Pan-retroviral Nucleocapsid-Mediated Phase Separation Regulates Genomic RNA Positioning and Trafficking

Anne Monette1,*, Meijuan Niu1, Lois Chen1,2, Shringar Rao1,3, Robert James Gorelick4, Andrew John Mouland1,2,5,6,*

1HIV-1 RNA Trafficking Laboratory, Lady Davis Institute at the Jewish General Hospital, Montréal, QC H3T 1E2, Canada
2Department of Microbiology and Immunology, McGill University, Montréal, QC H3A 2B4, Canada
3Department of Biochemistry, Erasmus University Medical Center, Ee634, PO Box 2040, 3000CA Rotterdam, the Netherlands
4AIDS and Cancer Virus Program, Frederick National Laboratory for Cancer Research, Frederick, MD 21701, USA
5Department of Medicine, McGill University, Montréal, QC H3G 2M1, Canada
6Lead Contact

SUMMARY

The duality of liquid-liquid phase separation (LLPS) of cellular components into membraneless organelles defines the nucleation of both normal and disease processes including stress granule (SG) assembly. From mounting evidence of LLPS utility by viruses, we discover that HIV-1 nucleocapsid (NC) protein condenses into zinc-finger (ZnF)-dependent LLPSs that are dynamically influenced by cytosolic factors. ZnF-dependent and Zinc (Zn2+)-chelation-sensitive NC-LLPS are formed in live cells. NC-Zn2+ ejection reverses the HIV-1 blockade on SG assembly, inhibits NC-SG assembly, disrupts NC/Gag-genomic RNA (vRNA) ribonucleoprotein complexes, and causes nuclear sequestration of NC and the vRNA, inhibiting Gag expression and virus release. NC ZnF mutagenesis eliminates the HIV-1 blockade of SG assembly and repositions vRNA to SGs. We find that NC-mediated, Zn2+-coordinated phase separation is conserved among diverse retrovirus subfamilies, illustrating that this exquisitely evolved Zn2+-dependent feature of virus replication represents a critical target for pan-antiretroviral therapies.

Graphical Abstract

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*Correspondence: anne.monette@mail.mcgill.ca (A.M.), andrew.mouland@mcgill.ca (A.J.M.).

AUTHOR CONTRIBUTIONS
A.M. and A.J.M. conceived of the study; A.M., M.N., S.R., and L.C. performed experiments; R.J.G. generated all recombinant retroviral NC proteins and provided key resources; A.J.M. and R.J.G. secured funding; A.M. and A.J.M. drafted the manuscript; and all authors revised and edited final version.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.03.084.

DECLARATION OF INTERESTS
The authors declare no competing interests.
In Brief

Monette et al. discover a high degree of conservation of zinc-finger embedded, intrinsically disordered prion-like domains across retrovirus Gag proteins. These domains within the Gag Nucleocapsid regulate the formation of zinc-dependent liquid-liquid phase condensates and stress granules in HIV-1-expressing cells to induce repositioning of the viral genomic RNA.

INTRODUCTION

Eukaryotic cells have liquid-like but spatially organized, membraneless compartments such as stress granules (SGs), P bodies (PBs), and nucleoli, enriched in multivalent proteins having disordered and modular domains, and which are concentrated away from the aqueous environment by a process called liquid-liquid phase separation (LLPS) (Ambadipudi et al., 2017; Bergeron-Sandoval et al., 2016). Phase separation initiates LLPS and is emerging as one of the underlying principles of cell organization governing cell function and survival (Uversky, 2017), such that protective SGs assemble in response to environmental and genotoxic stressors (Franzmann and Alberti, 2019; Kroschwald and Alberti, 2017). Following stress withdrawal, LLPS and SGs rapidly disassemble, and are thus centers of exquisite control in normal cell physiology.

Viruses depend on an exhaustive array of host cell components and like liquid droplets (Camus et al., 2013), LLPSs represent ideal platforms for many virus replication stages (Alenquer et al., 2019; Heinrich et al., 2018; Liu et al., 2014; Nikolic et al., 2017). In addition to co-opting cellular proteins, LLPS may promote proximity-dependent interactions.
and the assembly of viral factories for virus expression (Nikolic et al., 2017; Novoa et al., 2005). Numerous viruses, however, inhibit SG assembly to evade the inherently antiviral nature of these ribonucleoprotein assemblies required for protein synthesis (Poblete-Durán et al., 2016). SG blockade by viruses is achieved by modification, cleavage, sequestering, or redistribution of SG-proteins and translation initiation factors (Zhang et al., 2019). HIV-1 has evolved multiple mechanisms to block the assembly of canonical and non-canonical SGs. For example, HIV-1 blocks SG assembly induced by heavy metals as well as SG assembly induced by the expression of key SG-inducing proteins, G3BP1 and TIAR (Valiente-Echeverría et al., 2014). HIV-1 achieves this blockade via interactions between the viral structural precursor polyprotein (pr55Gag), and several host factors such as eukaryotic elongation factor 2, G3BP1, Cyclophilin A, and eIF4E (Cinti et al., 2016; Valiente-Echeverría et al., 2014). A single point mutation, in fact, in the C-terminal capsid (CA) domain of pr55Gag, prevents host factor binding and disables the blockade to SG assembly, likely by changing the conformation of this pr55Gag domain (Valiente-Echeverría et al., 2014). Complete regulation of this mechanism is increasingly puzzling from demonstrations that the nucleocapsid (NC) domain of pr55Gag promotes Gag- and CA-resistant SGs (Rao et al., 2018), and suggests that an equilibrium exists between SG assembly and disassembly during HIV-1 replication.

NC is among the viral protease (PR) cleavage products generated from pr55Gag, also yielding matrix (MA) and capsid (CA) proteins. This 55-amino acid (7-kDa) protein is a major component of infectious virus cores, with ~2,000 HIV-1 NC molecules coating the dimeric, viral genomic RNA (vRNA) (Darlix et al., 1995). NC has two zinc-coordinating finger (ZnF) domains in CCHC conformation controlling several steps of retroviral replication from transcription to vRNA selection for packaging (Muriaux and Darlix, 2010). The highly conserved amino acids essential for the many functions of retroviral NC ZnFs, along with their inability to produce viable escape mutants from drug targeting, poise them as ideal therapeutic targets (Goebel et al., 2001; Rice et al., 1995, 1997b).

NC is characterized as a nucleic acid chaperone from its ZnF-dependent roles in nucleic acid binding (single-stranded DNA and RNA), condensing, annealing, and strand transfer (Muriaux and Darlix, 2010). It localizes to the cytoplasm, and to the nucleus on account of its nuclear localization signals (Anton et al., 2015; Lochmann et al., 2013). Like NC, other cellular RNA-binding proteins and chaperones (e.g., G3BP1, TIAR, TIA-1, DDX6, TDP-43, FUS/TLS, and hnRNPs) are SG and PB components (Kedersha et al., 2016; Poblete-Durán et al., 2016), may be Zn²⁺-regulated (Garnier et al., 2017; Rayman et al., 2018), and form LLPSs due to their low-complexity, intrinsically disordered prion-like domains (PrLDs) (Kedersha et al., 2016; Maharana et al., 2018). The entire NC is a PrLD at the C terminus of pr55Gag, where NC exerts its functions via its ZnFs (Darlix et al., 2011; Dick and Vogt, 2014). Retroviral HIV-1, SIV, and MuLV NCs are also important for virion structure, whereby NC-mutated virions are immature and have aberrant particle morphology (Cimarelli et al., 2000; Muriaux et al., 2004; Tanchou et al., 1998; Yovandich et al., 2001). Rabies virus (RABV), vesicular stomatitis virus (VSV), Ebola, and measles virus proteins have PrLDs and use LLPS mechanisms for host-defense shielding replication dynamics in membraneless inclusions within viroplasms (Heinrich et al., 2018; Nikolic et al., 2017).
From demonstrations that other viral proteins form LLPS, and from shared similarities that NC has with other proteins that bind RNA, possess PrLDs, and induce SG assembly and LLPS, we investigated the possibility that NC function may be triggered by its ability to phase separate. We performed biochemical and cellular experiments to show that NC condenses into LLPSs in vitro and in living cells. NC-LLPSs displayed fundamental properties of a liquid phase, including a spherical shape from surface tension, an ability to fuse and separate, and rapid internal and cellular diffusion (Heinrich et al., 2018). We observed that NC-LLPS and the HIV-1 blockade to SG assembly are Zn²⁺-dependent, blocked by NC ZnF mutagenesis or Zn²⁺ chelation, to promote aberrant cellular localization of NC, Gag, and the vRNA, retention of Gag, and reduced virus production. In silico methods mapped conserved Gag protein PrLD and ZnF positioning across retroviruses. We demonstrated that both HIV-1 Gag and NC from several retroviral subfamily members undergo Zn²⁺-dependent LLPS. Finally, we have observed that NC is generated in cells by PR. Our results point to a pan-retrovirus NC-specific, membraneless, LLPS mechanism nucleating virus assembly, and controlled by reversible, Zn²⁺-dependent secondary structures supported by PrLDs. In this report, we provide insight on how Zn²⁺-dependent NC-LLPSs promote vRNA trafficking, vRNA packaging, and virus release.

RESULTS

NC Condenses into ZnF-Dependent LLPSs Dynamically Regulated by the Cellular Environment

To establish that HIV-1 NC condensed into LLPSs, increasing concentrations of purified Zn²⁺-loaded and green-fluorescence-labeled NC protein (Wu et al., 1996) were mixed with buffers containing Ficoll or dextran as crowding agents (Alberti et al., 2019; Boehning et al., 2018; Maharana et al., 2018). Laser microscopy and differential interference contrast (DIC) validated that green fluorescent NC-LLPSs had expected spherical morphology. NC-LLPSs could be seen at 5 µM of NC protein with Ficoll, but were more numerous and spherical at the optimal concentration of 10 µM, with homogeneous NC-LLPS sizes ranging from 0.5 to 2 µm (Alberti et al., 2019) (5 versus 10 µM of NC, p < 0.0001) (Figure 1A).

LLPSs can be produced in vitro using synthetic macromolecular crowding agent Ficoll, hypothesized to mimic the crowded cellular environment (Alberti et al., 2019). To predict whether NC could initiate formation of LLPS in cells, we performed in vitro experiments replacing Ficoll with cellular homogenate with estimated equimolar concentration of total protein to NC. Cell homogenate readily induced formation of NC-LLPSs (Figure 1B), with these being more diverse in size (ranging from 0.5 to 3 µm) and less spherical, supporting that these NC assemblies may represent NC-LLPS and NC-RNPs containing cytosolic proteins or nucleic acids. With NC proteins greatly outnumbering any single cellular protein from homogenates in these formulations, comparison of visible DIC aggregates with green NC aggregates confirmed that 97.63% of these were NC-positive and distinguishable from cell debris (Figures 1B and 1C).

Unlike static NC-LLPSs generated by in vitro Ficoll-induced LLPS experiments, those formed by cell homogenates were dynamic, exhibiting liquid-phase properties including round shape induced by surface tension, fluidity, rapid internal diffusion, and interaction.
and fusion forming novel droplets (Baase et al., 2010; Brangwynne et al., 2009; Heinrich et al., 2018) (Figures 1C–1G; Video S1). Live cell footage was used to track NC-LLPS movement, where the homogenate induced these to travel total distances of 16.67 ± 1.28 µm and linear distances of 4.04 ± 0.65 µm in 30 s (Figures 1E–1G). NC-LLPS displacements had average speeds of 0.56 ± 0.04 µm/s, ranging from 0.17 to 1.67 µm/s, thus moving at rates proportional to their sizes (Figure 1H).

We have recently demonstrated that HIV-1 NC induces SG assembly (Rao et al., 2018), despite NC being cleaved from pr55Gag during virus maturation, and therefore not typically found as an abundant entity in cells. To test if the NC moiety within full-length Gag inhibits or participates in LLPS in cells, mCherry-Gag expressing cellular homogenates were used to observe that albeit at lower abundance, mCherry-Gag co-condensed with NC-LLPSs, suggesting that NC within Gag may stimulate LLPS activity prior to its cleavage by PR (Figure 1I).

Low complexity PrLDs and ZnFs are hallmarks of phase-separating proteins (Maharana et al., 2018). Like NC, PrLD-containing proteins are intrinsic to SGs and can multimerize into Zn$^{2+}$-dependent LLPS processes of aggregate-prone neurodegenerative disease makers TIA-1, TDP-43, FUS/TLS, Tau, amyloid-β, synucleins, and SOD1 (Caragounis et al., 2010; Garnier et al., 2017; Pfaender and Grabrucker, 2014; Rayman et al., 2018; Shelkovnikova et al., 2012). Gag multimerization requires NC with intact ZnFs and an RNA scaffold (Burniston et al., 1999; Campbell and Vogt, 1995; El Meshri et al., 2015). To test whether NC ZnFs were responsible for NC-LLPS, we performed in vitro experiments comparing wild-type (WT) NC to those with mutated ZnF motifs (SSHS-SSHS) (Guo et al., 2000) or the mutated linker region (R7A/R10A/K11A) (Wu et al., 2014a). Although linker mutant LLPSs were not as spherically shaped as WT NC-LLPSs, their overall numbers did not change (Figure 1J). In contrast, the ZnF NC mutant could not form LLPS (p < 0.0001), suggesting that the NC-LLPS may be Zn$^{2+}$ dependent. Therefore, we used the Zn$^{2+}$ chelator TPEN (N,N,N’,N’-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine), which inhibits LLPS and SG by TIA-1 (Rayman et al., 2018), and 1,6-Hexanediol (HEX), which inhibits LLPS by hnRNP A1 and TDP-43 (Babinchak et al., 2019; Molliex et al., 2015) and should interfere with maintenance of the Zn$^{2+}$-induced globular, hydrophobic plateau of NC ZnFs. We also tested the oxidizer arsenite (ARS), which causes release of Zn$^{2+}$ from ZnFs, thus disrupting SG assembly in cells (Rayman et al., 2018), and which selectively binds and unfolds C3H ZnFs (Zhao et al., 2012). All three chemicals disrupted NC-LLPSs (p < 0.0001) (Figure 1K), confirming that NC-LLPSs are Zn$^{2+}$-dependent.

**Zn$^{2+}$ Chelation Inhibits NC-SG Assembly and Disrupts NC-vRNA-Gag RNP Formation**

We have previously reported that NC induces translational arrest and Gag- and CA-resistant SGs containing TIAR-1, G3BP1, eIF3, PABP, and poly(A) mRNAs (Rao et al., 2018). We have herein recapitulated that Gag did not disrupt NC-LLPS granules, but it rather colocalized with NC-LLPS granules, perhaps through Gag/NC-NC interactions (Figure 1I). From observations that NC-LLPS were Zn$^{2+}$ dependent (Figures 1J and 1K), and demonstrations that NC ZnF mutants have reduced SG assembly (Yu et al., 2016), we tested NC-SG sensitivity to Zn$^{2+}$ chelation, as previously shown for TIA-1 (Rayman et al., 2018).
Dose-escalating TPEN treatment (0–40 µM) of HeLa cells transfected with the NC-Renilla Luciferase (RLuc) expression constructs showed that TPEN reversed both NC-RLuc-G3BP1 colocalization and NC-induced SGs with no effect on expression of NC-RLuc (Figure 2A). TPEN also modified the formerly diffused cytoplasmic NC-RLuc localization to one mostly localizing closer to the plasma membrane (Figure 2A).

To visualize effects of TPEN on NC and cellular Zn\(^{2+}\), we loaded HeLa cells with a Zn\(^{2+}\)-fluorescent probe FluoZin prior to TPEN treatment. This provided evidence that Zn\(^{2+}\) strongly localized with NC-RLuc and G3BP1 in absence of TPEN, and that while TPEN did not diminish overall cellular Zn\(^{2+}\) content, it resulted in dispersion and reduced colocalization with NC-RLuc (−drug versus 40 µM of TPEN (Figure 2B; STAR Methods for statistics). These data provide evidence that TPEN successfully chelates Zn\(^{2+}\) from NC and NC-G3BP1 SGs but does not deplete it from cells. From observations that G3BP1-FluoZin colocalization was also diminished by TPEN treatment—despite G3BP1 not containing ZnFs, and no reports supporting the possibility of it binding Zn\(^{2+}\), where G3BP1 rather assembles into SGs via RNA binding and acetylation dynamics (Gal et al., 2019; Irvine et al., 2004)—we performed dose-escalating TPEN treatment (0–40 µM) of HeLa cells transfected with pG3BP1-GFP (Valiente-Echeverría et al., 2014). These experiments demonstrated that TPEN treatment had no significant effects on G3BP1-GFP-induced SGs also populated by SG marker TIAR (Figure S1), confirming earlier demonstrations that ARS-induced SGs are insensitive to TPEN (Rayman et al., 2018).

We sought to investigate the effects of TPEN on NC, vRNA, and Gag distribution in WT HIV-1 expressing cells. HeLa cells were cotransfected with NC-RLuc and the WT proviral construct pNL4–3, then treated with 20 µM of TPEN. We first examined the effect of TPEN on association of NC with DDX6, since DDX6 is a component of the HIV-1 Gag-vRNA packaging initiation complex (Barajas et al., 2018). Despite TPEN causing differences in DDX6 signal intensity and its colocalization with NC-RLuc in HIV-1 negative conditions, TPEN had no effect on NC-RLuc-DDX6 or Gag-DDX6 colocalization in HIV-1 expressing cells (Figure 2C). These results are in agreement with reports proposing that association of NC or Gag with DDX6 in early virion assembly intermediates are indirect, where the DDX6 helicase may rather unwind the vRNA for Gag accessibility (Reed et al., 2012). We did observe, however, that coupled HIV-1 expression and Zn\(^{2+}\) chelation by TPEN led to a fraction of NC-RLuc localizing to nuclei (Figure 2C).

Interactions between PrLDs and RNA are believed to drive LLPS (Maharana et al., 2018), and NC ZnFs are required for selectivity and packaging of the vRNA (Gorelick et al., 1990). Thus, to investigate whether TPEN treatment could alter the interactions between the vRNA and NC, we cotransfected HeLa cells with pNC-RLuc and WT proviral pNL4–3. Combined immunofluorescence/fluorescence in situ hybridization of the full-length vRNA (Vyboh et al., 2012) and NC-RLuc showed reduced colocalization (p = 0.0002) (Figure 2D). This effect was not seen for NC-RLuc-Gag or vRNA-Gag colocalization, perhaps because these associations are assisted by members of larger RNPs or because Gag is itself expressed from the vRNA. TPEN treatment also caused nuclear retention of the vRNA and NC-RLuc (Figure 2D), suggesting that the Zn\(^{2+}\)-loaded NC domain may promote nuclear egress of NC-vRNA RNPs for virus production. Protein sequence analysis of components of HIV-1
vRNA export complexes revealed that they do not possess ZnFs and are thus not expected to be affected by Zn$^{2+}$ chelation.

Zn$^{2+}$ Chelation Inhibits the HIV-1 Blockade to SG Assembly and Induces vRNA Repositioning to SGs

Numerous divergent viruses including HIV-1, Ebola, and Zika block SG assembly (Amorim et al., 2017; Cinti et al., 2016; Le Sage et al., 2017; Valiente-Echeverría et al., 2014). For HIV-1, the CA domain may be responsible for the SG blockade, whereas WT NC, but not NC ZnF mutants, promotes SGs (Rao et al., 2018; Yu et al., 2016). The Zn$^{2+}$-loaded Gag NC domain may thus also promote LLPS toward growth of Gag-vRNA RNPs for virus assembly, whereas the CA domain and other cellular proteins interacting with NC-ZnF may keep SG assembly in check to guarantee that viral RNPs do not grow uncontrolled, producing dysfunctional viral particles. Thus, we sought to determine whether Zn$^{2+}$ chelation could reverse the HIV-1 blockade of SG assembly. Infected Jurkat T cells treated with 20 µM of TPEN showed that Zn$^{2+}$ chelation increased G3BP1 signal intensity in ARS-positive and -negative conditions (p < 0.0001), increased vRNA-G3BP1 colocalization (p < 0.0001), and increased Gag expression (ARS+ versus ARS+TPEN+; p = 0.0017, 95% confidence interval (CI) −1.139.0 to −220.1, 1-way ANOVA, Tukey post-test) (Figure 3A). vRNA was also visualized in HeLa cells transfected with pNL4–3 and treated with ARS and TPEN (20 or 40 µM). TPEN consistently increased the G3BP1 signal intensity in ARS+ and negative conditions (p < 0.0001), increased vRNA-G3BP1 colocalization (p < 0.0001), and increased Gag expression (ARS+ versus ARS+TPEN+; p = 0.0010, 95% CI −2.483.0 to −469.3, 1-way ANOVA, Tukey post-test) (Figure 3B). As shown in earlier work (Abrahamyan et al., 2010; Valiente-Echeverría et al., 2014), HIV-1 reduced the size and number of ARS-induced G3BP1+ SGs (pcDNA3.1+ARS versus pNL4–3+ARS, p < 0.0001, two-tailed t test), whereas TPEN treatment abrogated the SG blockade by HIV-1 (pNL4–3+ARS versus pNL4–3+ARS+TPEN (20 µM), p = 0.0037, two-tailed t test) (Figure 3B). Importantly, TPEN also caused vRNA localization to SGs in ARS-treated cells (Figure 3B). Conversely, TPEN treatment had a lesser effect on G3BP1-Gag colocalization (p = 0.0261), and little influence on Gag-vRNA colocalization (Figure 3B) in both HeLa and Jurkat T cells. Together, these results show that Zn$^{2+}$ chelation of NC abrogates the SG blockade by HIV-1, leading to vRNA repositioning to SGs.

Zn$^{2+}$ Chelation Causes Cellular Retention of Gag and Decreased Virus Production

Our observations that Zn$^{2+}$ chelation abrogated the SG blockade by HIV-1, vRNA accumulation in SGs, but concomitant increased Gag expression, appeared confounding since SGs usually contain translationally silent mRNAs (Anderson and Kedersha, 2009). To address this, we transfected HeLa and HEK293T cells with pNL4–3, or cotransfected with pNL4–3 and NC-RLuc and treated with TPEN, ARS, or HEX, and collected cell lysates for western blotting and viral supernatants for virus quantification. NC-RLuc caused decreased Gag expression (pNL4–3 versus pNL4–3/NC-RLuc; p = 0.0008, 95% CI 27.12 to 122.2, 1-way ANOVA, Tukey post-test) (Figure S2; Figure 4A), supporting that NC induces SG assembly and represses vRNA translation favoring its encapsidation (Rao et al., 2018). As previously observed (Figures 3A and 3B), and consistent between HeLa and HEK293T cells, TPEN caused a striking increase in Gag expression (p < 0.0001) (Figure
Consistent with previous work (Rao et al., 2018), NC-RLuc expression in both cell types resulted in decreased virus production (p < 0.0001) (Figure 4B). Decreased virus production from TPEN treatment was also observed in HIV-1 infected Jurkat T cells (pNL4–3 versus pNL4–3+TPEN; p = 0.0003, 95% CI 9.012 to 18.84, 1-way ANOVA, Tukey post-test, data not shown). Decreased virus production by TPEN suggests that Zn$^{2+}$ is required for NC-LLPS, NC-SGs, and limiting Gag expression for virus production. These findings suggest that Zn$^{2+}$-dependent NC-LLPSs may act by binding and arresting vRNA translation favoring egress of packaging initiation complexes slated for encapsidation and release as infectious viral particles.

**NC ZnF Mutants Reverse HIV-1 Blockade on SG Assembly and Induce vRNA Repositioning to SGs**

Our observations that the HIV-1 blockade to ARS-induced SG assembly was consistently abrogated by Zn$^{2+}$ chelation (Figures 3A and 3B), and led to vRNA repositioning (Figure 3B), prompted us to determine if Zn$^{2+}$-chelation phenotypes were specific to NC ZnF function. HeLa cells transfected with the NC ZnF-mutated pNL4–3 proviral constructs (i.e., pNL4–3 NC-C15S-C49S) (Guo et al., 2000) were treated with ARS and TPEN. Strikingly, mutated NC ZnF led to a complete reversal of HIV-1 blockade of SG assembly that was unaltered by TPEN treatment (p < 0.0001) (Figures 5A and 5B, where Figure 5B compares conditions tested to those in Figure 3). As formerly observed from TPEN treatment (Figure 3B), NC ZnF mutants also caused the vRNA to be restricted to G3BP1$^+$ SGs (Figure 5A). As observed with SG-inducing NC-RLuc (Figure 2D), vRNA was localized to the nucleus in untreated cells, and this was further pronounced in ARS-treated cells (Figure 5A), providing evidence that NC ZnFs are required for nuclear export and/or cytoplasmic retention of the vRNA for virus assembly. Other prominent NC ZnF mutant phenotypes were loss of G3BP1-vRNA, G3BP1-Gag, and vRNA-Gag colocalization in ARS-negative conditions (Figure 5A), supporting that NC ZnF nucleates HIV-1 Gag-vRNA RNPs.

**Clinical HIV-1 Zn$^{2+}$ Ejectors Induce the Nuclear Retention of the vRNA**

From our observations that TPEN treatment caused nuclear localization of NC and the vRNA (Figures 2C, 2D, and 3B), as also validated by TPEN treatment of the NC-ZnF mutant (Figure 5A), we examined 19 essential and associated Rev-RRE-CRM1-vRNA nuclear export proteins (e.g., Rev, CRM1, DDX1, DDX3, DHX9 (RHA), eIF5a, RanGTP, RIP, Sam68) (Hofmann et al., 2001; Maares and Haase, 2016; Yedavalli et al., 2004), to find that none possessed ZnFs and should not be affected by TPEN treatment. We then tested whether the nuclear vRNA phenotype could be repeated using clinical HIV-1 NC ZnF-selective targeting Zn$^{2+}$ ejectors. From numerous clinical HIV-1 Zn$^{2+}$ ejectors, we chose Azodicarbonamide (ADA) and 2,2'-dithiobisbenzamide-1 (DIBA-1) (Rice et al., 1995, 1997b) from their entry into Phase I/II testing, specific NC-selectivity with no affinity for cellular protein ZnFs (Goebel et al., 2001; Huang et al., 1998), and from interests in their repurposing as microbicides (Mori et al., 2015). HeLa cells transfected with pNL4–3 were treated with 100 µM of ADA or 50 µM of DIBA-1. Indeed, both drugs led to nuclear retention of the HIV-1 vRNA (both −drug versus +drug, p < 0.0001) (Figure 5C), in similar proportions to ZnF mutant proviral constructs (pNL4–3 versus pNL4–3 NC-C15S-C49S, p
Pan-retrovirus NC Proteins Have Overlapping ZnFs and PrLDs and Undergo Zn\(^{2+}\)-Dependent LLPS

HIV-1 NC overexpression thus triggers Zn\(^{2+}\)-dependent SGs (Figures 2A and 2B), and purified HIV-1 NC protein undergoes Zn\(^{2+}\)-dependent LLPS (Figures 1A and 1J). Envisaging this phenomenon exists across retrovirus subfamily NCs, we used predictive software to identify ZnF-containing PrLDs, because we expected that their proximities would regulate NC-LLPSs. This notion is supported by studies of PrLDs across eukaryotic viruses with a high proportion identified in retroviruses (Tetz and Tetz, 2018). To map proximity of retrovirus Gag PrLDs and ZnFs, POND\(\text{R}\) and PLAAC algorithms were used to generate PrLD alignment maps of pan-retrovirus NC and Gag amino acid sequences (Lancaster et al., 2014; Peng et al., 2006, 2005; Romero et al., 2001). Predicted PrLDs for HIV-1 Gag and NC were validated by previous studies (Xue et al., 2012), while others were validated by the MobiDB database (Piovesan et al., 2018). The accuracy of predictive software programs was tested using proteins with established presence or absence of PrLDs (Figure S3A) (Baase et al., 2010; Wang et al., 2018; Ward et al., 2004). All retrovirus Gag proteins displayed two conserved PrLDs of similar length and location: one encompassing the NC domain and extending to C-terminal end of Gag, and another within the Gag N-terminal CA domain and late (L) domains, as supported by previous reports (Figure 6A) (Deshmukh et al., 2015; Freed, 2002; Liang et al., 2003).

The molecular composition of membraneless organelles includes ZnFs or Zn\(^{2+}\)-binding domains also found in disordered proteins forming amyloidogenic segments (Alberti et al., 2019; Garnier et al., 2017; Gomes and Shorter, 2019). We mapped retroviral ZnFs onto predicted PrLDs to find them positioned within predicted C-terminal PrLDs, with exception of Spumaviruses lacking traditional ZnFs, instead having functionally equivalent RNA recognition motifs (RRMs) (Linial, 1999; Müllers, 2013), also concentrated within predicted PrLDs (Figure 6A). Thus, proximity of ZnFs and PrLDs are highly conserved throughout Retroviridae Gag proteins, suggesting that all could form Zn\(^{2+}\)-dependent NC-LLPSs.

We tested purified Zn\(^{2+}\)-loaded NC proteins from several closely and distantly related retroviral genera for their ability to LLPS (Weiss, 2006), including HIV-1, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), human T-lymphotropic virus type 1 (HTLV-1), Rous sarcoma virus (RSV), and murine leukemia virus (MuLV) (Guo et al., 2000; Post et al., 2016; Stewart-Maynard et al., 2008; Wu et al., 2014b, 1996; Yovandich et al., 2001) (Figure 6B). As expected, all pan-retroviral NC proteins could initiate LLPSs at similar sizes of ~1 µm (Figure 6C), despite differences in their lengths, sequences, linker regions, or number of ZnFs (Figure 6D).

From our observations that NC proteins produced the most spherical LLPS at first thaw, we next used mechanical and chemical methods to test if pan-retrovirus NC-LLPS were also Zn\(^{2+}\) dependent (Figure 1K), including Zn\(^{2+}\)-oxidizing freeze/thaw cycles, and Zn\(^{2+}\) drugs TPEN, ADA, and DIBA-1. Both freeze/thaw cycles and 20 µM of TPEN disabled
NC-LLPSs (p < 0.0001 for all cases) (Figure 6E; Figure S3B). Both ADA (100 µM) and DIBA-1 (50 µM) potently disabled HIV-1 and SIV NC-LLPSs (all, p < 0.0001) (Figure 6E; Figure S3B), as supported by reports of these inhibiting multiple replication steps of HIV-1, HIV-2, and SIV (Rice et al., 1997a, 1995, 1997b). Both drugs also disabled single-ZnF-containing MuLV NC-LLPSs (~drug versus ADA, p < 0.0001; ~drug versus DIBA, p = 0.0300) (Figures 6D and 6E; Figure S3B), as supported by reports that the DIBA-2 congener inactivates HIV-1 and MuLV (Figure 6A) (Rein et al., 1996; Rice et al., 1995). DIBA-1 also potently disabled HTLV-1 NC-LLPS (p < 0.0001), and modestly disabled RSV NC-LLPS (p = 0.0294) (Figure 6E; Figure S3B), although to our knowledge, DIBA drug variants have yet to be tested on either retrovirus. Finally, to address whether the NC domain within full-length Gag also contributes to LLPS, we tested purified Gag protein for its ability to form condensates, where titration experiments demonstrated Gag-LLPS to form starting at 10 µM, but optimally at 20 µM (p < 0.0001), and where Gag-LLPSs were also observed to be sensitive to mechanical (freeze/thaw; p < 0.0001), general (TPEN; p < 0.0001), and specific (ADA and DIBA; p < 0.0001) Zn\textsuperscript{2+} ejection treatments (Figure S3C). These data demonstrate that divergent pan-retroviral NC and full-length HIV-1 Gag proteins undergo Zn\textsuperscript{2+}-dependent LLPS.

ZnF-Dependent and Zn\textsuperscript{2+}-Chelation-Sensitive NC-LLPS Are Dynamically Formed in Live Cells

NC-LLPSs are thus not specific to HIV-1 (Figures 6C and 6D), and rather represent a possible conserved pan-retrovirus replication mechanism. Based on our findings that cell homogenates induce dynamic NC-LLPSs (Figures 1C–1H), we then performed experiments to observe Zn\textsuperscript{2+}-dependent NC-LLPS in real-time. HeLa cells were transfected with WT or mutated ZnF NC-YFP for live cell imaging 4 h later, where 3–4 µm \WT NC-YFP cytoplasmic structures were already apparent in some cells at this time point (Figure 7A; Video S2). In other cells, we observed de novo formation of WT NC-YFP structures, growing from 0.5 µm to 1.5–2 µm during 9 min of video capture (Figure 7A; Video S2). NC-YFP-LLPSs demonstrated dynamics similar to in vitro cell homogenate-stimulated NC-LLPSs (Figures 1C–1H), observed to travel, interact, and fuse with each other (Figure 7A; Video S2).

To test the Zn\textsuperscript{2+} dependency of NC-YFP-LLPSs, and to test whether Zn\textsuperscript{2+} chelation was reversible, we performed live cell experiments alternating regular medium and TPEN treatment (Figure 7B). We observed that 20 µM of TPEN dissolved pre-established NC-LLPSs and NC-SGs in 6 min, and that replacement of TPEN medium with unconditioned medium restored NC-LLPSs and NC-SGs in 20 s, indicating that effects of Zn\textsuperscript{2+} chelation were rapidly reversible (Figure 7C), as supported by TPEN displacing but not depleting cellular Zn\textsuperscript{2+} (Figure 2B). Importantly, in unconditioned medium, NC-YFP localized primarily to cytoplasmic LLPSs and SGs, whereas TPEN treatment not only reduced the size and abundance of NC-LLPSs/SGs, but also led to a diffuse phenotype and nuclear localization of NC-YFP (Figure 7D), as supported by earlier experiments (Figures 2C and 2D). Conversely, the ZnF mutant NC-YFP construct (i.e., NC-C15S-C49S-YFP) mostly localized to nuclei and nucleoli, and could not form LLPSs or SGs at steady state or under any treatment tested (Figures 7E and 7F). Adjusting microscope objectives permitted us
to observe that in cells expressing higher levels of mutant ZnF NC-YFP, also localizing to the cytoplasm, LLPSs or SGs could not form at steady state or under any treatment tested (Figures 7G and 7H). Altogether, these results demonstrate that de novo-expressed NC undergoes dynamic LLPS and SG assembly in cells, and that these structures and NC localization are ZnF- and Zn$^{2+}$ dependent.

The Gag SP1/NC cleavage site is the most sensitive to PR, and mutations in NC/SP2 and SP1/NC cleavage sites are responsible for PR inhibitor (PI) resistance (Côté et al., 2001; Königű et al., 2013). Several reports provide evidence of active PR and GagPol polyprotein proteolytic products in the cytoplasm of infected cells in the absence of virus production and in high viral load patient-derived T cells (Freed et al., 1994; Hu et al., 2005; Kaplan and Swanstrom, 1991; Nie et al., 2007; Park and Morrow, 1991; Wen et al., 2016). Our observations of rapidly forming NC-LLPSs in live cell experiments suggest that basal NC cleavage by PR in cells may represent another event in HIV-1 replication. The unprocessed PR is 10,000-fold less sensitive to PIs than the targeted mature PR dimer (Pettit et al., 2004). Therefore, we overexpressed PR-GFP in cells cotransfected with pNL4–3 or pNC-RLuc, where we observed that NC and PR were extensively colocalized, and this was unaffected by the PI saquinavir (SAQ), suggesting that PR association with NC persists independently of proteolytic activity. SAQ treatment also produced PR- and NC-positive 0.5–1 µm of puncta co-staining for G3BP1, but not PB marker Dcp1 (Figures S4A and S4B). We examined HIV-1 PR protein sequence to find it devoid of predicted PrLDs, indicating that PR should not itself undergo LLPS, promoting the more likely scenario that its affinity with NC-SGs or NC-LLPSs is likely derived from its role in NC cleavage or perhaps in a mechanism protecting cells from PR-derived toxicities. Finally, to gain evidence warranting future studies of cellular NC cleavage by active PR, we treated pNL4–3 transfected cells with SAQ, and used two different Gag-specific antibodies to observe that SAQ treatment caused decreased puncta resembling viruses (Figure S4C). Supernatants were collected and production of infectivity was tested using an X-Gal staining assay in TZM-bl cells, demonstrating a complete loss of infectivity by SAQ treatment (p = 0.0001). Finally, to assess the effect of SAQ treatment on active PR in cells generating Gag-free NC, western blots of cytosolic Gag proteins were analyzed from cellular lysates extensively washed and treated with trypsin and EDTA removing bound, noninternalized virus particles (Yao et al., 1998), demonstrating that SAQ treatment efficiently blocks NC cleavage in cells, while imaging analysis demonstrates that SAQ treatment increases NC-Gag colocalization (Gag(rabbit), p = 0.0106; Gag(mouse), p = 0.0064) (Figure S4C), providing further evidence that NC is cleaved from Gag in untreated cells. These findings warrant future studies on possible roles for NC-LLPSs in the late stages of HIV-1 replication.

**DISCUSSION**

We are first to discover that both purified HIV-1 NC and Gag proteins induce LLPS and that both this phenomenon and NC-induced SGs are Zn$^{2+}$ dependent. NC-LLPS was induced in vitro with crowding agents or cytosol but also in cellulo. In live cell experiments, de novo-synthesized NC efficiently bound Zn$^{2+}$ to induce LLPSs and SGs. The rapid reestablishment of NC-SGs following TPEN washout highlights NC’s high affinity for Zn$^{2+}$. Indeed, zinc deficiency is the most prevalent micronutrient abnormality in HIV-1 infected and treated...
patients (Cárcamo et al., 2006; Koch et al., 1996a, 1996b; Visser et al., 2003; Bunupuradah et al., 2012; Jones et al., 2006; Wellinghausen et al., 2000) and it correlates with decreased CD4+ T cells, high viral load, and mortality (Baum et al., 2003; Bunupuradah et al., 2012; Cunningham-Rundles et al., 2005; Fufa et al., 2009; Graham et al., 1991; Irlam et al., 2010; Lai et al., 2001; Visser et al., 2003; Wellinghausen et al., 2000). While zinc supplementation delays disease progression (Baum et al., 2010; Cárcamo et al., 2006; Mocchegiani et al., 1999, 1995; Read et al., 2019; Zeng and Zhang, 2011), an excess also causes disease progression and mortality (Tang et al., 1993, 1996). This duality of Zn2+ homeostasis is also linked to the onset of neurodegeneration (Szewczyk, 2013). Our work highlights Zn2+ homeostasis duality, where virus-promoting NC-LLPSs require Zn2+, but an excess of NC leads to the assembly of translationally silent NC-SGs (Rao et al., 2018). Likewise, heavy metal neurotoxins cause transition of ‘‘protective’’ LLPS to pathological SGs and permanent aggregates in neurological diseases (Ash et al., 2019; Rayman et al., 2018; Trojsi et al., 2013).

Viruses have evolved a finely tuned requirement for Zn2+. We observed that pan-retroviral NC-LLPSs were sensitive to Zn2+ chelation. Indeed, Zn2+ is the most common metal binding to viral proteins and is a key cofactor for DNA and RNA viruses (Chaturvedi and Shrivastava, 2005; Lazarczyk and Favre, 2008). Aside from NC, HIV-1 Integrase (IN), Tat, and Vif also require Zn2+ for folding, varied functions, and host resistance (Frankel et al., 1988; Garber et al., 1998; Huang and Wang, 1996; Lazarczyk and Favre, 2008; Lee and Han, 1996; Lee et al., 1997; Luo et al., 2005; McEuen et al., 1992; Misumi et al., 2004; Paul et al., 2006; Xiao et al., 2007). Viruses outcompete cellular proteins for cellular Zn2+ via their highly conserved (CCHC) anisotropic ZnFs distinct from canonical eukaryotic (CCHH) ZnFs (Bess et al., 1992; Laity et al., 2001), providing the opportunity to target viral ZnFs and not host protein ZnFs (Beerheide et al., 1999; Rice et al., 1993).

We observed dramatic effects on vRNA positioning, Gag expression, and virus production by Zn2+ chelators and ejectors, highlighting the importance of Zn2+ bioavailability for retroviral NC function. As the second most abundant metal essential for >300 enzymes, metalloproteins (MTs), and Zn2+ transporters (ZNTs), Zn2+ is tightly and unevenly controlled across organs (Chaturvedi and Shrivastava, 2005; Colvin et al., 2008; Friedman et al., 1984; Lazarczyk and Favre, 2008). Indeed, MT and ZNT expression and functions are modified by numerous viruses (Ilbäck et al., 2004; Mindaye et al., 2017; Raymond et al., 2010; Read et al., 2018; Zilliox et al., 2006) and HIV-1 shifts cell and organ Zn2+ bioavailability for inflammation and apoptotic resistance (Joshi and Guidot, 2011; Raymond et al., 2010).

This work also demonstrates that Zn2+ chelation/ejection mediates the relocalization NC and the vRNA to the nucleus. NC-G3BP1 SGs were found to be enriched in Zn2+, and TPEN treatment shows that NC-SGs are Zn2+ dependent. Despite nuclear repositioning of both NC and vRNA by TPEN in the proviral context, this chemical also stunts their interaction, suggesting it may be Zn2+-dependent and nuclear in origin. Although components of the Rev-RRE-CRM1 export complex do not possess ZnFs, we could not exclude the possibility that vRNA retention by TPEN was specific for NC and therefore tested the NC-Zn2+-selective clinical compounds, ADA and DIBA-1. The presence of nuclear NC is not without...
precedence, as it possesses a nuclear localization signal (Yu et al., 2016), and is responsible for early Zn\(^{2+}\)-dependent events stimulating integration (Levin et al., 2010; Poljak et al., 2003), and mediates nucleolar retroviral Gag localization (Lochmann et al., 2013). In its apo form, NC remains disordered like FG-repeat-containing nucleoporins (Yu et al., 2016), providing yet another passport for its nucleocytoplasmic shuttling (Yamada et al., 2010).

Viral infection imposes cellular stress. To maintain a non-hostile environment hosting replication, various viruses have evolved different strategies to block SG assembly (Poblete-Durán et al., 2016), and usurping SG proteins for their own functions (Abrahamyan et al., 2010; Thomas et al., 2009). We show that NC does not cause SG assembly when HIV-1 is co-expressed, suggesting that accessory viral proteins may chelate Zn\(^{2+}\) from NC, or that vRNA binding to NC outcompetes its propensity to multimerize. We also find that HIV-1 NC ZnF mutants cannot block SG assembly, where vRNA accumulates in ARS-induced SGs. This establishes that the Zn\(^{2+}\)-dependent NC-vRNA association is required for SG blockade by HIV-1, otherwise possibly initiated by expression of this foreign vRNA.

Indeed, numerous studies on a broad range of viruses have long demonstrated that most induce phase separation for replication. Across time and virus type- or family-centric literature, widely varying terms and functions have been ascribed to classify these phase-separating viral replication compartments, including virus factories, viroplasm, or mini-organelles generating subcellular microenvironments concentrating viral components into membrane-bound or membraneless inclusions for replication and protection against virus defenses (Netherton and Wileman, 2011; Novoa et al., 2005). Cytoplasmic inclusion bodies produced later by aggregation of structural proteins or nucleocapsids are also importantly similar to pathologic aggresomes causing neuronal dysfunction (Novoa et al., 2005; Wileman, 2006). Negri body viral factories described for RABV and VSV have been re-examined for their LLPS properties (Heinrich et al., 2018; Nikolic et al., 2017). Viral proteins from divergent influenza A, hendra, measles, and herpes simplex viruses also appear to use LLPS for virus replication (Alenquer et al., 2019; McSwiggen et al., 2019; Zhou et al., 2019). In a greater perspective, the association between viral nucleoproteins and vRNA appears responsible for LLPS (Alberti et al., 2019; Kondo et al., 2013; Lifland et al., 2012; Nikolic et al., 2019; Zhou et al., 2019), and the association between ZnF and RING finger proteins lead to the assembly of virus factories, virus-like particles, and inclusion bodies (Brick et al., 1998; Fehling et al., 2012; Hanslip et al., 2006; Hoenen et al., 2012; Mathur et al., 2014; Nerenberg et al., 2005; Senkevich et al., 1995). Our work demonstrates that Gag and pan-retrovirus NC proteins induce Zn\(^{2+}\)-dependent LLPS and SGs, supporting the notion that translational silencing of vRNA ushers it into trafficking cytoplasmic RNP for virus assembly.

A fundamental principle underlying biological molecules undergoing LLPS is multivalency and the capacity to interact with multiple nucleic acids and proteins simultaneously (Li et al., 2012). This is in line with the pleiotropic, flexible, disordered retroviral NC nucleic acid chaperones performing a myriad of functions during early and late stages of viral replication (Darlix et al., 2014). Similar to NC composition and function, it is widely accepted that proteins that undergo LLPS typically contain PrLDs and can bind multivalent DNA and RNA scaffolds through ZnFs or RNA-recognition motifs (RRMs). From our investigation...
of previously mapped domains of proteins undergoing LLPS, we observed that all have juxtaposed or overlapping PrLDs and RRM and that the longest proteins, like Gag, which we also find to form LLPS, have both RNA-binding RRM and ZnFs within PrLDs (Wang et al., 2018).

Large in silico and meta-analyses show disproportionately higher PrLDs in viruses relative to eukaryotes and in retroviruses and Gag proteins relative to other viruses (Pushker et al., 2013; Tetz and Tetz, 2018). It is speculated that viral protein PrLDs facilitate multiple inter-protein interactions for LLPS viral protein condensates, providing a competitive advantage over host RNPs requiring the same machineries for replication (Tarakhovsky and Prinjha, 2018). Other speculations based on degree of outer virus shell disorder, where HIV-1 MA, CA, and NC are more disordered than their orthologs, suggest that disorder assists “shapeshifting” immune evasion strategies (Goh et al., 2019). From our discovery that HIV-1 NC undergoes Zn\(^{2+}\)-dependent LLPS, we conducted computational studies to map conserved and overlapping PrLDs and ZnFs. These analyses revealed that NC proteins from divergent retrovirus subfamilies also generate Zn\(^{2+}\)-dependent LLPS.

HIV-1 resistance to RT, PR, and integrase (IN) targeting drugs creates an urgent need for new drug strategies (Das and Arnold, 2013). From its high conservation among all viral clades and its many essential functions during replication, NC ZnF is maintained as a prime target for therapeutic intervention (Mori et al., 2015). Despite many Zn\(^{2+}\) ejectors showing strong antiviral activity against a spectrum of strains without eliciting resistance, the difficulty in identifying those whose selectivity outweighs toxicity drives their repurposing as potent microbicides and ongoing efforts to identify non-covalent NC inhibitors (Bernacchi et al., 2007; Breuer et al., 2012; de Rocquigny et al., 2008; Goebel et al., 2001; Goudreau et al., 2013; Mori et al., 2012; Musah, 2004; Pustowka et al., 2003; Raja et al., 2006; Rice et al., 1993; Shvadchak et al., 2009; Srivastava et al., 2004; Stephen et al., 2002; Turpin et al., 2008; Vercruysse et al., 2012; Wallace et al., 2009; Warui and Baranger, 2009). Our discovery of NC- and Gag-induced LLPS sheds new light on the molecular basis for selection and packaging of vRNA and expands our understanding of mechanisms governing NC activity to assist in perfecting promising NC-ZnF-targeting compounds.

**STAR METHODS**

**LEAD CONTACT AND MATERIALS AVAILABILITY**

This study did not generate new unique reagents. Further request for information on methods and reagents should be directed to Lead Contact, Andrew J. Mouland (andrew.mouland@mcgill.ca).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell culture**—Adherent HeLa cells and HeLa-derived TZM-bl cells originated from a human female cervix, while adherent HEK293T cells originated from a human female embryonic kidney. HeLa and HEK293T cells [American Type Culture Collection (ATCC)], and TZM-bl cells (NIH AIDS Reagent Program) were grown and maintained in Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO Thermo Fisher Scientific #) supplemented
with 10% fetal bovine serum (FBS) (Hyclone), 1% penicillin/streptomycin (Invitrogen) at 37°C and 5% CO₂. The CD4+/CXCR4+ Jurkat CE6.1 T cell line (ATCC) originates from peripheral blood and was grown and maintained as suspension culture in RPMI 1640 (Life Technologies) supplemented with 10% FBS (Hyclone) and 1% penicillin/streptomycin (Life Technologies) at 37°C and 5% CO₂.

METHOD DETAILS

**Recombinant DNA**—pNL4–3 was obtained from NIH AIDS Reference and Reagent Program (ARRP). pNL4–3 NC C15S/C49S has been previously described (Guo et al., 2000). Gag-mCherry was provided by Paul Bieniasz (Rockefeller University, USA) (Jouvenet et al., 2008). pRLuc-N1 (Packard BioScience/Perkin-Elmer Life Sciences) and the construction of p2-p1/Rluc and NC-p1R7-YFP and NC-p1C15–49S-YFP have been previously described (Chatel-Chaix et al., 2008, 2004). pG3BP1-GFP was provided by Imed Gallouzi (McGill University, Canada (Tourrière et al., 2001). pcDNA3.1 was purchased from Invitrogen. pRF-EGFP was provided by Rongtuan Lin (Solis et al., 2011), and pEGFP-C1 was purchased from Clonetech.

**Antibodies and fluorescent probes**—Primary antibodies used were as follows: mouse anti-p24 (IF, 1:250; WB, 1:10,000; NIH AIDS ARRP); rabbit anti-DDX6 (IF, 1:200; Bethyl Laboratories #A300–461A), rabbit anti-G3BP1 (IF, 1:1,000; WB, 1:1,000; (provided by Imed Eddine Gallouzi, McGill University, Canada (Gallouzi et al., 1998)), goat anti-TIAR (1:200; Santa Cruz Biotechnology #sc-1749), rabbit anti-Renilla Luciferase (IF, 1:200; WB, 1:1000; MBL International #PM047), sheep anti-Digoxigenin-AP, Fab fragments (IF, 1:250; Roche #11093274910), rabbit anti-phospho-eIF2α (Ser51) (WB, 1:1,000; Cell Signaling Technology #9721), rabbit anti-eIF2α (WB, 1:1,000; Cell Signaling Technology #9722), rabbit anti-cleaved caspase-3 (Asp175) (WB, 1:1,000; Cell Signaling Technology #9661), rabbit anti-beta Actin (1:5,000; Abcam #ab8227), and rabbit anti-GFP (WB for YFP, 1:5000 Novus Biologicals # NB600–308). For IF, secondary antibodies used were as follows: Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor® 488 (1:500; Invitrogen-Thermo Fisher Scientific #A-21202); Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor® 488 (1:500; Invitrogen-Thermo Fisher Scientific #A-21206); Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor® 594 (1:500; Invitrogen-Thermo Fisher Scientific #A-21207); Donkey anti-Sheep IgG (H+L) Cross-Adsorbed, Alexa Fluor® 594 (1:500; Invitrogen-Thermo Fisher Scientific #A-31573); Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor® 647 (1:500; Invitrogen-Thermo Fisher Scientific #A-31573); Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor® 647 (1:500; Invitrogen-Thermo Fisher Scientific #A-21447). For western blotting secondary antibodies used were as follows, goat anti-mouse, goat anti-rabbit, or donkey anti-goat IgG polyclonal antibodies conjugated to horseradish peroxidase (HRP) (Rockland Immunochemicals). FluoZin-3, AM, cell permeant (Thermo Fisher Scientific # F24195) was used to visualize Zn²⁺ in cells.

**Proteins**—Methods and analyses of NC protein purification and preparation have been previously described for WT and mutant NC HIV-1 (Guo et al., 2000; Wu et al., 2014a, 1996), and WT RSV, MuLV, HTLV-1 (Stewart-Maynard et al., 2008), SIV (Post et al.,
NC proteins lyophilized from acetonitrile, water and trifluoroacetic acid, and containing 1 equivalent of Zn$^{2+}$ per finger were dissolved in commercial D-PBS (Wisent), aliquoted and stored at −80°C until further use. Recombinant HIV-1 Gag protein purchased from Abcam (ab109969) was diluted in D-PBS (Wisent), as recommended by the manufacturer. Purified Gag was produced using Escherichia coli expression systems and purified using nickel-affinity columns, which maintain purified protein Zn2+ content and secondary structures permitting functional and structural studies of Zn2+ binding proteins (Colombo et al., 2013; Zhang et al., 2017).

Creation and imaging of liquid-liquid phase condensates—Formation of NC-LLPS protein samples was monitored by DIC and fluorescence microscopy. For examination of fluorescently labeled NC-LLPS, purified NC proteins were labeled using the Alexa Fluor 488 Microscale Protein Labeling Kit (Thermo Fisher Scientific, #A30006) according to manufacturer’s instructions. Freshly labeled Retroviral NC and HIV-1 Gag proteins were mixed to final concentrations of 0, 1, 5, 10, and 20 µM, using buffers containing 20 mM HEPES, 220mM NaCl, pH 7.4, and with either 150 mg/ml ficoll (Lymphocyte Separation Medium, Corning, #25–072-CV), or 10% dextran (Sigma-Aldrich #9004–54-0) added as molecular crowding agents. A technical consideration is that both labeled and non labeled NC proteins can only produce perfectly spherical and homogeneously sized LLPS after one round of freezing at 80°C, and thereafter gradually lose this ability as a result of their loss of Zn$^{2+}$ produced by freeze thaw cycles. For the induction of in vitro LLPS using cellular homogenate instead of ficoll as crowning agent, HeLa cells were grown in 12-well plates, and 9 × 10^6 cells were washed with PBS, then collected from plates using cell scrapers and spun down at 1,500 rpm for 10 mins at 4°C. Cell pellets in Eppendorf tubes were resuspended in 50 µL of PBS, and were homogenized using Eppendorf® micropestles (Sigma-Aldrich #Z317314). For LLPS experiments, 1 µL of cellular homogenate was added to formulations, for an estimated cellular contents of 15,000 cells, and thus approximately 1 × 10^{14} proteins per reaction (based on predictions of 10^1′ proteins per mammalian cell). The number of NC proteins per reaction was estimated to be 3.5 × 10^{13}. For imaging of LLPSs from purified proteins in LLPS formulations, 6 µL of sample mixtures were loaded onto 25 × 75 mm × 1mm thick glass slides (Thermo Scientific, # 640–004T, and covered with 18 mm ø No. 1 cover glasses (VWR VistaVision™, VWR International), and were sealed with clear enamel (Revlon). NC-LLPSs were observed by microscopy 10 mins later, for both green fluorescence coinciding with clearly discernible spheres using differential interference contrast. This was performed using a Leica DM16000B laser confocal microscope equipped with a WaveFX spinning disk confocal head (Quorum Technologies), HCX PL APO / 63×, Oil / 0.60–1.40 NA BL objective, 525/50 nm ex/em filter to detect Alexa Fluor 488, and a Hamamatsu EM-CCD digital camera for image capture. Scanning was performed and digitized at a resolution 1,024 × 1,024 pixel. Image processing and analyses were performed by Imaris software v. 8.1.2 (Bitplane/Andor). For imaging of dynamic LLPS created using cellular homogenate, 525/50 nm ex/em filter was used to detect Alexa Fluor 488 positive NC-LLPS spheres, and then DIC and time-lapse microscopy videos were recorded to capture their motion and surrounding cell lysate. Imaris imaging software (Bitplane/Andor) was used for measurements of LLPS particle sizes, shapes, and displacement distances.
**Cell transfection**—For adherent HeLa and HEK293T cells, 4 × 10^5 cells were seeded directly into 12-well plates (VWR) for western blotting, and 1.5 × 10^5 cells were seeded onto sterile coverslips (18 mm ø No. 1 German cover glasses, VWR VistaVision™, VWR International) deposited into 12-well plates for imaging. 0.8 × 10^5 cells were seeded per chamber of 4-chamber wells (Lab-Tek®II Chambered #1.5 German Coverglass System; ThermoFisher) for live cell imaging microscopy. Cells were transfected 24 hr later with 2 µg plasmid DNA per well using JetPrime (PolyPlus) according to the manufacturer’s instructions. pcDNA3.1 was used as control for transfections with pNL4–3, and pRLuc-N1 was used as control for experiments using p2-p1/RLuc (i.e., NC-RLuc). Jurkat T cells were transfected with 3 µg of plasmid DNA per 1 × 10^6 cells using JetPrime (PolyPlus) for 12 days prior to treatments or collection.

**Reagents used for treatment and Zn^{2+} chelation and detection**—Cells were treated with 500 µM sodium arsenite (ARS) (NaAsO₂; Sigma-Aldrich # S7400) for 1 hr prior to collection or fixing onto cover glasses. Cells were treated with indicated concentrations of TPEN (N,N',N'-Tetakis(2-pyridylmethyl)ethylidenediamine) (10, 20, or 40 µM) (Sigma-Aldrich # P4413), azodicarbonamide (ADA; 50µM, Sigma-Aldrich #A96606) or 2,2'-dithiobisbenzamide-1 (DIBA-1; 100µM, Sigma-Aldrich #PZ0634) for 1 hr prior to cell harvest. For visualization of Zn^{2+}, cells were loaded with 1 µM FluoZin-3 AM (Thermo Fisher Scientific) and 0.02% Pluronic F-127 (Thermo Fisher Scientific # P3000MP) for 40 min, washed, and given fresh media. NC-LLPSs were treated with 500 µM ARS, 20 µM TPEN, or 3.5% 1,6-hexanediol (HEX) (Sigma-Aldrich #240117). The HIV-1 PR inhibitor saquinavir (SAQ) was obtained from the Division of AIDS, NIH through the NIH AIDS Research Reference and Reagent Program, and was used from the time of transfection to the time of collection.

**Fluorescence in situ hybridization, immunofluorescence**—FISH/IF co-analyses on adherent and suspension cells were performed exactly as described previously (Monette et al., 2009; Vyboh et al., 2012). Briefly, for suspension cells, sterile 18 mm ø No. 1 cover glasses (VWR) were treated with 0.1% poly-L-lysine solution (Sigma) overnight at 4°C. Cover glasses were dropped into wells, and cells were allowed to settle onto these for 1 h at 37°C prior to fixing cells onto cover glasses. For fixing or cells onto cover glasses, cells were washed once in D-PBS (Wisent) and fixed with 4% paraformaldehyde for 20 min. Fixed cells were then washed with D-PBS, quenched in 0.1 M glycine for 10 min, washed with D-PBS, permeabilized in 0.2% Triton X-100 for 5 min and washed twice with D-PBS. A digoxigenin-labeled RNA probe was synthesized in vitro in presence of digoxigenin-labeled UTP (Roche). To stain the vRNA, cells were DNase (Invitrogen) treated for 15 min (25 U per coverslip), then incubated in hybridization solution for 16–18 h at 42°C (50% formamide, 1 mg/ml tRNA, 2 × SSPE, 5 × Denharts, 5 U RNaseOut (Invitrogen), 50 ng probe). Cells were then incubated in 50% formamide for 15 min at 42°C and incubated twice in 2 × SSPE for 5 min each at 42°C. Cells were briefly washed in PBS before being blocked in 1 × blocking solution (Roche). Primary antibodies were applied for 1 h at 37°C, and then washed for 10 min in PBS followed by secondary antibodies for 1 h. Cells were washed for 20 min in PBS before being mounted on glass slides using ProLong Gold.
Antifade Reagent with DAPI (Life Technologies). Negative isotype-matched antibodies were used to control staining specificity.

**Microscopy and imaging analyses**—Laser confocal microscopy was performed using a Leica DM16000B microscope equipped with a WaveFX spinning disk confocal head (Quorum Technologies) and HCX PL APO / 40 ×, Oil / 0.75–1.25 NA CS and HCX PL APO / 63 ×, Oil / 0.60–1.40 NA BL objectives, and images were acquired with a Hamamatsu EM-charge coupled device digital camera. Scanning was performed and digitized at a resolution 1,024 × 1,024 pixel. For multi-color image capture, AlexaFluor-647, −594, 488, conjugated secondary antibody emissions were sequentially captured with 665–715, 570–620, and 500–550, bandpass filters, followed by 435–485 nm [for 4′,6-diamidino-2-phenylindole (DAPI) staining], and then DIC image capture. For live cell experiments, imaging capture experiments of NC-YFP transfected cells began 4 hr post transfection. Multiwell chambered cell culture dishes (LabTek) were transferred to a closed stage-top incubator, preheated to 37°C and infused with 5% CO₂. Transfected cells were located, and still images of YFP (YFP ET540/30 m filter) and DIC were captured. To avoid bleaching YFP signal during video recording, signal intensity was first observed to set live cell imaging capture to low laser power (i.e., 47%) and exposure time (i.e., 561 ms), and boosted sensitivity (i.e., 255). Videos were captured using a heated 63 × NA 1.40 objective. Images were recorded from laser-scanned cell layers with a thickness of 1 µm and were digitized at a resolution of 1024 × 1024 pixels. Raw .liff files were exported by the Volocity software (Perkin Elmer) for import into Imaris and ImarisColoc software v. 8.1.2 (Bitplane/Andor) used for generation of new colocalization channels, and .csv exports of quantitative measurements of mean signal intensity values used for downstream data harmonizing and statistical analyses using Excel (Microsoft) and GraphPad v6.1 (Prism), respectively. The steady state localization of NC-YFP was first recorded for 9 minutes. For TPEN experiments, we first, focused on live cells having established NC-YFP structures, and took still capture DIC images of starting cell populations, then rapidly changed unconditioned medium for medium containing 20 µM TPEN, and begun recording live cell videos to observe NC-YFP behavior for 360 s. TPEN containing medium was then replaced with unconditioned medium, to again record NC-YFP behavior for another 360 s. Unconditioned medium was again replaced with medium containing 20 µM TPEN, and NC-YFP behavior was recorded again for 360 s. Finally, DIC image were recorded. Videos were exported as .avi files from Volocity software (Perkin Elmer).

**Western blot analysis**—Cells were collected at indicated times post transfection. Cells were first washed with D-PBS, then lysed in ice-cold lysis buffer (100 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 0.5% Nonidet P-40, protease and phosphatase inhibitor cocktail [Roche]). For cell free virus analyses, cells were washed twice with ice-cold D-PBS, treated with 0.1% trypsin–0.02% EDTA 2 × for 5 min at 37°C, and washed twice with D-PBS prior lysis buffer (Yao et al., 1998). Cell lysates were quantified using the Bradford assay (Bio-Rad), used according to manufacturer instructions, and 20 µg of total cell lysates were denatured in Laemmlie sample buffer and incubated for 5 min at 95°C prior to loading into SDS-PAGE protein separating gels. Gels were transferred onto polyvinylidene difluoride membranes (company). Membranes were blocked with 5% non-fat
milk in Tris-buffered saline and 0.5% Tween 20 (TBST) prior to incubation with primary antibodies listed. Membranes were washed 3 times with TBST, and then probed with secondary HRP-conjugated secondary antibodies listed. Proteins of interest were detected using the Western Lightning Plus-ECL reagent kit (Perkin-Elmer). Signal intensity was quantified by ImageJ (NIH).

**Virus quantification from supernatants**—Culture supernatants from HeLa, HEK293T and Jurkat T cells were harvested and passed through a 0.2 µm filter (VWR) to remove cellular debris and centrifuged at 3,000 rpm for 20 mins. HIV-1 virus-p24 antigen levels in culture supernatants were assessed using the 96-well plate format HIV-1 p24 Antigen Capture Assay ELISA kit (Advanced Biosciences Laboratories, #5421).

**Infectivity assay**—Viral titer in cell supernatants was quantified using the X-gal staining assay in TZM-bl cells as described previously (Rao et al., 2019; Xing et al., 2016), where TZM-bl cells seeded onto 96-well plates were subjected to dilutions of supernatants from each condition for 48 hours, and were then fixed using 1% paraformaldehyde, washed, and treated with X-Gal for the detection of β-galactosidase.

**Informatics**—A Predictor of prion-like domains (PLAAC; [http://plaac.wi.mit.edu/](http://plaac.wi.mit.edu/)), a predictor of Natural Disordered Regions (PONDR; [http://www.pondr.com/](http://www.pondr.com/)), and a protein disorder database (MobiDB; [http://mobidb.bio.unipd.it/](http://mobidb.bio.unipd.it/)) were used to identify and validate the positions of PrLDs cellular proteins, HIV-1 PR protein, and Gag proteins from various retroviruses, and to examine their distributions relative to NC ZnF positioning. For PLAAC, the Gag FASTA sequences were obtained from the NCBI protein sequence database and applied to the software using default parameters. From software outputs, predicted PrLDs having > 0.8 score were mapped out. For PONDR, the Gag FASTA sequences were obtained the same way as PLAAC, however the name of the retrovirus was manually typed out in the ‘Protein name’ section. In the ‘Predictor’ section, VLXT and VSL2 were selected, but the ‘Output options’ remained untouched. For a more stringent cut-off, only the regions that peaked above 0.8 on the PONDR Score were considered during the mapping process. Several HIV-1 sequences from different strains were analyzed using both PLAAC and PONDR. These predictions were subsequently validated using MobiDB. The full name of the retrovirus in addition to the key word ‘Gag’ was typed into the search engine. The consensus PrLDs in the overview tab were used to validate the PrLD maps that were scaled and finalized using Illustrator. Finally, the ZnF placements were determined using the NCBI protein sequence database and scaled for each Retrovirus. For Spumaviruses lacking traditional ZnF motif, predefined RGG/RG motifs were mapped instead using a protein sequence analysis tool perry.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All experiments were performed in triplicate with similar results, unless otherwise indicated in figure legends as n = #. Statistical analyses for each figure panel are presented below. Three independent observers validated phenotypes resulting from all experimental conditions tested. Cellular imaging statistics reported for MFI and calculated SG-positive cell numbers are from observation of average of n = 120 cells per condition tested. SGs were
identified as G3BP1+ foci, and cells were scored as SG-positive when they had at least three G3BP1+ foci ranging in size from 200 to 300 nm in diameter (Jain et al., 2016; Thomas et al., 2009; Wheeler et al., 2016), as measured using Imaris software. LLPS imaging statistics result from counting an average of \( n = 2,000 \) LLPS per condition tested, or 10 \( 63\times \) frames where LLPSs were diminished or absent as a result of applied treatments. LLPS dynamics statistics were calculated from the analysis of an average of \( n = 20 \) LLPS per condition tested. Statistical analyses were performed using Prism V6.01, GraphPad, where one-way ANOVA (with Tukey’s post-test) and 95% CI was used for multiple comparisons, and an unpaired 2-tailed Student’s t test with 95% CI was used to compare two groups. Data are presented as mean ± SEM or SD, as indicate in figure legends, and \( P \) values of less than 0.05 were considered to indicate a statistically significant difference.

Figure 1A, boxplot: \( p < 0.0001, \) 95% CI −250.7 to −161.7

Figure 1B, boxplot: NC -ficoll versus NC -ficoll+cell lysate: \( p < 0.0001, \) 95% CI −399.0 to −260.5 Figure 1J, boxplot: NC WT versus NC ZnF mutant, \( p < 0.0001, \) 95% CI 141.3 to 264.7

Figure 1K, boxplot: all \( p < 0.0001, \) no drug versus TPEN, 95% CI 178.6 to 253.2; no drug versus HEX, 95% CI 122.3 to 196.8; no drug versus ARS, 95% CI 53.6 to 117.1. ****, \( p < 0.0001, \) 1-way ANOVA, Tukey post-test.

Figure 2A: boxplots (right) \( (p < 0.0001; \) no drug versus 40 \( \mu M \) TPEN; 95% CI 445.3 to 1271.0); boxplots (bottom right) \( (p < 0.0001; \) no drug versus 40 \( \mu M \) TPEN; 95% CI 4.586 to 12.14).

Figure 2B: drug versus 40 \( \mu M \) TPEN, \( p = 0.0045, \) 95% CI −612.2 to −73.33, 1-way ANOVA, Tukey post-test)

Figure 2D: boxplot \( (p = 0.0002, \) 95% CI 218.8 to 785.6).

**, \( p < 0.01; \) ***, \( p < 0.001; \) ****, \( p < 0.0001; \) 1-way ANOVA, Tukey post-test.

Figure 3A: G3BP1 signal intensity \( (\) ARS + versus ARS+TPEN +; \( p < 0.0001, \) 95% CI −737.8 to −380.7, 1-way ANOVA, Tukey post-test), and vRNA-G3BP1 colocalization \( (\) ARS + versus ARS + TPEN +; \( p < 0.0001, \) 95% CI −385.8 to −170.9).

Figure 3B: TPEN increases G3BP1 signal intensity \( (\) ARS+ versus ARS+TPEN[40 \( \mu M \]) +; \( p < 0.0001, \) 95% CI −2624.0 to −827.3); vRNA-G3BP1 colocalization \( (\) ARS+ versus ARS+TPEN[40 \( \mu M \])+; \( p < 0.0001, \) 95% CI −1219.0 to −494.1); \( (\) ARS+ versus ARS+TPEN[40 \( \mu M \])++; \( p = 0.0261, \) 95% CI −911.4305 to −38.66125). *, \( p < 0.05; \) **, \( p < 0.01; \) ***, \( p < 0.001; \) ****, \( p < 0.0001; \) 1-way ANOVA, Tukey post-test.

Figure 4A: Gag expression induced by TPEN treatment \( (\) pNL4–3 versus pNL4–3+TPEN; \( p < 0.0001, \) 95% CI −143.1 to −47.97). Data are mean ± SEM.

Figure 4B: Bar graph pNL4–3 versus pNL4–3+TPEN; \( p < 0.0001, \) 95% CI 33.61 to 76.79; pNL4–3 versus pNL4–3+HEX, \( p < 0.0001, \) 95% CI 33.63 to 81.98; pNL4–3 versus pNL4–
3+ARS, p < 0.0001, 95% CI 48.20 to 91.55; pNL4–3/pRLuc versus pNL4–3/pNC-RLuc; p < 0.0001, 95% CI 42.90 to 86.24). Data are mean ± SD. *, p < 0.05; ***, p < 0.001; ****, p < 0.0001; 1-way ANOVA, Tukey post-test.

Figure 5B: Bar graph NC ZnF mutant cannot block SG assembly relative to WT pNL4–3 (p < 0.0001, 95% CI −81.97 to −54.97); bar graph (+40 µM TPEN; p < 0.0001, 95% CI −49.91 to −20.33) Data are mean ± SD.

Figure 5D: boxplots nuclear retention of the vRNA (-drug versus +ADA, p < 0.0001, 95% CI −510.0 to −262.3; -drug versus DIBA, p < 0.0001, 95% CI −430.4 to −260.8; -drug versus TPEN, p < 0.0001, 95% CI −354.3 to −116.8; pNL4–3 versus pNL4–3 NC-C155-C49S, p < 0.0001, 95% CI −523.1 to −247.1). *, p < 0.05; ***, p < 0.001; ****, p < 0.0001; 1-way ANOVA, Tukey post-test.

Figure 6E: boxplots (right) (HIV-1, -drug versus ADA, p < 0.0001, 95% CI 169.8 to 217.8; HIV-1, -drug versus DIBA, p < 0.0001, 95% CI 105.6 to 159.7; SIV, -drug versus ADA, p < 0.0001, 95% CI 89.49 to 141.5; MuLV, -drug versus ADA, p < 0.0001, 95% CI 93.35 to 147.1; MuLV -drug versus DIBA, p = 0.0300, 95% CI 2.012 to 55.77; HTLV-1, -drug versus DIBA, p < 0.0001, 95% CI 97.48 to 179.1; RSV, -drug versus DIBA, p = 0.0294, 95% CI 3.053 to 82.06). *, p < 0.05; ****, p < 0.0001; 1-way ANOVA, Tukey post-test.

DATA AND CODE AVAILABILITY

This study did not generate any unique datasets or code. The retroviral Gag and other protein sequences examined for PrLDs and ZnFs were obtained through the NCBI Reference Sequence Database (RefSeq) and GenBank: HIV-1: AAD39400.1; SIV: AEK79593.1; FIV: CAA48157.1; EIAV: ACT31322.1; HTLV-1: AAB20767.1; RSV: CAA48534.1; MuLV, RefSeq: NP_040332; HTLV-2: CAA73488.1; BLV: AAA42784.1; MVV: AAB25459.1; HIV-2: AAA76840.1; ALV: AJG42160.1; SRV: BAD89356.1; MMTV: AAF31472.1; FFV: AGC11912.1; SFVagm = RefSeq: YP_001956721.2; SFVcpz: AAA19977.1; HFV: GenBank: CAA68998.1; FeLV: AAA43054.1; GALV: ALV83305.1; PERV: CAA76581.1; BFLV: AKW77106.1; SnRV = RefSeq: NP_043925.1; WDSV, RefSeq: NP_045938.1; HERV-W = AAF74213.1; HERV-K: CAA69289.1; JSRV: CAA01899.1; TIA1, GenBank: EAW99824.1; hnRNP A2B1, RefSeq: XP_005249786.1; hnRNPA0, RefSeq: NP_006796.1; hnRNPA1L2, RefSeq: NP_001011725.1; DAZAP1, RefSeq: NP_061832.2; RNAPolIII, GenBank: AAT12524.1; HEWL, PDB: 1LSZ_A; BPT1, RefSeq: NP_001001554.2; CALM1, GenBank: AAD45181.1; UB, GenBank: AA36789.1; REV, GenBank: AYF56141.1; CRM1, GenBank: CAA69905.2; DDX1, RefSeq: NP_004930.1; DDX11, RefSeq: NP_085911.2; DDX17, GenBank: CAG30318.1; DDX18, GenBank: CAG33341.1; DDX21, RefSeq: NP_004719.2; DDX24, RefSeq: NP_065147.1; DDX3, GenBank: AAC34298.1; DDX4, RefSeq: NP_077726.1; DDX5, RefSeq: NP_001307524.1; DDX36, GenBank: AAH36305.1; DHX9, RefSeq: NP_001348.2; Eif5A, GenBank: AAH80196.1; PIMT, GenBank: AAH07501.1; RanGTP, GenBank: CAG29343.1; RHA, GenBank: AML93444.1; RIP, GenBank: AAH46349.1; Sam68, RefSeq: NP_006550.1.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank members of the late Mark Wainberg’s lab for p24 ELISA support; Donald Johnson and Cathy Hixson for producing recombinant NC; Imed Gallouzi, Colin Crist, Solene Jamet, Henry Yu, Stéphane Richard, and Rongtuan Lin for reagents and advice; and Chia-Hao Chang and Christian Young for microscopy assistance. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl cells (Cat#8129) from Dr. John C. Kappes and Dr. Xiaoyun Wu. M.N. was supported by generous funding from the Lady Davis Institute at the Jewish General Hospital (Montreal, Canada). This work was supported by operating grants FRN-56974 and FRN-162447 from the Canadian Institutes of Health Research (to A.J.M.) and by U.S. Federal funds from the NCI, NIH, directly and under contract no. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S.A. Government. The funders had no role in study design, data collection and interpretation, or the decision to submit for publication.

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Cell Rep. Author manuscript; available in PMC 2022 March 30.
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Highlights

• Retroviral Gag proteins have conserved intrinsically disordered prion-like domains
• Pan-retrovirus family nucleocapsid proteins induce liquid-liquid phase separation
• Nucleocapsid protein phase separation and stress granule assembly is Zn$^{2+}$ dependent
• Zn$^{2+}$ chelators and ejectors induce nuclear repositioning of the genomic RNA

Cell Rep. Author manuscript; available in PMC 2022 March 30.
Figure 1. Zn$^{2+}$-Dependent HIV-1 NC Phase Separation Is Dynamically Influenced by the Cellular Microenvironment

(A) LLPS titration of NC protein (green) mixed with phase separation buffer and Ficoll, visualized by laser microscopy and DIC, with corresponding boxplots (n = 3).

(B) Ficoll-substituted cell homogenates inducing NC-LLPSs (green), with corresponding boxplots (n = 3).

(C) NC-LLPSs (cyan arrows) versus cellular RNP aggregates (black arrows) from cell homogenate experiments (n = 3).

(D) Dynamic (strong cyan), interacting (blue), and fusing (yellow) behaviors of cell homogenate-induced NC-LLPS (n = 3). (C and D) Colored circles define examples of LLPS behaviors.

(E–G) Traced distances traveled by (E) dynamic (strong cyan), (F) interacting (blue, purple), and (G) (red, green) fusing (yellow) NC-LLPS (n = 3); x-y distance (µm), linear distance traveled; total distance (µm), sum total distances traveled.
(H) Boxplots of NC-LLPS travel rates colored as in (E) to (G) \( n = 3 \).

(I) Co-condensation of NC-LLPS and mCherry-Gag from cell homogenate \( n = 3 \).

(J) Effect of linker or ZnF mutant NC proteins on NC-LLPSs, with corresponding boxplots (right) \( n = 3 \).

(K) Effect of drugs on NC-LLPSs, with supporting boxplots (right) \( n = 3 \).

Statistical analysis details are described in STAR Methods. Boxplot horizontal lines indicate median, and whiskers are minimum to maximum.

NC, nucleocapsid; NC-AF488, AlexaFluor 488-labeled NC; DIC, differential interference contrast; tot, total; t, time; s, second; avg., average; ns, non-significant.
Figure 2. HIV-1 NC-SGs and NC-vRNA-Gag RNPs Are Disrupted by Zn\textsuperscript{2+} Chelation

(A) HeLa cells transfected with pNC-RLuc (p2-p1/Rluc) (red) and treated with TPEN (0–40 µM), reversing NC-mediated G3BP1\textsuperscript{+} SGs (cyan), with corresponding boxplots (right) of decreased NC-RLuc-G3BP1 colocalization by TPEN (top right) (n = 3), and decreased NC-induced SGs by TPEN (bottom right) (n = 3). White dashed cell outlines indicate cells that do not express NC-RLuc. Yellow dashed boxes and corresponding close-up images (right) demonstrate nuclear NC-RLuc via NC-RLuc-DAPI colocalization.

(B) HeLa cells transfected with pNC-RLuc (red) and treated with TPEN (40 µM), with effects on Zn\textsuperscript{2+} distribution (green, FluoZin-3) and reversal of NC-mediated G3BP1\textsuperscript{+} SGs (cyan) (n = 3).

(C) HeLa cells co-transfected with pNC-RLuc and proviral construct pNL4–3, and treated with TPEN (20 µM), for effects on NC (green), Gag (red), and DDX6 (cyan) localization, with corresponding boxplots (bottom) (n = 4).
(D) HeLa cells co-transfected with pNC-RLuc and pNL4–3, and treated with TPEN (20 µM), for effects on NC (green), Gag (cyan), and vRNA (red) localization, with corresponding boxplots (bottom) showing decreased NC-vRNA colocalization by TPEN (n = 4). Statistical analysis details described in STAR Methods. Boxplot horizontal lines indicate median, and whiskers are minimum to maximum. µm, micron; RLuc, Renilla Luciferase; ∩, colocalization (intersection); MFI, mean fluorescence intensity; ns, non-significant.
Figure 3. Zn\textsuperscript{2+} Chelation Reverses the HIV-1 SG-Blockade and Induces vRNA Localization to SGs

(A) Infected Jurkat T cells treated with ARS (500 µM) and/or TPEN (20 µM) for effects on SG assembly and localization of G3BP1 (cyan), vRNA (red), and Gag (green), with corresponding boxplots (bottom), showing TPEN increases G3BP1 signal intensity (ARS + versus ARS+TPEN+), and vRNA-G3BP1 colocalization (ARS + versus ARS+TPEN+) (n = 3).

(B) HeLa cells transfected with pNL4–3 and treated with ARS (500 µM) and/or TPEN (20 or 40 µM) for effects on SG assembly and localization of G3BP1 (cyan), vRNA (red), and Gag (green), with corresponding boxplots (bottom), showing TPEN increases G3BP1 signal intensity (ARS+ versus ARS+TPEN, 40 µM), vRNA-G3BP1 colocalization (ARS + versus ARS+TPEN, 40 µM), but that TPEN in ARS-treated cells has a lesser effect on G3BP1-Gag colocalization (ARS + versus ARS+TPEN, 40 µM) or Gag-vRNA colocalization (ns, non-significant) (n = 4). Statistical analysis details described in STAR Methods. Boxplot
horizontal lines indicate median, and whiskers are minimum to maximum. RLuc, Renilla Luciferase; ∩, colocalization (intersection); MFI, mean fluorescence intensity.
Figure 4. Zn$^{2+}$ Chelation Leads to Cellular Retention of HIV-1 Gag and Decreased Virus Production

(A) Bar graph from western blots (Figure S2) of effects of drugs on Gag expression, showing increased Gag by TPEN treatment (pNL4–3 versus pNL4–3+TPEN) (n = 3).

(B) Bar graph of effects of drugs on virus production or on pNC-RLuc expression (pNL4–3 versus pNL4–3+TPEN; pNL4–3 versus pNL4–3+HEX; pNL4–3 versus pNL4–3+ARS; pNL4–3/pRLuc versus pNL4–3/pNC-RLuc) (n = 4); avg, average. Statistical analysis details described in STAR Methods.
Figure 5. HIV-1 NC ZnF Mutants Reverse the HIV-1 SG-Blockade and Induce vRNA Localization to SGs

(A) HeLa cells transfected with pNL4–3 NC ZnF mutant (NC-C15S-C39S) and treated with ARS (500 µM) and/or TPEN (40 µM) for effects on SG assembly and localization of G3BP1 (cyan), vRNA (red), and Gag (green), where NC ZnF mutant cannot block SG assembly and restricts the vRNA to SGs and nuclei (n = 4).

(B) Bar graph comparing SG numbers in HeLa cells across conditions tested, including data presented in Figure 3B; demonstrating that the pNL4–3 NC ZnF mutant cannot block SG assembly relative to WT pNL4–3 transfected cells, which is unaltered by TPEN (40 µM) (n = 4).

(C) HeLa cells transfected with WT pNL4–3 and treated with ADA (100 µM) or DIBA-1 (50 µM) for their effects on localization of G3BP1 (cyan), vRNA (red), and Gag (green), where both drugs cause the nuclear retention of the vRNA (n = 3).
(D) Boxplots of vRNA-DAPI colocalization comparing effects of ADA or DIBA-1 with other conditions tested; demonstrating that all Zn$^{2+}$ drugs cause nuclear retention of the vRNA (−drug versus +ADA; −drug versus DIBA; −drug versus TPEN; pNL4–3 versus pNL4–3 NC-C15S-C49S) (n = 3). Statistical analysis details described in STAR Methods. Boxplot horizontal lines indicate median, and whiskers are minimum to maximum; ∩, colocalization (intersection); MFI, mean fluorescence intensity.
Figure 6. Pan-Retrovirus NC Proteins Condense into Zinc-Dependent LLPSs
(A) Predicted Gag PrLDs (blue), mapped ZnFs (orange), and RRMs (yellow).
(B) Phylogeny of retrovirus subfamily members, NC-LLPS tested, colored; modified from Linial (1999) and Weiss (2006).
(C) Boxplots of pan-retrovirus NC-LLPSs (see also Figure S3B) (n = 4).
(D) Sequences, lengths, and ZnF-Zn$^{2+}$ positions (blue) of NCs tested for LLPS.
(E) Pan-retrovirus NC-LLPSs (left), with boxplots (right) of their disruption by Zn$^{2+}$ depletion treatments, with all highly sensitive to freeze/thaw or TPEN treatment (all p < 0.0001), but some differently affected by ADA and DIBA-1 (HIV-1, −drug versus ADA; HIV-1, −drug versus DIBA; SIV, −drug versus ADA; SIV, −drug versus DIBA; MuLV, −drug versus ADA; MuLV −drug versus DIBA; HTLV-1, −drug versus DIBA; RSV, −drug versus DIBA) (n = 3). Statistical analysis details described in STAR Methods. Boxplot horizontal lines indicate median, and whiskers are minimum to maximum.
are defined in the Key Resources Table; PrLDs, prion-like domains; ZnFs, zinc fingers; RRMss, RNA-recognition motifs; aa, amino acid; DIC, differential interference contrast; ns, non-significant.
Figure 7. ZnF-Dependent Dynamically Formed Cellular NC-LLPSs Are Zn$^{2+}$-Chelation Sensitive

(A) Still images of NC-YFP experiment videos (see Videos S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, and S12) of HeLa cells transfected with WT NC-YFP, with established NC-SGs (top), and de novo NC-LLPS assemblies (white arrows, bottom) (n = 3).

(B) Methodology of NC-YFP TPEN treatments.

(C) Video of HeLa cells transfected with WT NC-YFP, where TPEN treatment disrupts NC-SGs and is reversible (n = 3).

(D) Locations of TPEN-sensitive NC-SGs (white arrows), and DIC images demonstrating morphology is unaffected by treatments.

(E) As in (C), for nuclear phenotype of ZnF-mutated NC-YFP unaffected by TPEN treatment (n = 3).

(F) As in (D), where TPEN has no effect on ZnF mutant NC-YFP or morphology.
(G) As in (C) and (E), for cytoplasmic phenotype of ZnF-mutated NC-YFP unaffected by TPEN treatment (n = 3).
(H) As in (D) and (F), where TPEN has no effect on ZnF mutant NC-YFP or morphology.
WT, wild-type; MUT, mutant; YFP, yellow fluorescent protein; t, time; s, second.
## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-HIV-1 p24 Monoclonal | NIH AIDS Reagent Program | Cat#3537 |
| Anti-HIV-1 SF2 p24 Polyclonal | NIH AIDS Reagent Program | Cat#4250 |
| Goat antiserum against NCp7 | Robert Gorelick, Institute National Cancer | N/A |
| Rabbit anti-DDX6 | Bethyl Laboratories | Cat#A300–461A, RRID:AB_2277216 |
| Rabbit anti-G3BP1 | Imed Gallouzi, McGill University | Gallouzi et al., 1998 |
| Goat anti-G3BP1 | Santa Cruz Biotechnology | Cat#sc-70283, RRID:AB_2107230 |
| Goat anti-TIAR | Santa Cruz Biotechnology | Cat#sc-1749, RRID:AB_6352508 |
| Rabbit anti-Renilla Luciferase | MBL International | Cat#PM047, RRID:AB_1520866 |
| Sheep anti-Digoxigenin-AP, Fab fragments | Sigma | Cat#11093274910, RRID:AB_514497 |
| rabbit anti-phospho-eIF2α | Cell Signaling Technology | Cat#9721, RRID:AB_330951 |
| rabbit anti-eIF2α | Cell Signaling Technology | Cat#9722, RRID:AB_230924 |
| rabbit anti-cleaved caspase-3 | Cell Signaling Technology | Cat#9661, RRID:AB_2341188 |
| rabbit anti-beta Actin | Abcam | Cat#ab2277, RRID:AB_2305186 |
| rabbit anti-GFP | Novus Biologicals | Cat#NB600–308 |
| Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor® 488 | Invitrogen | Cat#A-21202, RRID:AB_10003058 |
| Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor® 488 | Invitrogen | Cat#A-21206, RRID:AB_2535792 |
| Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor® 594 | Invitrogen | Cat#A-21207, RRID:AB_141637 |
| Donkey anti-Sheep IgG (H+L) Cross-Adsorbed, Alexa Fluor® 594 | Invitrogen | Cat#A-31573, RRID:AB_2716768 |
| Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor® 647 | Invitrogen | Cat#A-31573, RRID:AB_2536183 |
| Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor® 647 | Invitrogen | Cat#A-21447, RRID:AB_141844 |
| goat anti-mouse IgG (H+L) Polyclonal Antibody (HRP (Horseradish Peroxidase)) | Rockland Immunochemicals | Cat#ROCK610–1319, RRID:AB_219659 |
| goat anti-rabbit IgG (H+L) Polyclonal Antibody (HRP (Horseradish Peroxidase)) | Rockland Immunochemicals | Cat#ROCK611–1322, RRID:AB_219723 |
| donkey anti-goat IgG (H+L) Polyclonal Antibody (HRP (Horseradish Peroxidase)) | Rockland Immunochemicals | Cat#ROCK711–703, RRID:AB_840935 |

### Chemicals, Peptides, and Recombinant Proteins

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HIV-1/NL4–3 (NCp7-WT) | Robert Gorelick, National Cancer Institute | Wu et al., 1996 |
| HIV-1/NL4–3 (NCp7-SSHS/SSHS mutnt) | Robert Gorelick, National Cancer Institute | Guo et al., 2000 |
| HIV-1/NL4–3 (NCp7-linker mutant) | Robert Gorelick, National Cancer Institute | Wu et al., 2014a |
| EIAV (NCp11-WT) | Robert Gorelick, National Cancer Institute | Stewart-Maynard et al., 2008 |
| FIV (NCp10-WT) | Robert Gorelick, National Cancer Institute | Wu et al., 2014b |
| HTLV-1 (NCp15-WT) | Robert Gorelick, National Cancer Institute | Stewart-Maynard et al., 2008 |
| MuLV (NCp10-WT) | Robert Gorelick, National Cancer Institute | Stewart-Maynard et al., 2008 |
| RSV (NCp12-WT) | Robert Gorelick, National Cancer Institute | Stewart-Maynard et al., 2008 |
| SIV/Mne (NCp8-WT) | Robert Gorelick, National Cancer Institute | Post et al., 2016 |
| HIV-1 Gag protein | Abcam | Cat#ab109969 |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| FluoZin-3, AM, cell permeant | Thermo Fisher Scientific | Cat#F24195 |
| Alexa Fluor 488 Microscale Protein Labeling Kit | Thermo Fisher Scientific | Cat#A30006 |
| DMEM | GIBCO / Thermo Fisher Scientific | Cat#11965–118 |
| RPMI 1640 | Invitrogen | Cat#11875–119 |
| FBS | Wisent | Cat#080–150 |
| Penicillin-streptomycin | Wisent | Cat#450–201-EL |
| HEPES | VWR | Cat#CA-EM5320 |
| Ficoll (Lymphocyte Separation Medium) | Corning | Cat#25–072-CV |
| Dextran | Sigma | Cat#1179741 |
| JetPrime (PolyPlus) | VWR | Cat#CA89129–924 |
| Sodium Arsenite (NaAsO2) | Sigma | Cat#S7400 |
| TPEN (N,N,N′,N′-Tetakis(2-pyridylmethyl)ethylenediamine) | Sigma | Cat#P4413 |
| 1,6-hexanediol | Sigma | Cat#240117 |
| Saquinavir | NIH AIDS Reagent Program | Cat#4658 |
| Trypsin/EDTA | Thermo Fisher Scientific | Cat#25200056 |
| Pluronic F-127 | Thermo Fisher Scientific | Cat#P3000MP |
| Poly-L-lysine solution 0.1% (w/v) in H2O | Sigma | Cat#P98920 |
| D-PBS, 1X | Wisent | Cat#311–425-CL |
| Formamide | VWR | Cat#CAFX0420–8 |
| Sodium Chloride | EMD Chemicals | Cat#Sx0420–5 |
| Tris | Bioshop Canada | Cat#TR001.5 |
| EDTA-free Protease inhibitor tablets | Roche | Cat#11873580001 |
| Paraformaldehyde | Polysciences, Inc. | Cat#18814 |
| tRNA | Invitrogen | Cat#15401–021 |
| Triton-X | OmniPur, EMD Millipore | Cat#9400 |
| Denhardt’s Solution (50X) | Thermo Fisher Scientific | Cat#750018 |
| DNasel | Invitrogen | Cat#18047–019 |
| RNase OUT | Invitrogen | Cat#10770019 |
| Fluorescent Antibody Enhancer Set for DIG Detection #4 (blocking solution) | Roche | Cat#1768506 |
| ProLong Gold Antifade Mountant with DAPI | Invitrogen / Thermo Fisher Scientific | Cat#P36931 |
| DIG RNA Labeling Kit | Sigma | Cat#11277073910 |
| Transcription T7 RNA Polymerase | Invitrogen | Cat#18033–019 |

**Critical Commercial Assays**

| Pierce ECL Plus western blotting Substrate | Thermo Fisher Scientific | Cat#32132 |
| Bradford protein assay | BioRad | Cat#500–0006 |
| HIV-1 p24 Antigen Capture Assay ELISA kit | Advanced Biosciences Laboratories | Cat#5421 |

**Deposited Data**

| HIV-1 | NCBI protein sequence database | GenBank: AD39400.1 |
| SIV | NCBI protein sequence database | GenBank: AEK79593.1 |
| FIV | NCBI protein sequence database | GenBank: AA48157.1 |
| EIAV | NCBI protein sequence database | GenBank: ACT31322.1 |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HTLV-1              | NCBI protein sequence database | GenBank: AAB20767.1 |
| RSV                 | NCBI protein sequence database | GenBank: AA48534.1 |
| MuLV                | NCBI protein sequence database | RefSeq: NP_040332. |
| HTLV-2              | NCBI protein sequence database | GenBank: A73468.1 |
| BLV                 | NCBI protein sequence database | GenBank: AAA42784.1 |
| MVV                 | NCBI protein sequence database | GenBank: AAB25459.1 |
| HIV-2               | NCBI protein sequence database | GenBank: AAA76840.1 |
| ALV                 | NCBI protein sequence database | GenBank: AIG42160.1 |
| SRV                 | NCBI protein sequence database | GenBank: AD89356.1 |
| MMTV                | NCBI protein sequence database | GenBank: GA31472.1 |
| FFV                 | NCBI protein sequence database | GenBank: GC11912.1 |
| SFVagm              | NCBI protein sequence database | RefSeq: YP_001956721.2 |
| SFVcpz              | NCBI protein sequence database | GenBank: AA19977.1 |
| HFV                 | NCBI protein sequence database | GenBank: CA8A898.1 |
| FeLV                | NCBI protein sequence database | GenBank: AA43054.1 |
| GALV                | NCBI protein sequence database | GenBank: ALV83305.1 |
| PERV                | NCBI protein sequence database | GenBank: CA876581.1 |
| BFV                 | NCBI protein sequence database | GenBank: AWK77106.1 |
| SnRV                | NCBI protein sequence database | RefSeq: NP_043925.1 |
| WDSV                | NCBI protein sequence database | RefSeq: NP_045938.1 |
| HERV-W              | NCBI protein sequence database | GenBank: AA84213.1 |
| HERV-K              | NCBI protein sequence database | GenBank: CA869289.1 |
| JSRV                | NCBI protein sequence database | GenBank: CA801899.1 |
| TIA1                | NCBI protein sequence database | GenBank: EAW99824.1 |
| hnRNP A2B1           | NCBI protein sequence database | RefSeq: XP_005249786.1 |
| hnRNP a0            | NCBI protein sequence database | RefSeq: NP_006796.1 |
| hnRNP A1L2          | NCBI protein sequence database | RefSeq: NP_001011725.1 |
| DAZAP1              | NCBI protein sequence database | RefSeq: NP_061832.2 |
| RNApol II           | NCBI protein sequence database | GenBank: AA12924.1 |
| HEWL                | NCBI protein sequence database | PDB: 1LSZ_A |
| BPT1                | NCBI protein sequence database | RefSeq: NP_001001554.2 |
| CALM1               | NCBI protein sequence database | GenBank: CA485181.1 |
| UB                  | NCBI protein sequence database | GenBank: AA836789.1 |
| REV                 | NCBI protein sequence database | GenBank: AYF56141.1 |
| CRM1                | NCBI protein sequence database | GenBank: CA86905.2 |
| DDX1                | NCBI protein sequence database | RefSeq: NP_004930.1 |
| DDX11               | NCBI protein sequence database | RefSeq: NP_085911.2 |
| DDX17               | NCBI protein sequence database | GenBank: CA830318.1 |
| DDX18               | NCBI protein sequence database | GenBank: CA833341.1 |
| DDX21               | NCBI protein sequence database | RefSeq: NP_004719.2 |
| DDX24               | NCBI protein sequence database | RefSeq: NP_065147.1 |
| DDX3                | NCBI protein sequence database | GenBank: AAC34298.1 |
### REAGENT or RESOURCE | SOURCE | IDENTIFIER
--- | --- | ---
DDX4 | NCBI protein sequence database | RefSeq: NP_077726.1
DDX5 | NCBI protein sequence database | RefSeq: NP_001307524.1
DHX36 | NCBI protein sequence database | GenBank: AAH36035.1
DHX9 | NCBI protein sequence database | RefSeq: NP_001348.2
Eif5A | NCBI protein sequence database | GenBank: AAH80196.1
PIMT | NCBI protein sequence database | GenBank: AAH07501.1
RanGTP | NCBI protein sequence database | GenBank: CAG29343.1
RHA | NCBI protein sequence database | GenBank: AML93444.1
RIP | NCBI protein sequence database | GenBank: AAH46349.1
Sam68 | NCBI protein sequence database | RefSeq: NP_006550.1

### Experimental Models: Cell Lines

| Name | Source | Catalog Number | Reference |
--- | --- | --- | --- |
HeLa (ATCC® CCL-2) | American Type Culture Collection | N/A | Platt et al., 2009; Takeuchi et al., 2008; Wei et al., 2002; Derdeyn et al., 2000; Platt et al., 1998 |
HEK293T/17 (ATCC® CRL-11268) | American Type Culture Collection | N/A | |
Jurkat, C6–1 (ATCC® TIB-152) | American Type Culture Collection | N/A | |
TZM-bl | NIH AIDS Reagent Program | Cat#8129 | Jouvenet et al., 2001; Platt et al., 2009; Takeuchi et al., 2008; Wei et al., 2002; Derdeyn et al., 2000; Platt et al., 1998 |

### Recombinant DNA

| Name | Source | Catalog Number | Reference |
--- | --- | --- | --- |
pDNA3.1 | Thermo Fisher Scientific | Cat#V79020 | |
HIV-1 NL4–3 Infectious Molecular Clone (pNL4–3) | NIH AIDS Reagent Program | Cat#114 | |
Gag-mCherry | Paul Bieniasz, Rockefeller University, USA | | Jouvenet et al., 2001 |
pG3BP1-GFP | Imed Gallouzi, McGill University, Canada | | Tourniére et al., 2001 |
pNL4–3 NC-C15S-C49S | Robert Gorenlik, National Cancer Institute | | Guo et al., 2000 |
pRluc-N1 | BioSignal Packar | Cat#631000 | |
p2-p1/Rluc | Mouland Lab | | Chatel-Chaix et al., 2004 |
NC-p1<sup>R7</sup>-YFP | Mouland Lab | | Chatel-Chaix et al., 2008 |
NC-p1<sup>C15–49S</sup>-YFP | Mouland Lab | | Chatel-Chaix et al., 2008 |
pPR-EGFP | Rongtuan Lin, McGill University | | Solis et al., 2011 |
pEGFP-C1 | Clontech | Cat#6084–1 | |

### Software and Algorithms

| Name | Source | Reference |
--- | --- | --- |
Imaris software v. 8.1.2 | Oxford Instruments / Imaris | https://imaris.oxinst.com/ |
Velocity v. 6.3 | Perkin Elmer | https://www.perkinelmer.com/ |
ImageJ | National Institutes of Health (NIH) | https://imagej.nih.gov/ij/ |
GraphPad v. 6.1 | Prism | https://www.graphpad.com/scientific-software/prism/ |
Predictor of prion-like domains (PLAAC) | Lancaster et al., 2014 | http://plaac.wi.mit.edu/ |
Predictor of Natural Disordered Regions (POND) | Molecular Kinetics, Inc. (Peng et al., 2006, 2005; Romero et al., 2001) | http://www.pondr.com/ |
MobiDB: a database of protein disorder and mobility annotations | Piovesan et al., 2018 | http://mobidb.bio.unipd.it/ |
Protein sequence analysis tool | Philipp O. Tsvetkov | Garnier, et al. (2017) |

### Other

| Name | Source | Reference |
--- | --- | --- |
Leica DM16000B laser confocal microscope | Leica | N/A |

*Cell Rep. Author manuscript; available in PMC 2022 March 30.*
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| WaveFX spinning disk confocal head | Quorum Technologies | N/A |
| Hamamatsu EM-CCD digital camera | Hamamatsu | N/A |
| Hybridization Oven | Boekel Scientific | Model 24100 |
| Nitrocellulose membranes 0.2 µm | Bio-Rad | Cat#1620112 |
| Quick Spin Columns | Roche | Cat#11814427001 |
| 4-chamber Lab-Tek®II Chambered #1.5 German Coverglass System | Thermo Fisher Scientific | Cat#155382 |
| 12-well plates | VWR | Cat#CA62406–165 |
| Boil proof 1.5 mL tubes | DIAMED | Cat#DIATE610–3167 |
| Eppendorf® micropestles | Sigma | Cat#Z317314 |
| 25 × 75 mm x 1mm thick glass slides | Thermo Fisher Scientific | Cat#0640–004T |
| 18 mm ø No. 1 cover glasses | VWR | Cat#16004–300 |
| 0.2 µm Sterile syringe filters | VWR | Cat#28143–310 |