, I performed one-tailed Welch's unequal variances t-tests comparing the mean correlation coefficients of the 36 C-terminus residues with every other possible 36-residue region of the protein. I found that the C-terminus was significantly less well correlated than all other ranges tested, except those centered on residues 74 – 96 and 149-163, both of which correspond to predicted unstructured regions of the protein, and the latter of which was previously found to be unstructured by X-ray crystallography [166].

Based on these data, I concluded that the 36 C-terminal amino acids of VP3 comprised a predicted highly dynamic IDR. IDRs are frequently implicated as drivers of LLPS as their conformational plasticity is well-suited to the multivalent weak interactions that cause the phenomenon [31]. I therefore hypothesized that VP3 acts as a scaffold for IBDV VFs, with its IDR as a key driver of LLPS. Consistent with this hypothesis, I discovered that the rate of cytoplasmic puncta formation was significantly reduced in the presence of VP3 lacking the C-terminus, as compared to the presence of full-length wt VP3, with fewer punctate cells being

observed and fewer puncta per cell. Moreover, the puncta that did form in the absence of the VP3 C terminus were significantly smaller, had a reduced mean fluorescence intensity, and were less “round” or “smooth” than those formed from wt VP3. Additionally, using FRAP, I discovered that the mobile fraction in puncta that formed in the absence of the VP3 C terminus was significantly lower (0.291) than the puncta that formed in the presence of full-length wt VP3 (0.704) ($p < 0.01$), demonstrating that the VP3 Δ C puncta were less liquid-like. Taken together, these data demonstrated that the C terminal IDR of VP3 was a potent driver of cytoplasmic puncta formation and influenced their liquidity.

Interestingly, removal of the VP3 C terminus did not completely abolish cytoplasmic puncta formation, suggesting that the VP3 C terminal IDR was not strictly required for puncta formation. I also observed that exogenous VP3 could be recruited to IBDV VFs in infected cells, even in the absence of the C terminus. Such behavior precluded the C-terminus from being a requisite for VF compatibility. Moreover, as VP3 is known to dimerize, it is possible that when VP3 Δ C was recruited to the VFs of infected cells, it may have formed heterodimers with full-length VP3 provided by the virus. LLPS requires multivalent intermolecular contacts, frequently three or more [31], and although the two protruding C-termini of the dimer were appealing candidates as contact sites to ensure multivalency associated with LLPS, the ability of the VP3 Δ C to be recruited to VFs in infected cells suggested that deletion of at least one C-terminus did not remove this required valency. Therefore, I hypothesize that the VP3 C-terminal IDR might interface with other client biomolecules, thus influencing the physical properties of the resultant structures, for example by affecting the kinetics of the recruitment of biomolecules to VFs, or the packing and diffusive environment within the structures. Furthermore, the interaction

of the VP3 C terminus with VP1 is known to substantially promote VP1's polymerase activity [164], leading to vRNA synthesis. As our results suggest that vRNA is required for the formation of puncta with liquid-like properties, the VP3 C-terminus may also influence the liquidity of VFs by promoting vRNA synthesis. Simply put, the VP3 C-terminal IDR could be important in modulating the liquidness of VFs.

Interestingly, I observed minimal differences in the dynamic behaviors of the puncta formed in the presence of full-length wt VP3 and in the presence of VP3 Δ C: both were freely mobile in the cytoplasm and appeared to undergo fusion events, and both were sensitive to treatment with small aliphatic diols, which are means of designating LLPS [139]. The liquid-like properties of these puncta are shared with both the VFs formed during IBDV infection, as well as the observed behaviors of other virally-induced biomolecular condensates such as ReoV VFs and rotavirus viroplasms [43,44,95,149]. It remains unknown why some properties of the VP3 puncta were more significantly altered by the removal of the VP3 C-terminal IDR than others, however, as these live cell imaging assays were applied qualitatively in our study, it was not possible to draw quantitative conclusions as to how the presence or absence of the VP3 C terminus affected these behaviors. In addition, because aliphatic diols broadly perturb the hydrogen-bonding environment of the entire cell, they caused notable toxicity in DF-1 cells, even at low concentrations. To mitigate cytotoxicity, I treated cells with 1,3-PD, a less-toxic compound in the same class as the more commonly used 1,6-HD, however, responses varied too widely between cells and between puncta within a single cell to quantitatively distinguish differences in

the behavior of puncta formed in the presence of the full-length VP3 or VP3 Δ C, which is a limitation of our study.

The most quantitative assay that measured the physical properties of the puncta was FRAP, and as mentioned above, these data were consistent with the VP3 C terminus promoting the liquidity of the puncta, as the mobile fraction measured for puncta formed in the presence of the full-length VP3 was significantly higher than in the puncta formed by VP3 Δ C. Interestingly, I also observed that the recovery of the VP3 Δ C puncta exhibited a shorter recovery half-life (0.341 seconds), whereas the recovery of the wt VP3 puncta was longer (3.517 seconds). Although both were very rapid in the context of FRAP, a faster recovery half-life did not intuitively pair with a substantially reduced mobile fraction, as rapid recovery is suggestive of liquidity, whereas a reduced mobile fraction is suggestive of rigidity. However, it is important to note that in our system, the mNG fluorophore was fused to the client protein VP1, rather than the scaffold protein VP3. Previous studies have indicated that VP3 directly binds VP1 via the 10 C-terminal amino acids of VP3 [170]. As these are absent in the Δ C system, it is therefore possible that the unexpectedly rapid recovery half-life in the VP3 Δ C puncta was due to the presence of a small amount of unbound VP1-mNG that was able to move more readily as it was not tethered to VP3, even if the rest of the puncta was more rigid. However, further work is needed to confirm this effect.

Using AlphaFold, I have predicted the full-length structure of VP3 for the first time, and identified a candidate IDR in the C-terminal 36 amino acids of the protein. In MD simulations, I validated that the C-terminal domain is predicted to be significantly less well ordered than the

structured portions of the protein, indicating that it is a *bona fide* IDR. To determine the role of the C-terminus in the formation of IBDV VFs, I generated a truncation mutant of VP3, VP3 Δ C, which lacked the 36-residue C-terminus. I found that although puncta still formed in the absence of the C-terminus, they did so at a greatly reduced rate, indicating that the VP3 C-terminus is an important driver of VF formation. Furthermore, I found that puncta that form in the absence of the C-terminus exhibit substantially reduced liquidity as compared to those that form in the presence of full-length VP3, indicating that the C-terminus is an important determinant of the physical properties of phase-separated VFs.

Chapter 6: Discussion and Conclusions

The field of LLPS biology is of growing interest in cell biology as a number of cellular structures, including the nucleolus, P-bodies, SGs, and the NPC, are either entirely formed by LLPS or derive essential aspects of their functionality from the phenomenon. LLPS is also exploited by a number of viruses across families and genome structures, including the *Flaviviridae*, *Picornaviridae*, *Coronaviridae*, and *Reoviridae*, among others. Previously, the Broadbent lab discovered that the VFs of the *Birnaviridae*, such as IBDV, are also formed through LLPS. In the present work, I set out to determine how IBDV forms its VFs and drives the LLPS phenomenon.

6.1. Biological implications of my findings

First, I developed a suite of biological and analytical tools for generating, perturbing, and measuring VF-like biomolecular condensates that form via LLPS. I developed a system to fluorescently label VFs in live cells using the “spike-in” modality, which facilitated the collection of time-resolved data, including the dynamics of VFs during infection and in response to treatment with 1,3-PD. The live-cell labeling system also permitted the quantitative analysis of VF liquidity by FRAP. I also developed a computer vision-based pipeline for the analysis of VF geometry in micrographs of fixed cells, including the calculation and comparison of geometric metrics for VFs.

Next, I determined that VP3 is necessary but not sufficient to induce the formation of phase-separated structures. Furthermore, I found that while VP3 alone does not readily phase separate, VP3, in combination with VP1 and likely vRNA, constitutes the minimal component set for

phase separation in DF-1 cells. Even among the dsRNA viruses, there is diversity in the minimal component set for phase separation. In ReoV, μ NS is capable of phase separation independently, and appears to nucleate spontaneously in the cytoplasm. In rotavirus, the viroplasm minimal component set is bipartite, consisting of NSP2 and NSP5. Previously, it was unclear whether the VF-forming behavior of IBDV was more mechanistically analogous to ReoV or rotavirus, and our findings indicate that IBDV VF formation is more similar to viroplasm formation, both in the multipartite nature of the minimal component set, and indirect reliance on other structures for nucleation (via PI3P anchoring in the case of IBDV). This not only improves the understanding of IBDV VFs, but also provides a useful platform for the further study of IBDV phase separation dynamics, as it will be possible to use the reduced component set to more readily interrogate the effects of modifications to viral proteins on LLPS.

I also made the novel finding that IBDV VFs increase in liquidity with time, unlike what has been reported for other dsRNA viruses, for example, rotaviruses. Our discovery that the minimal component set for IBDV VF phase separation comprises VP3, VP1, and vRNA suggests a potential mechanism for increased liquidity of IBDV VFs over the course of infection. VP1, the viral RdRp, synthesizes vRNA, including vRNA encoding VP3 and VP1. VP3 binds VP1 and acts as an activator, greatly increasing the rate of vRNA synthesis. As previously discussed, this suggests that VP1, VP3, and vRNA may form a positive feedback loop in biosynthesis, leading to VF growth and changes in physical properties throughout infection.

Using our understanding of the components that induce phase separation in IBDV VFs, I expanded our understanding of phase separation at the molecular level. As I identified VP3 as

necessary but not sufficient for phase separation, I investigated the structure of VP3, and used computational biology to identify a candidate IDR within the C-terminus of the protein. I then utilized MD simulations to robustly characterize the disordered nature of this protein region, providing a detailed characterization of a VP3 region that had previously been excluded from crystallographic investigations of the protein. In the context of LLPS biology, IDRs are frequently implicated as key drivers of phase separation. As they freely explore their conformational space, they adopt numerous transient configurations that are conducive to the weak, transient interactions that drive LLPS at the molecular level. It is tempting, therefore, to ascribe strict necessity to IDRs and IDPs when they appear in phase-separated structures. However, there is no biological necessity for disorder in phase separation. To avoid assigning this assumption to VP3, I created a truncated variant of VP3 lacking the C-terminal disordered region, and analyzed it both in isolation and in combination with other viral components. I determined that the C-terminal IDR is an important promoter of VF phase separation and contributes to the liquidity of the structures, further highlighting the importance of IDRs in promoting LLPS. However, VF-like structures still formed in the absence of the IDR, albeit at a reduced rate and with reduced liquidity. Although the possibility of phase separation in the absence of disorder is known, the two phenomena co-occur so frequently that a counterexample such as the VP3 IDR is valuable to the study of LLPS by virtue of its uncommon nature.

This work has also contributed to the study of *Birnaviridae* biology. Although members of the family, including IBDV and IPNV, are major agricultural and aquacultural pathogens, respectively, birnaviruses remain significantly understudied as compared to related dsRNA viruses in the Reoviridae family, such as mammalian and avian reoviruses and rotavirus. In cases

where understudied viruses have better-studied counterparts, it is common to infer their biology from the well-characterized counterparts. Although this can provide insight and directions for investigation, direct study is superior when possible. I have therefore contributed to the study of birnaviruses by reducing what must be inferred from rotaviruses and reoviruses. Our results provide an example of the value of such direct study: rotavirus biomolecular condensates, termed “viroplasms”, undergo a hardening process over the course of infection, becoming less liquid with time. Our data indicate that the opposite is true for IBDV VFs, whose condensates become more liquid as measured by fluorescence recovery after photobleaching (FRAP) throughout the course of infection. Although it remains unknown what effect this may have on the replication dynamics of IBDV compared with rotavirus, identifying the difference between the two serves both to directly expand understanding of birnavirus biology and to motivate further investigations to reduce reliance on inference from related viruses.

6.2. Potential applications of my findings

The characterization of viral replication at the molecular level is beneficial beyond academic interest. Although IBDV vaccine development is supported by a robust industry, many commercial vaccines still rely on the development of live-attenuated strains. By the nature of live attenuation by serial passage, antigenic integrity may be traded for attenuation. Our work has identified a nonstructural protein, VP3, as directly influencing the formation of IBDV’s replicative bodies, and identified the unstructured C-terminal IDR as a determinant of the properties of those structures. Furthermore, I validated previous findings that deletion of the IBDV C-terminus prevents rescue of viable virus by reverse genetics. In combination, these results make the VP3 C-terminus an attractive site for targeted attenuation at the molecular level.

This may facilitate the development of live-attenuated vaccines without the requirement for serial passage.

Condensate disruption has yet to gain widespread recognition as a means of antiviral drug development, in part because of how frequently the phenomenon occurs throughout the cell. Drugs and therapies designed to target the LLPS phenomenon broadly are likely to disrupt essential cellular condensates. Any potential antiviral drugs targeting phase-separated structures must therefore target the components that are unique to the condensates of interest. Identification of necessary scaffolds such as VP3 is particularly valuable in this context, as drugs or biologics that target VP3 and prevent recruitment or necessary interactions have the potential to disrupt condensate formation. As the VFs are known to be the sites of vRNA synthesis, their disruption is an attractive target for antiviral therapies. Although direct interest in antiviral therapies against IBDV is somewhat limited, IBDV provides a convenient, readily cultured platform for developing condensate-targeting antiviral drugs.

Finally, birnaviruses are of interest as therapeutic agents in themselves. For example, IBDV has been identified as a candidate agent for use in oncolytic viral therapy (OVT) in the treatment of several cancers, including glioblastomas, B-cell carcinomas, and colorectal carcinomas in murine models [172,173]. IBDV is an attractive candidate for this approach as the virus is not zoonotic and so does not readily infect mammalian cells, but is still taken up by cancer cells where it causes cell lysis, as well as triggering cell death pathways and an inflammatory response that drives the influx of immune cells that could also aid in combatting the cancer. The field therefore benefits from a robust understanding of the cell biology of IBDV infection, to facilitate the

enhancement of viral replication in the tumor microenvironment. Hence, basic/fundamental study of IBDV-cell interactions may aid in the development of efficacious OVTs in the future. In particular, the study of LLPS VFs may contribute to these developments. The tumor microenvironment is biochemically unique, and LLPS condensates are sensitive to environmental conditions and composition. A comprehensive understanding of the drivers of VF formation may therefore have particular utility for improving viral replication within tumors.

6.3. Limitations

This work is not without limitations. Most experiments were conducted in DF-1 cells, which though of avian origin and convenient for microscopy, are not the same as the B-cells preferentially infected by IBDV *in vivo*. Although an immortalized B-cell line (DT40) exists, it is extremely challenging to conduct microscopy studies in the thin cytoplasm surrounding the nucleus of spherical DT40 cells in suspension. I did verify the behavior of VFs in HD11 cells, an immortalized macrophage cell line. Though not the B-cells preferentially infected by IBDV, *in vivo* macrophage infection is documented in infected birds, hence HD11s were selected as a more biologically relevant platform to confirm the effects I studied in DF-1s. Moreover, our experiments were performed using only the lab-adapted PBG98 strain of IBDV. However, I verified that the VP3 C-terminus was present and conserved across a panel of 88 field strains.

The experiments I performed also inherently involved tagged or modified viral proteins, which are known to influence phase-separation behavior and the resulting properties of biomolecular condensates, even in “spike-in” experiments. I attempted to mitigate this by tag selection, screening a number of tags of different types, and selecting mNG based on its performance in

our screen and in similar experiments performed by others. I also designed experiments to only compare “like to like”, where tags were kept consistent between groups under comparison, and designed experiments to avoid the assumption that the tag does not influence condensate properties.

6.4. Future work

I primarily determined the role of the VP3 C-terminus by truncation of VP3 at residue 221, creating VP3 Δ C. Removal of the entire C-terminus, though effective in elucidating the role of the region, is a relatively coarse screen. In future work, modifications could be made to the C-terminus and the resultant condensate screened for changes in physical properties by the methods I have developed for this work. I have screened several variants of the VP3 C-terminus by MD simulation. Although I did not identify any mutations that introduced statistically significant differences in predicted flexibility, this could reflect a limitation of the point mutations screened, or the difficulty in discerning statistically significant differences in the mobility of highly disordered proteins. Furthermore, although I was not successful in attempts to rescue viable IBDV with the VP3 Δ C mutation, it might be possible to rescue virus with a different C-terminal mutation. If any such mutations were found to modulate viral replication, such rescue might enable the development of novel attenuated vaccine strains.

Moreover, although this work has expanded our understanding of IBDV VFs and the role of VP3 in multiple ways, it has also raised new questions that could be pursued in future studies. For example, I do not know whether processes other than viral genome replication take place within the VF. To address this, I would need to comprehensively assay VF composition, and although I

have determined the minimal component set for IBDV VF formation, the true compositions of condensates are typically more complex than their minimal components. Because the composition of the heavy phase is determined by “solubility”, any number of viral or cellular biomolecules may selectively partition into VFs. Although the interactome of VP3 has been characterized by mass spectrometry [108], condensates lack membranes and are ephemeral, with properties and composition strongly influenced by environmental conditions. Because cells are lysed in preparation for mass spectrometry, this subsequent change in environmental condition may affect the detected interactome and inferred condensate composition. Furthermore, the interactions which drive phase separation are inherently weak and transient. Although this can be mitigated somewhat with chemical crosslinking, interactome analysis by immunoprecipitation relies on the interactions in question surviving both extraction and multiple washings, which may reduce sensitivity for low-quantity, low-affinity interactions. It is more attractive therefore to employ a proximity labeling technique, wherein condensate-resident biomolecules are labeled *in-situ* with an indelible biotin tag, which can be later separated and identified by mass spectrometry. Although it was not included in this thesis, I also developed multiple biotin ligase and peroxidase-conjugated constructs with VP1 and VP3 to address this. On the basis of this initial work, VP3 conjugated to APEX2 is an attractive option for *in-situ* proximity labeling of VF components. Subsequent affinity capture of biotinylated proteins and analysis by mass spectrometry could provide the most comprehensive survey to date of IBDV VF composition, providing insights into the roles of VFs in viral replication, and provide a springboard for diverse investigations of these roles.

6.5. Summary

In summary, in this work, I investigated the formation and properties of IBDV VFs at the molecular level. First, I developed a suite of biological and analytical tools for the generation, perturbation, and measurement of VF-like biomolecular condensates that form through LLPS. Using this suite of tools, I found that IBDV VFs increase in liquidity with time, unlike what has been reported for other dsRNA viruses, for example, rotaviruses. Furthermore, I utilized our in-house reverse genetics system alongside several purpose-made expression plasmids and discovered that the multifunctional viral protein VP3 is necessary but not sufficient to induce phase separation, and that the minimal component set required for phase separation comprises VP3, VP1, and viral RNA. I also applied modern computational methods, including AlphaFold and MD simulations, to the study of VP3 and determined that VP3 encodes a highly dynamic C-terminal tail that constitutes an IDR. Finally, building on these findings, I made the novel determination that the VP3 C-terminal IDR is an important driver of VF formation and a determinant of VF physical properties, but is not absolutely required for condensate formation. This work presents the most complete characterization of the biophysical properties of the IBDV VF structures to date. Moreover, this work has generated a suite of tools that will facilitate future investigations. Finally, this work also contributes to the LLPS field at large, as expanding of our understanding of LLPS biology is most readily accomplished by the identification and characterization of new instances of the phenomenon.

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