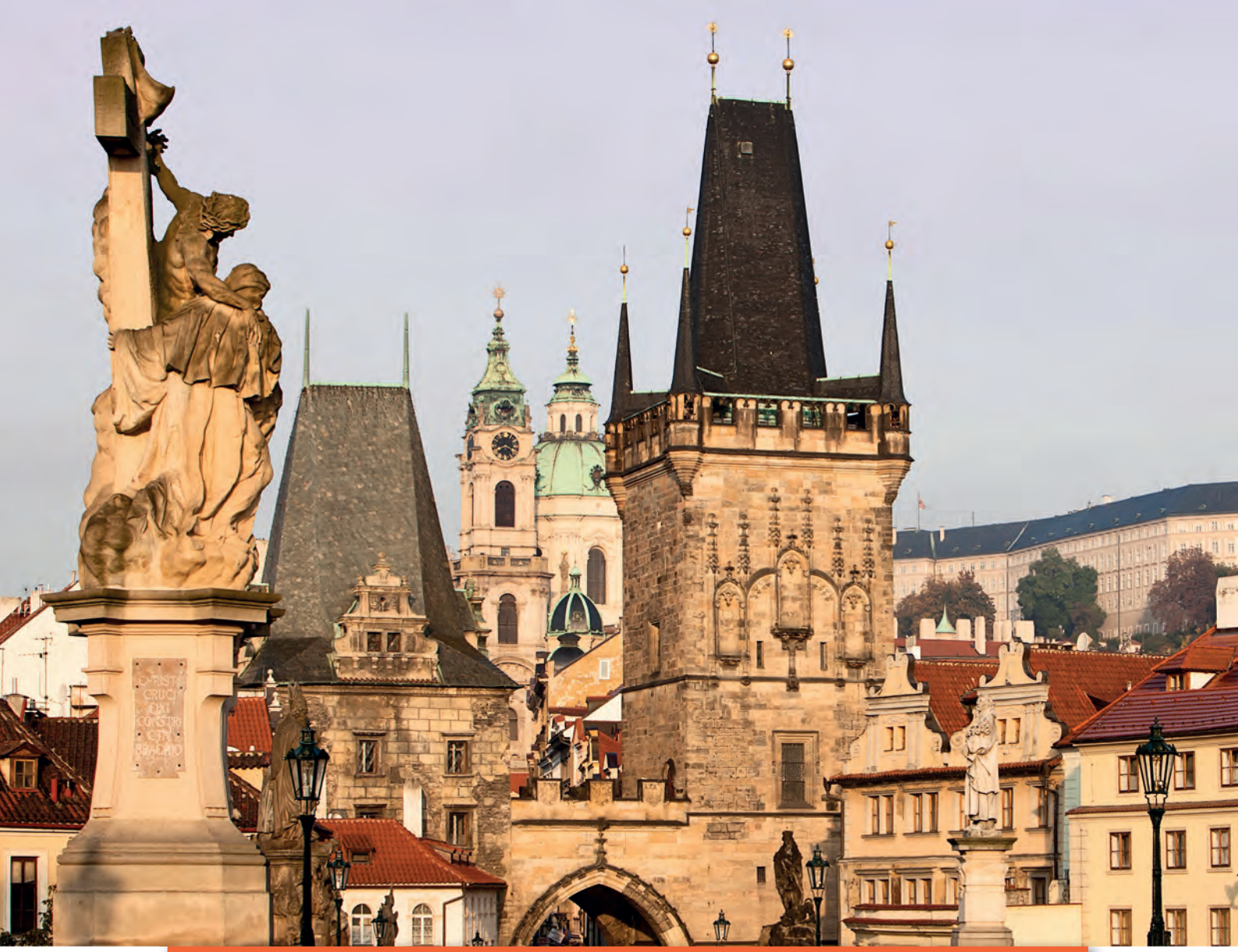

Program and Book of Abstracts

13TH INTERNATIONAL RETROVIRAL SYMPOSIUM

Assembly, Maturation and Uncoating

September 16 – 19, 2025 / Prague, Czech Republic

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The project National Institute of virology and bacteriology (Programme EXCELES, ID Project No. LX22NPO5103) – Funded by the European Union – Next Generation EU.

National Institute
of Virology and Bacteriology

TUESDAY 16TH SEPTEMBER	
16:00 – 20:00	REGISTRATION
18:00 – 19:00	Dinner
19:00 – 19:10	Welcome & Conference Opening
19:10 – 20:20	**Session I: Envelope Incorporation, Structure, and Function**
WEDNESDAY 17TH SEPTEMBER	
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10:35 – 10:55	Coffee Break
10:55 – 12:30	**Session III: Virus Assembly-I**
12:30 – 13:30	Lunch
13:30 – 15:05	**Session IV: Virus Assembly-II**
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15:30 – 17:10	**Session V: Virus Release and Maturation**
18:00 – 20:00	Boat trip with dinner
THURSDAY 18TH SEPTEMBER	
09:00 – 09:55	**Session VI: Therapeutic Strategies**
09:55 – 10:20	Coffee Break
10:20 – 11:20	**Session VII: Capsid Structure and Function**
11:20 – 12:10	**Keynote I**
12:10 – 13:30	Lunch
13:30 – 15:30	Prague Tour
15:30 – 17:15	Explore Prague on own
17:30 – 18:10	**Session VIII: Single Molecule and other Biophysical Approaches**
18:10 – 19:00	**Keynote II**
19:00 – 20:00	Dinner
20:00 – 22:00	Poster session
FRIDAY 19TH SEPTEMBER	
09:00 – 10:20	**Session IX: Host Factors**
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10:50 – 12:15	**Session X: Capsid Trafficking and Nuclear Events**
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13th International Retroviral Symposium

Assembly, Maturation and Uncoating

Prague, Czech Republic

September 16 – 19, 2025

Program

Abstracts

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symposium chair

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University of Utah, USA,
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GENERAL INFORMATION

Registration Information

Category	Early Bird (by 25 June, 2025)	Late Fee (from 26 June, 2025)
Regular	600 €	750 €
Student	450 €	550 €
Invited Speaker	0 €	0 €

Registration Desk Hours

(Registration desk is located in the National Library of Technology at the foyer)

Date	Time
Tuesday, 16 September, 2025	16:00 – 20:00
Wednesday, 17 September, 2025	08:00 – 17:00
Thursday, 18 September, 2025	08:00 – 20:00
Friday, 19 September, 2025	08:30 – 13:00

Welcome to the 13th International Retroviral Symposium in Prague, Czech Republic!

We are delighted to host this year's Symposium in the historic and culturally rich city of Prague – one of Europe's most scenic destinations. With its stunning architecture, vibrant atmosphere, and long tradition of scientific excellence, Prague provides an inspiring backdrop for both academic exchange and personal exploration.

Our goal is to bring together researchers from diverse scientific fields to deepen our understanding of the biochemical, biophysical, and virological mechanisms that govern the retroviral life cycle. The complex nature of these viral processes requires a multidisciplinary approach, and we are excited to create a collaborative platform where experts can share insights and push the boundaries of retrovirology research.

This year's symposium will focus on critical aspects of retroviral assembly, maturation, and uncoating, with participants from disciplines such as virology, biochemistry, high-resolution microscopy, and biophysics. Our discussions aim to advance the fight against HIV-1/AIDS and other retroviral diseases through innovative research and new therapeutic strategies.

Please note that we welcome abstracts on retroviruses and other RNA viruses.

Key discussion topics will include:

- Envelope Incorporation, Structure, and Function
- RNA Nuclear Trafficking, Packaging, and Translation
- Virus Assembly and Morphogenesis
- Novel Therapeutic Strategies
- Capsid Trafficking, Uncoating, and Integration
- Capsid Protein Structure and Function
- Maturation and Viral Particle Release
- The Role of Host Factors in Viral Replication

This symposium is a unique opportunity for scientists at all levels – from principal investigators and research scientists to postdoctoral fellows, graduate students, and biotechnology professionals – to come together and advance the scientific progress needed to combat retroviral diseases.

Tomáš Ruml, Michaela Rumlová, Saveez Saffarian, Karin Musier-Forsyth

PROGRAM

The symposium will be held in Balling Hall

Tuesday 16th September 2025

16:00 – 20:00 Registration

18:00 – 19:00 Dinner

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Paul Spearman

19:50 – 20:05 Structure of HIV-1 Env Glycoprotein on Virions Reveals an Alternative Fusion Subunit Organization and Native Membrane Coupling
Jacob T. Croft

20:05 – 20:20 HIV-1 Env Tail Interactions with the Matrix Subunit Revealed by Cryo-electron Tomography and Molecular Dynamic Simulations
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Juan R. Perilla

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Irene Gialdini (Lamb)

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Hélène Dutartre

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Delphine Muriaux

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Virgile Rat

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10:50 – 12:15 Session X: Capsid Trafficking and Nuclear Events

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Vinay K. Pathak

11:45 – 12:00 HIV-1 Uncoating Inside Nuclear Speckles Strongly Enhances Integration into Speckle-associated Chromatin Domains
Ryan C. Burdick

12:00 – 12:15 Deciphering the Biogenesis of HIV-induced CPSF6 Puncta
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12:15 – 12:30 Poster/Talk Awards & Closing Remarks

ABSTRACT BOOK

Lectures are sorted by Program,
Posters are sorted alphabetically according to the authors' surnames.

Texts of abstracts have not undergone neither linguistic nor editorial correction.

ORAL PRESENTATIONS

PHOSPHATIDYLINOSITOL (4,5)-BISPHOSPHATE PROMOTES INCORPORATION OF HOST TRANSMEMBRANE PROTEINS AND ENV INTO HIV-1 PARTICLES

Ricardo de Souza Cardoso and Akira Ono

Department of Microbiology and Immunology, the University of Michigan Medical School, Ann Arbor, Michigan, USA

The acidic phospholipid phosphatidylinositol-(4,5)-bisphosphate (PIP2), which is enriched at the plasma membrane (PM), plays crucial roles in HIV-1 assembly. Through interaction with the MA highly basic region (HBR), PIP2 recruits Gag to the PM. At the PM, Env is recruited to the HIV-1 assembly sites by a mechanism that is not fully understood. However, genetic, biochemical, and cell-based studies showed that its cytoplasmic tail (CT) plays a role in Env incorporation into HIV-1. Besides Env, several host transmembrane proteins (HTMPs) are incorporated into HIV-1 particles. We recently demonstrated that the HTMPs CD43, PSGL-1, and CD44, which contain a juxta-membrane polybasic sequences (JMPBS), are incorporated into HIV-1 via a PIP2-dependent mechanism. Therein, PIP2 likely serves as a negatively charged entity recruiting the positively charged protein regions, the MA HBR of Gag and the JMPBS of HTMPs. Interestingly, the HIV-1 Env CT also contains a basic residue-rich sequence. To test whether PIP2 contributes to the efficient incorporation of Env, we used two orthogonal approaches to deplete cellular PIP2 and examined their effects on Env incorporation and infectivity. We found that PIP2 depletion of virus-producing cells reduces both Env level and infectivity per virus particles. Interestingly, when cells are treated with an inhibitor of a PIP2-generating enzyme, PI4P-5-kinase Ia, at low concentrations that do not affect Env incorporation, reduction in infectivity was still observed. Together, these data support a novel role(s) for PIP2 in HIV-1 assembly, where PIP2 facilitates newly produced virus particles to acquire and/or maintain infectivity.

Supported by NIH R37AI071727.

CELL TYPE-SPECIFIC INCORPORATION OF HIV-1 ENVELOPE GLYCOPROTEIN INTO PARTICLES REQUIRES THE TUBULAR RECYCLING ENDOSOME

Grigoriy Lerner[(1)], Lingmei Ding[(2)], Boris Anokhin[(2)], Kathleen Candor[(3)], and Paul Spearman[(2)]

(1) Laboratory of Neurogenetics, NIA, NIH, Bethesda, MD
(2) Infectious Diseases, Cincinnati Children's Hospital and University of Cincinnati, Cincinnati, OH USA
(3) Immunology Graduate Program, University of Cincinnati and Cincinnati Children's Hospital, Cincinnati, OH USA

The HIV-1 envelope glycoprotein (Env) is incorporated into particles during assembly on the plasma membrane (PM). Env has a long cytoplasmic tail (CT) that is required for particle incorporation in cell types that include H9, Jurkat, CEM, and primary T cells and macrophages, termed nonpermissive cells, while 293 T, MT-4, and Cos-7 cells are permissive for incorporation of Env with a truncated CT. We have shown that Env labelled on the cell surface rapidly enters the tubular recycling endosome compartment (TRE). Trafficking of Env to the TRE was dependent upon motifs within the long CT previously implicated in Env recycling and particle incorporation. Depletion of TRE components MICAL-L1 or EHD1 led to defects in Env incorporation, particle infectivity, and viral replication. Remarkably, disruption of the TRE only altered Env incorporation in cell types previously defined as nonpermissive for incorporation of CT-deleted Env, including monocyte-derived macrophages, and was not observed in 293 T, HeLa, or MT-4 cells. To further understand the requirement for the Env CT in nonpermissive cells, we performed cell-cell fusion experiments between permissive and nonpermissive cell types. Remarkably, heterokaryons were permissive for truncated Env incorporation. This work identifies the TRE as an essential component of Env trafficking and particle incorporation in nonpermissive cells. Permissive cell types, in contrast, demonstrate a dominant phenotype in cell-cell fusions, suggesting the existence of a pathway for truncated Env incorporation that is independent of the TRE and is reduced or absent in nonpermissive cells.

Supported by NIH R01 AI150486.

STRUCTURE OF HIV-1 ENV GLYCOPROTEIN ON VIRIONS REVEALS AN ALTERNATIVE FUSION SUBUNIT ORGANIZATION AND NATIVE MEMBRANE COUPLING

Jacob T. Croft, Hung N. Do, Klaus N. Lovendahl, Daniel Leaman, Pooja Jain, Katelyn Chase, Cynthia Derdeyn, Michael B. Zwick, Sandrasegaram Gnanakaran*, Kelly K. Lee*

In contrast to other viruses, effective vaccines for Human Immunodeficiency Virus type 1 (HIV-1) have yet to be developed. The high glycosylation and sequence diversity of the HIV-1 Envelope protein (Env), the sole viral surface antigen, make it a challenging target for the immune system. Furthermore, a detailed characterization of the authentic, functional Env trimer on virions has remained elusive. While engineered, native-like Env trimers in many ways recapitulate functional Env, differences in antigenic profile and dynamic behavior by single-molecule FRET have been reported. We used Cryo-electron tomography of intact HIV-1 virus-like particles (VLPs) and determined the structure of the Env ectodomain by subtomogram averaging, revealing differences in conformation of the membrane-proximal regions compared to stabilized, solubilized Env. We also used Hydrogen-Deuterium Exchange Mass Spectrometry (HDX-MS) and Molecular Dynamics (MD) simulations to investigate the dynamic behavior of membrane-bound Env, identifying a novel interaction with the membrane. In mapping HDX-MS data onto the new structural model, we propose an allosteric network connects the membrane-interacting motifs along the gp120-gp41 interface thus modulating conformational sampling of activated, open and closed states. Finally, using subtomogram averaging we demonstrate that disruption of the membrane results in relaxation of Env to the familiar, SOSIP-like structure. These studies provide new insight to the conformation and dynamics of Env in its native context.

HIV-1 ENV TAIL INTERACTIONS WITH THE MATRIX SUBUNIT REVEALED BY CRYO-ELECTRON TOMOGRAPHY AND MOLECULAR DYNAMIC SIMULATIONS

Jacob T. Croft[1,†], Hung N. Do[2,†], Klaus N. Lovendahl[1], Daniel P. Leaman[3], Pooja Jain[4], Katelyn Chase[1], Cynthia A. Derdeyn[4], Michael B. Zwick[3], Sandrasegaram Gnanakaran[2,*], Kelly K. Lee[1,*]

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2. Theoretical Biology & Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM, USA 87545
3. Department of Immunology & Microbiology, The Scripps Research Institute, La Jolla, CA, USA 92037
4. Department of Lab Medicine & Pathology, University of Washington, Seattle, WA, USA 98195

Assembly of HIV-1 particles is a dynamic process involving the interplay between Gag proteins, viral RNA, membrane, and the Env glycoprotein. Our understanding of this process has been fragmented, often relying upon examination of each component in isolation. Here we have used cryo-electron tomography (cryo-ET) to directly image Env and Gag organization in intact viral particles, revealing unanticipated positioning of Env CT over the MA lattice rim. This finding contrasts with the prevailing model that depicts Env CT protruding through the prominent central holes in the MA lattice. Tomographic reconstruction with sub-tomogram averaging has resolved the intact Env cytoplasmic tail and density connecting the tail to MA subunits in immature HIV-1 viral particles. Molecular Dynamics (MD) simulations were applied to assess the structural impact of CT-MA engagement, providing more detailed insight into this critical assembly interaction. Additionally, clustering analysis of Env in mature and immature particles directly resolved how the Env cell entry machinery, which performs pivotal functions of receptor binding and membrane fusion, reorganizes to generate infectious, entry-competent viral particles. Lastly structural mass spectrometry was applied to compare Env displayed on immature vs mature particles, thus enabling previously hypothesized models for contributions of Tail-Gag interactions and maturation state on the Env ectodomain to be tested. These studies provide new insights into the HIV-1 assembly process and the changes that convert viral particles into their infectious forms.

Supported by US NIH grant R01-AI179697

WHY IS THE RECRUITMENT OF DHX15 INTO THE MPMV PARTICLE CRUCIAL FOR GENOMIC RNA PACKAGING?

Alžběta Dostálková, Marina Kapisheva, Ivana Křížová, Jana Racková, Michaela Rumlová

Department of Biotechnology, University of Chemistry and Technology, Prague, Czech Republic

Retroviruses exploit host cell machinery for replication, including the recruitment of RNA helicases. This study uncovers a distinctive mechanism by which the betaretrovirus Mason-Pfizer monkey virus (M-PMV) hijacks the host DEAH-box RNA helicase DHX15 via a virus-encoded G-patch motif. We demonstrate that this retroviral G-patch acts as a functional cofactor, mimicking cellular G-patch proteins and enabling the relocation of nuclear DHX15 to the cytoplasm, where it is incorporated into assembling virions. Through the use of mutagenesis, interactome analysis, CRISPR-Cas9 engineering and structural modelling, we demonstrate that the DHX15–G-patch complex is vital for the efficient packaging of viral genomic RNA (gRNA). Furthermore, our results show that DHX15 interacts directly with the M-PMV gRNA within the constitutive transport element (CTE) region, significantly enhancing reverse transcription efficiency and viral infectivity. Disruption of the G-patch motif or depletion of DHX15 markedly reduces gRNA incorporation, impairs reverse transcription and abrogates infectivity. These results highlight a sophisticated strategy of host factor recruitment and a previously unrecognised function of a cellular helicase in betaretroviral replication.

THE REGULATION OF HIV-1 UNSPLICED RNA FUNCTIONS

Wei-Shau Hu[(1)], Olga Nikolaitchik[(1)], Zetao Cheng[(1)], Saiful Islam[(1)], Joseph Kanlong[(2)], Jonathan Kitzrow[(1)], Akhil Chameettachal[(1)], Alice Duchon[(1)], Vinay K. Pathak[(3)], Karin Miser-Forsyth[(2)]

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(3) Viral HIV Dynamics and Replicaiton Program, National Cancer Institute, Frederick, Maryland 21702

HIV-1 unspliced RNA serves two critical roles in viral replication: it serves as a translation template to generate structural proteins and enzymes, and it serves as the viral genome when packaged into virions. Recent studies demonstrate that HIV-1 uses multiple transcription start sites to generate several unspliced RNA species, including two major transcripts referred to as 3G RNA and 1G RNA. Intriguingly, these two 99.9% identical RNA species are functionally distinct. The 1G RNA is selected over 3G RNA for packaging into virions as the genome, and the 3G RNA is translated more efficiently than the 1G RNA. We have shown that the 5' untranslated regions of 3G RNA and 1G RNA fold into two different ensembles that affect their functions. Furthermore, co-expression of the two RNA species is important for HIV-1 replication fitness. These studies reveal that HIV-1 utilizes alternative transcription start sites to regulate RNA functions and optimize viral replication.

RNA STRUCTURES REGULATING THE PACKAGING AND TRANSLATION OF HIV-1 SPLICED VIRAL RNAS

A.S. Gribling-Burrer [(1)], Ulyana Myerkina [(1)], Patrick Bohn [(2)], Kharytonchyk S [(3)], Alice Telesnitsky [(3)], R. P. Smyth [(1)]

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(3) Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109-5620

HIV-1 produces more than 50 capped and polyadenylated transcripts that must be tightly regulated to ensure successful replication. These transcripts fall into three classes: unspliced (US), partially spliced (PS), and fully spliced (FS) RNAs. While US RNA functions both as genomic RNA and as a template for structural and enzymatic proteins, PS and FS RNAs encode regulatory and accessory proteins but are efficiently excluded from virion packaging. The molecular basis of this functional segregation remains poorly understood.
Using isoform resolved long-read RNA structural probing, we previously showed that the 5'UTRs shared by US and spliced RNAs adopt distinct conformations. In spliced RNAs, the $Pr55^{Gag}$ binding site is unstructured, potentially explaining their exclusion from packaging. Here, we combined RNA structure probing with translation inhibitors to assess the role of ribosome engagement on 5'UTR RNA structure. Whereas US RNA showed minimal structural rearrangement under puromycin (ribosome dissociation), cycloheximide (elongation arrest), or harringtonine (initiation arrest), spliced RNAs exhibited marked increases in DMS reactivity upon cycloheximide or harringtonine treatment, but not with puromycin. These changes are consistent with ribosome-induced unfolding.
Intriguingly, ribosome-induced unfolding was also observed in highly translated cellular mRNAs but was absent in transcripts translated via non-scanning mechanisms. Since US RNA remains actively translated yet does not undergo such remodeling, our findings raise the possibility that HIV-1 US RNA is translated through a non-canonical mechanism.

ROLES FOR THE HIV-1 GAG NUCLEOCAPSID DOMAIN IN VIRAL RNA SUBCELLULAR TRAFFICKING AND TRANSLATION

Lesko S. L.[1,2], Bruce J. W.[1,2], Scott S.[1,2], McLaughlin O.[1,2], Whitworth I.[3], Smith L.[3], Suzuki A.[2], Kutluay S.[4], Kharytonchyk S.[5], Telesnitsky A.[5], Summers M. F.[6,7], Sherer N. M.[1,2]

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4 Department of Microbiology and Immunology, Washington University, St. Louis, MO, USA
5 Department of Microbiology and Immunology, University of Michigan-Ann Arbor, Ann Arbor, MI, USA
6 Department of Chemistry and Biochemistry, University of Maryland-Baltimore County, Baltimore, MD, USA
7 Howard Hughes Medical Institute, University of Maryland-Baltimore County, Baltimore, MD, USA

HIV-1's unspliced (US) RNA can function in the cytoplasm either as an mRNA template for Gag and Gag-Pol protein synthesis or as a genomic RNA dimer bound by Gag and packaged during virion assembly. The 5'-untranslated Leader region of the US RNA carries a 7mG cap and is comprised of multiple stem-loop structures that regulate Gag/Gag-Pol translation, genome dimerization, and Gag association during packaging. Here, we describe an integrated visual system for studying the viral and cellular regulation of US RNA cytoplasmic fate based on multicolor reporter viruses that allow us to make single cell measurements of US, partially spliced (PS) and completely spliced (CS) HIV-1 RNA translation rates in conjunction with measurements of US RNA subcellular trafficking and the efficiency of genome packaging into virions. Using this system, we confirm that 5' Leaders engineered for a "locked" cap-sequestered conformation (^{Cap}1G) are translated poorly and better packaged relative to locked cap-exposed (^{Cap}3G) 5' Leader US RNAs. We also demonstrate that Gag's preferred with ^{Cap}1G US RNAs mediated through Gag's Nucleocapsid (NC) RNA-binding domain plays a major role in both driving ^{Cap}1G US RNA into the packaging pathway as well as in suppressing the translation of ^{Cap}1G US RNA relative to ^{Cap}3G US RNAs. Taken together, our results are consistent with US RNA cap-sequestration and Gag NC representing major determinants of HIV-1 translation efficiency, with Gag's NC domain playing a key role in regulating ^{Cap}1G US RNA fate. We will also discuss our efforts to adapt this system to study subcellular compartmentalization of US, PS, and CS viral RNAs and their interactions with specific viral and host regulatory factors.

LIVE-CELL IMAGING REVEALS HOW, WHEN, AND WHERE GAG SELECTS HIV-1 RNA FOR PACKAGING

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During virus assembly, Gag must select HIV-1 unspliced RNA from an abundant pool of cellular RNAs as the virion genome. How, when, and where Gag selects HIV-1 RNA for packaging is currently unclear. By using neighboring transcription start sites, HIV-1 generates two major transcripts with three (3G RNA) or one guanosine (1G RNA) at the 5' end. Despite only differing by 2 nucleotides, 1G RNA is selectively packaged into virions over 3G RNA. To understand how HIV-1 selects the virion RNA genome, we generated cell lines infected with two modified HIV-1 viruses, one expressing 3G RNA and one expressing 1G RNA. These two RNA species can be differentially labeled by fluorescent proteins or detected by RNAscope probes. We examined the distribution of the two RNAs in the cytoplasm and at the plasma membrane using spinning-disk confocal microscopy and total internal reflection fluorescence microscopy, respectively. Regardless of Gag expression, these two RNA species were distributed randomly in the cytoplasm. Live-cell imaging experiments showed that in the absence of Gag, both 3G and 1G RNA can reach the plasma membrane without preference. However, upon Gag expression, 1G RNA accumulated at the plasma membrane even before detectable Gag puncta appeared, and preferentially associated with assembling Gag particles once puncta became visible. These findings demonstrate that while both 1G and 3G RNA can traffic to the assembly site, and selective packaging of 1G RNA begins rapidly starting from its initial anchoring to the plasma membrane, where it nucleates HIV-1 particle assembly.

COMPUTATIONAL INSIGHTS INTO PH-DRIVEN CONFORMATIONAL SHIFTS IN HIV-1 CAPSID ASSEMBLY

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HIV-1's genome is packed inside a conical protein shell made of capsid proteins (CA), which protects it and modulates key events during viral replication. CA is integral to the life cycle and fitness of the virus and its sensitive to changing physiological conditions during cell trafficking and nuclear import, including local changes in pH. It has been posited that the dynamics of the central CA pore at the center of pentamers and hexamers regulate the permeability of small molecules, like nucleotides, which are essential for viral DNA synthesis. Previous studies suggest that structural elements such as the β-hairpin in the N-terminal domain and the protonation state of key residues like His12, govern the pore dynamics. Furthermore, it has been proposed that shifts in the protonation states of H12 under varying pH conditions can induce conformational changes in the CA assembly. In the present study, we leverage constant pH molecular dynamics simulations -- a unique computational approach -- to model protonation equilibria and capture the effects of varying pH on residue-specific microenvironments. By simulating CA hexamers and pentamers at different pH conditions, we calculate the pKa values of the key residues H12 and its titratable neighbor, Glu45. We also determine the structural changes in these targeted environments resulting from varying protonated states of H12 and E45. We further investigate how the protonation states of these residues influence pore dynamics, focusing on the β-hairpin structure, and identifying regions in the CA assembly where pH-induced conformational changes affect structural integrity, pore size, shape, and permeability. These findings provide novel insights into the role of pH- in regulating CA dynamics; the latter having an effect in nucleotide translocation and the overall assembly stability. We finally offer a new perspective for the design of future experimental studies to establish the role of pH during HIV-1 replication.

CYTOSOLIC INTERACTIONS OF HIV-1 GAGPOL AND GAG UNVEILED BY FLUORESCENCE FLUCTUATION SPECTROSCOPY

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The HIV-1 assembly process is driven by the structural protein Gag, which forms small oligomers in the cytoplasm that are then trafficked to the cell membrane. Although Gag alone is sufficient to form viral particles, the incorporation of GagPol, a polyprotein composed of Gag and viral enzymes, into virions is essential for infectivity. The Gag-to--GagPol ratio is critical for infectivity, but the mechanisms regulating their cytosolic interaction remain poorly understood. To investigate the formation of cytoplasmatic Gag:GagPol complexes at the single molecule level, we developed two fluorescently labelled constructs: a single labelled GagPol (*GagPol.mVenus*), with the integrase domain replaced by mVenus, and a double labelled GagPol polypeptide, where both Gag and GagPol polypeptides are tagged (*Gag.mCherry2-Pol.Venus)*. Co-expressing *GagPol.mVenus* with a Gag-mCherry2 fusion protein enables us to study GagPol:Gag *inter*-molecular interactions, while the double labelled construct allows us to simultaneously probe Gag and GagPol to precisely quantify their ratio. To characterize the mobility and stoichiometry of GagPol and evaluate its interaction with Gag we applied fluorescence fluctuations techniques such as raster and temporal image correlation spectroscopy. Our results showed that similarly to Gag, GagPol exists in the cytoplasm as a dual species: a faster monomeric species and a slower species, diffusing on the seconds timescale. Temporal image cross-correlation spectroscopy analyses revealed that this second species interacts with Gag oligomers, suggesting GagPol's participation in early, cytoplasmatic step of HIV-1 assembly. Overall, this study provides valuable tools to monitor Gag and GagPol dynamics and offers insights into the mechanisms of HIV-1 assembly regulation.

GAG-GAGPOL REVISTED

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During transcription of the viral proteins of HIV, the main structural protein, Gag, occasionally undergoes a reading frame shift that leads to expression of additional domains. The construct is referred to as GagPol and, upon proteolysis, leads to formation of the HIV protease, reverse transcriptase and integrase proteins. Canonically, the ratio of Gag to GagPol is given as 20:1 and this ratio is critical for infectivity. However, the ratio has been determined using ensemble methods and little is known about its variability and regulation. Hence, we are reinvestigating the ratio of Gag to GagPol to look at the cellular and particle variability of this ratio and investigate where and how it is controlled. To this end, we have developed a dual-labeled construct of the GagPol protein with one label in the Gag region and one in the GagPol region. By using dual-color quantitative fluorescence microscopy methods, we are now in a position to investigate the secrets of the Gag to GagPol ratio.

KINETICS OF HIV-1 GAG VIRION ASSEMBLY ON THE PLASMA MEMBRANE

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The mechanism by which HIV-1 Gag selectively packages dimeric viral RNA (vRNA) into immature virions is still unclear, especially given the low levels of vRNA relative to host cell mRNAs in the cytoplasm. The overall long-term goal of this work is to test the hypothesis that Gag assembles faster on the vRNA dimer than on a random mRNA monomer at the plasma membrane (PM), thereby leading to selective packaging. We have developed a system of differential equations that describe individual virion growth on the PM, as well as Gag virion production and release from the cell. As a first step toward our goal, we theoretically describe the kinetics of virion assembly on random mRNA. An unusual feature of this assembly is that it is dictated by the rate of Gag synthesis. We predict that the virion assembly time is inversely proportional to the Gag synthesis rate with the existence of two virion assembly regimes. When Gag synthesis occurs at a rate faster than the "critical" rate, assembly is rate-limited by virion nucleation. In contrast, when Gag synthesis occurs slower than the critical rate, assembly is rate-limited by virion growth. We hypothesize that these two assembly regimes correspond to two mechanisms of vRNA dimer packaging, allowing for its high efficiency in a broad range of Gag expression levels. We also predict testable dependencies of the virion assembly time on the strength of Gag/PM binding and Gag-Gag interactions, and on the probability of Gag assembly nucleation on the PM.

HTLV-1 HIJACKS THE NEURONAL SNAP25 PROTEIN TO ENSURE VIRAL TRANSMISSION

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One striking feature of the HTLV-1 deltaretrovirus is the low infectivity of free viral particles, making viral transmission almost exclusively dependent on cell-to-cell contacts. Such contacts allow the transfer of the viral biofilm, a cell--surface structure characterized by the accumulation of virions within a carbohydrate-rich matrix. The mechanisms governing the formation of the viral biofilm, the vesicular trafficking of viral proteins to the viral biofilm as well as its transfer to target cells remain poorly understood.
In this context, we searched for cellular proteins involved in vesicular trafficking of CD4+ T lymphocyte and which expression is altered upon HTLV-1 infection. We show, using several HTLV-1 chronically infected T-cell lines, that HTLV-1 infection of CD4+ T-cells correlates with the unexpected expression of the neuronal SNARE protein SNAP25, known for its role in vesicle secretion.
By combining high-resolution microscopy and biochemical analyses, we demonstrate that SNAP25 is required for viral biofilm-mediated infectivity of infected T-cells, without impairing formation of conjugates with target cells. The absence of SNAP25 results in reduced accumulation of viral particles in the cell-associated viral biofilms. In addition, we highlight that SNAP25 is present within the viral biofilm at the surface of infected T-cells, and is mainly localized in vesicles distinct from virions, suggesting that HTLV-1 globally reshapes T-cell vesicles secretion that get trap in the viral biofilm.
Altogether, our findings uncover for the first time how HTLV-1 induces and repurposes the expression of a neuronal SNARE protein in CD4+ T-cells to promote efficient viral production into the biofilm.

ULTRASTRUCTURAL INSIGHTS INTO PURIFIED HTLV-1 BIOFILM BY CRYO-ELECTRON TOMOGRAPHY

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Human T-cell leukemia virus type 1 (HTLV-1) is unique among (retro)viruses in forming a dense extracellular biofilm-like matrix on the surface of infected cells, thought to promote efficient cell-to-cell transmission. However, the biofilm's architecture and its role in virion organization remain poorly understood. We investigate the ultrastructural organization of HTLV-1 biofilms, focusing on virion morphology, Gag maturation, and interactions with biofilm components. To this end, we optimized cryo-focused ion beam (cryo-FIB) milling to produce thin lamellae of biofilm for cryo-electron tomography (cryo-ET) imaging.
Our findings reveal a complex extracellular environment enriched in viral particles, membranous vesicles, and extracellular matrix components. Virions display variability in size and core morphology, including single and double cores, as well as structures suggestive of maturation intermediates. Some cores exhibit canonical mature capsid lattices, while others contain unidentified intraviral densities, possibly reflecting intermediate stages of Gag cleavage, capsid formation, or RNA condensation. Strikingly, virions are frequently clustered and tethered to one another or surrounding membranes by thin, linear densities. These densities are consistent in size and shape with the host restriction factor tetherin, suggesting a structural role in virion retention within the biofilm. Intriguingly, although tetherin typically restricts viral release, prior studies suggest HTLV-1 may exploit tetherin to stabilize biofilm-associated viral assemblies and facilitate transmission.
Altogether, our approach provides novel insights into the ultrastructure of HTLV-1 biofilms, revealing how virion assembly and maturation may be uniquely adapted to this context and paving the way for further structural studies of the biofilm-associated HTLV-1.

INSIGHTS FROM MULTISCALE COMPUTER SIMULATION: HIV-1 CAPSID ASSEMBLY AND NUCLEAR PORE ENTRY (PLUS THE LEN-IP6 BATTLE)

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Multiscale computational methodology provides a systematic connection between atomic-level interactions and a more coarse-grained but computationally efficient representation. As such, we have systematically explored highly complex macromolecular assembly phenomena. One significant pay-off from this effort concerns the key insights gained into the processes underlying certain important aspects of the HIV-1 virus replication cycle. A prime example is seen in studies of the assembly of the viral capsid from over one thousand proteins: a phenomenon involving a billion atoms or more and over timescales that cannot be approached through all-atom molecular dynamics simulation alone. In that spirit, results will be presented concerning the fascinating kinetics of HIV-1 capsid assembly, the key role of the IP6 anion in that process, and the competition between the IP6 anion and the anti--viral drug Lenacapavir (LEN) in their binding with the capsid lattice. In addition, new perspectives on the entry of the assembled HIV-1 capsid into the nuclear pore complex, including the role of LEN in disrupting that process, will be presented. As time allows, results will further be highlighted on the role of the ribonucleoprotein (RNP) complex in capsid assembly and the modeling of the reverse transcription process initiated within the capsid.

INTERROGATING HUMAN RETROVIRUS ASSEMBLY BY COMPARATIVE ANALYSIS

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Many aspects of the retrovirus particle assembly pathway remain to be fully understood. We are presently engaged in a comparative analysis among human retroviruses in order to gain greater insights into virus particle assembly sites as well as the morphology of released particles. First, using a parallel analysis, we have found that the matrix (MA) domain of HTLV-1 is distinct in having strong membrane binding affiities relative to HIV-1 and other retroviruses except that of MLV MA, implicating similarities between HTLV-1 and MLV. Second, we have used point spread function engineering to investigate the dynamics and kinetics of Gag puncta biogenesis at the top membrane of cells. This analysis has found that the kinetics of formation and movement of punctate Gag was faster at the top membrane versus the bottom membrane. Finally, we have resolved the HTLV-1 immature CA lattice to 3.4 Å resolution by using single particle analysis (SPA), which has revealed that unlike that of the HIV-1 CA domain, the HTLV-1 CA possesses an ordered N-terminal domain (NTD) with a relatively flexible C-terminal domain (CTD). Cryo-ET has revealed clear lattice heterogeneity, notably the varying curvatures of the lattice and the varying distances between the CA layer and the membrane. Unlike lentiviral assembly, inositol hexakisphosphate (IP6) was found to be dispensable for both HTLV-1 immature particle assembly and for proper immature lattice formation.

EXPLORING THE ROLE OF HIV-1 GAG COMPACT FORM IN VIRAL ASSEMBLY

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The Gag polyprotein is the key player for HIV-1 assembly and budding of new particles in infected cells. Gag specifically packages two copies of viral gRNA in the cell cytoplasm and binds to the cell plasma membrane for budding. Previous studies, mainly in vitro, have suggested that gRNA and membrane selections are mediated by the compact form of Gag. This compact form binds to gRNA through its matrix (MA) and nucleocapsid (NC) domains. As the result of the competition of the membrane with gRNA for Gag binding, this compact form is then thought to convert into Gag extended form with NC bound to gRNA and MA to the plasma membrane. In this context, we evidenced and monitored the compact form of Gag in HeLa cells by confocal microscopy, using a bimolecular fluorescence complementation approach with a split-GFP bipartite system. Using wild-type Gag and Gag mutants, we showed that the compact form is highly dependent on the binding of MA and NC domains to RNA, as well as on interactions between MA and CA domains. In contrast, the accumulation of Gag compact form was found to only moderately depend on Gag multimerization. Finally, mutations impacting the formation of Gag compact form led to a strong reduction in viral particle production and infectivity, revealing its key role in the production of infectious viral particles.

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ANY ROLE OF F-ACTIN DEPOLYMERIZATION COFACTORS IN HIV-1 ASSEMBLY IN HOST CD4 T CELLS

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**presenting*

The Human Immunodeficiency Virus type 1 (HIV-1) assembles and buds at the plasma membrane of host CD4+ T lymphocytes, where its structural protein Gag self-assembles beneath the cell membrane. However, the cortical actin network directly under this membrane acts as a physical barrier for viral assembly (1). This cortical actin is a dense meshwork composed by filamentous actin (F-actin), which dynamics are modulated by actin regulators, allowing the polymerisation (from globular actin), depolymerisation, and branching. Previous studies showed that a decrease in actin density meshwork promotes HIV-1 assembly, due to Gag hijacking actin regulators, like Arpin, an inhibitor of the Arp2/3 complex involved in branched actin (2). Now, our work consists in investigating the possibility that Gag hijacks other actin regulators, which can trigger the depolymerisation of the F-actin. We analyze HIV-1 viral particle production under siRNA-mediated knockdown of key actin cofactors, as well as the interaction of Gag and mutants with these regulators, their potential incorporation into VLP and determine their spatial correlation with Gag and F-actin by super-resolution microscopy. Our results show that some actin depolymerizing cofactors interact with Gag at the cell membrane, can be incorporated into Gag-VLP, while others do not. We will see how their depletion may impact HIV-1 assembly and release in transfected cell lines and in infected primary T cells.

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DISTINCT ARCHITECTURE AND DYNAMICS OF GAG LATTICE IN IMMATURE HIV-1 VIRIONS VERSUS GAG VLPS VISUALIZED BY CRYOET AND MINFLUX MICROSCOPY

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The precise molecular mechanism triggering maturation within the HIV Gag lattice remains largely unknown. Specifically, the reasons behind the Gag lattice's incomplete coverage (typically 30 – 60 %) of the virion membrane and the role of lattice edges in initiating maturation are unclear.
In this study, we first utilized sub-tomogram averaging Cryo-ET to examine virus-like particles (Gag VLPs) assembled in HEK293 cells with humanized Gag. Our findings reveal that these VLPs incorporate more Gag and exhibit significantly enhanced lattice coverage (60 – 90 %). To confirm this difference isn't due to expression levels, we also analyzed released particles from pNL4.3(Ψ:(Δ 105-278 & Δ 301-332)(SS2-)(ΔENV))) and observed similar increased Gag incorporation and enhanced lattice coverage (60 – 90 %).
To investigate the dynamics of lattice edges in these virions and VLPs, we engineered Gag VLPs and pNL4.3(D25N) virions to incorporate a SNAP tag within Gag and Gag-Pol molecules. Cryo-ET analysis confirmed that SNAP tag incorporation did not alter the observed lattice coverage in these particles. Subsequently, we performed single--molecule tracking of Gag molecules in immobilized Gag VLPs and Gag-Pol molecules in immobilized immature virions using minFLUX. This allowed us to observe molecules binding to and dissociating from a larger, immobile lattice in both VLPs and virions.
Our data collectively demonstrate the presence of freely diffusing Gag and Gag-Pol molecules on the inner leaflet of the virion membrane and highlight that the Gag lattice coverage is likely regulated by HIV factors independent of Gag itself.

FUNCTIONAL AND STRUCTURAL CHARACTERIZATION OF SARS-COV-2 VIRUS-LIKE PARTICLES FOR ASSEMBLY AND ENTRY MECHANISM STUDIES

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SARS-CoV-2 virus-like particles (VLPs) serve as a biosafe and adaptable model system for investigating the molecular mechanisms of viral assembly, spike (S) protein incorporation, and host cell entry (Syed et al. 2021 Science). We previously assembled and engineered fluorescently labeled SARS-CoV-2 VLPs containing viral M, N, E, S and N-GFP structural proteins (Gourdelier et al. 2022 Sci. Reports). These VLPs enable real-time visualization of entry events, facilitating the analysis of membrane fusion and endocytic uptake under BSL-2 conditions.
In this context, we employ immuno-spotting and fluorescence microscopy, correlative atomic force microscopy and cryo-electron tomography to characterize the morphology and properties of VLP (containing M, N, E, S and viral RNA) and to evaluate VLP spike incorporation level and distribution. The VLP can be tagged with N-GFP and contain the Spike, but not all. The particles exhibit diameters ranging from 80 to 130 nm and displayed surface spike densities of 1 to 5 trimers per VLP. We also introduce a packageable pseudo-viral RNA into the VLP and measure their capacity to enter pulmonary target cells thanks to the delivery of a reporter gene. This multi-modal approach provides insights into VLP architecture and entry competence, contributing to a more comprehensive understanding of SARS-CoV-2 biology.
Our findings establish a versatile platform for studying entry dynamics and support the development of spike--targeted therapeutics.

PREDICTING LATTICE REMODELING DYNAMICS FROM GAG BINDING KINETICS

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The remodeling of the Gag lattice following budding from the plasma membrane is essential for maturation to infectious virions. The strength and speed of Gag interactions with itself, the membrane, and its cofactors must have evolved not only for successful maturation, but also to ensure productive assembly and membrane budding. We combine reaction-diffusion simulations with *in vitro* and *in vivo* experimental data to predict how the assembly and remodeling dynamics of the retroviral Gag immature lattice relies on binding to essential co-factors to ensure productive and robust assembly of this >2000 subunit structure. By studying the dynamic remodeling of the lattice within budded virions, we demonstrated that the incomplete structure of the lattice, covering only ~2/3 of the membrane surface, supports unbinding and rebinding events along the edge of the lattice, allowing protease dimerization events at the minutes timescale. We provide theoretical guidelines that can be used to both extract key kinetic parameters from bulk experimental studies, and to design experimentally testable conditions to enhance or inhibit self-assembly. Finally, we show how the remodeling of the membrane that must occur during budding places strong energetic selection on pathways of Gag self-assembly. Localization of Gag proteins to the membrane can provide an additional timescale for controlling self-assembly and ensuring successful growth. Our work offers models and tools to quantitatively assess how the kinetics and stoichiometry of component interactions controls targeted self-assembly in complex environments.

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THE CONSERVED HIV-1 SPACER PEPTIDE 2 TRIGGERS MATRIX LATTICE MATURATION

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During proteolytic maturation, the Gag membrane binding domain, MA, undergoes structural transition between two distinct, hexameric protein lattices. However, the triggering mechanism of that structural transition was unknown. We demonstrate that spacer peptide 2 (SP2), a conserved peptide of previously unknown function situated ~300 residues downstream of MA, released during virus maturation, binds to MA and induces structural maturation. By determining high-resolution structures of MA within virus particles using cryo-electron microscopy, we show that MA does not bind lipid into a side pocket as previously thought, but instead binds SP2 as an integral part of the protein-protein interfaces that stabilise the mature lattice. Analysis of Gag cleavage site mutants shows that the release of SP2 is required for MA maturation. By reconstituting MA maturation in vitro using purified components and lipids, we demonstrate that SP2 is sufficient to induce maturation of MA. SP2-triggered MA maturation correlated with faster fusion of virus with target cells. These results and further recent data to be presented, reveal a novel interaction between two HIV-1 components, provide a high-resolution structure of mature MA, establish and characterise the trigger of MA structural maturation, and assign function to the SP2 peptide.

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STRUCTURAL MATURATION OF THE MA LATTICE IS NOT REQUIRED FOR HIV-1 PARTICLE INFECTIVITY

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The HIV-1 matrix protein (MA) is a key factor in both the early and late stages of the retroviral replication cycle. During viral assembly, MA directs the HIV-1 Gag polyprotein to the host cell plasma membrane, and during maturation, the membrane-bound MA lattice undergoes a structural rearrangement. However, the role of MA in regulating lipid dynamics in the viral membrane, and how the structural maturation of the MA lattice affects viral infectivity have not yet been fully characterized.
Here, aided by structural information from cryo-EM, virological assays, and molecular dynamics (MD) simulations, we studied the maturation and lipid-protein interactions of the wild-type (WT) MA lattice, as well as infectivity--deficient L20K MA mutant and its revertant L20K/E73K/A82T MA. Our MD simulations revealed that both immature WT and L20K/E73K/A82T MA lattices induce static regions in the membrane, leading to lipid aggregation, with phosphatidylinositol phosphates (PIPs) and phosphatidylserine (PS) being the most correlated lipids.
Furthermore, cryo-EM and MD simulations revealed that the mature WT and L20K MA virions exhibit well-ordered MA lattices that are stabilized by a network of inter-trimer salt bridges. However, the L20K/E73K/A82T MA mutations disrupt these interactions, resulting in a structurally unstable MA lattice that is undetectable in cryo-EM. This suggests that an ordered mature MA lattice is not essential for viral infectivity. Our results provide molecular insights into the protein-lipid and protein-protein interactions within the MA lattice, increasing our understanding of MA's role in the retroviral replication cycle.

PROBING HIV-1 GAG CONFORMATIONAL DYNAMICS INSIDE THE VIRION: A SINGLE-MOLECULE FRET BASED APPROACH TO STUDY VIRAL MATURATION

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HIV-1 virions are released from infected cells in an immature, non-infectious state. Maturation involves proteolytic cleavage of the viral Gag polyprotein at specific positions, which occurs at distinct rates. Proteolysis liberates the matrix (MA), capsid (CA), and nucleocapsid (NC) domains, leading to a dramatic change in the architecture of the virion. Based on structural data on the immature virion, we hypothesize that Gag conformational dynamics and discontinuities in the Gag lattice control the accessibility of the cleavage sites to the viral protease, thereby determining the kinetics of cleavage.
We developed a strategy to visualize the conformational dynamics of individual Gag proteins inside the virion using single-molecule Förster resonance energy transfer (smFRET) imaging. This allows us to elucidate the role of Gag conformational dynamics during proteolysis and maturation and determine the mechanisms of action of maturation inhibitors. We used an amber codon suppression technique to incorporate non-canonical amino acids (ncAAs) at desired positions in Gag. The ncAAs are then labelled with membrane-permeable donor and acceptor fluorophores. We find that maturation inhibitors that target Gag or that disrupt the viral membrane lipid content mediate Gag conformational dynamics, which informs on their mechanisms of action. Furthermore, our observations reveal allosteric connections between distal domains of Gag within the immature lattice, which likely contributes to the control of the kinetics of proteolysis.

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LENACAPAVIR: THE FIRST-IN-CLASS HIV CAPSID INHIBITOR WITH TWICE YEARLY DOSING FOR HIV TREATMENT AND PREVENTION

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Capsid is a structural component of HIV viral particle consisting of 1,500 subunits of p24 protein assembled into a closed fullerene cone structure that plays an essential role in multiple stages of HIV lifecycle, making it an attractive target for the design of new antivirals. In 2006, Gilead initiated a project focused on the identification of novel small molecule antivirals targeting HIV capsid. After a decade of focused research based on innovative screening approaches and unorthodox medicinal chemistry-driven optimization of antiviral potency, metabolic stability and physico-chemical properties with > 4,000 individual compounds synthesized and profiled, we have identified lenacapavir (LEN), a molecule with unprecedented combination of pharmacological properties that enable unique twice-yearly dosing regimen. In clinical studies, LEN dosed subcutaneously once every 6 months demonstrated efficacy both for HIV treatment and prevention including 100% protection against HIV acquisition in adolescent girls and young women. In June 2025, FDA approved LEN for HIV prophylaxis across broad populations including pregnant women. With its efficacy and prolonged dosing interval LEN represents a breakthrough in the fight against HIV global epidemic. In addition, LEN has inspired an emerging shift towards long-acting drug approaches in some of the other global health areas such as TB and malaria.

MECHANISTIC ANALYSIS OF INSTI RESISTANCE CONFERRED BY NC MUTATIONS

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People with HIV (PWH) receiving HIV integrase (IN) strand transfer inhibitors (INSTIs) have been reported to experience virological failure (VF) in the absence of resistance mutations in IN. To elucidate INSTI resistance mechanisms and pathways, we performed long-term passaging of HIV-1 with an escalating concentration of the INSTI dolutegravir (DTG). Independent of viral strain and cell type, HIV-1 became resistant to DTG by sequentially acquiring mutations in Env and nucleocapsid (NC), with the occasional appearance of IN mutations. The selected NC mutations confer levels of INSTI resistance comparable to those observed with clinically relevant IN mutations. These NC mutants retained replication kinetics and preserved viral DNA ends essential for integration and INSTI binding. However, the NC mutations accelerate the kinetics of viral DNA integration, suggesting that NC mutations limit the window of time during which INSTIs bind intasomes and block integration. To assess the clinical relevance of results from our cell-culture selections, we analyzed samples from PWH experiencing VF on a DTG-containing regimen (ACTG A5381). Notably, plasma HIV RNA sequences from some individuals at VF showed NC mutations similar to those observed *in vitro*, with a subset also carrying IN mutations. Phenotypic analysis demonstrated that mutations in NC and IN act in concert to increase resistance to DTG. These results provide insights into the mechanism by which NC mutations reduce the susceptibility of HIV-1 to INSTIs and underscore the importance of genotypic analysis outside IN in individuals experiencing VF to INSTI-containing regimens.

LENACAPAVIR RESISTANCE DIMINISHES HIV-1 ADAPTABILITY IN CULTURE

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Several resistance-associated mutations (RAMs) have been observed in the capsid (CA) gene of HIV-1 in Lenacapavir (LEN)-treated individuals. The most potent RAM, M66I, imposes a substantial fitness cost on the virus. Given its high mutation rate, it is important to anticipate how HIV-1 might circumvent this M66I-induced fitness defect in LEN--treated individuals. To address this, we propagated M66I in T cells to select for second-site compensatory mutations. Because the codon encoding methionine differs from those encoding isoleucine by only one nucleotide, this strategy repeatedly led to reversion to WT (I66M). Therefore, we examined the ability of other M66 mutations to recapitulate the M66I phenotype, specifically its resistance to LEN and fitness defect. Serial propagation of several M66 mutants revealed that only M66L, which is not resistant to LEN, readily acquires second-site compensatory mutations in CA that restore viral fitness. In contrast, M66 mutants that demonstrate high-level resistance to LEN (e.g. M66V and M66I) were unable to adapt in culture. Our findings suggest that the adaptability of HIV-1 is reduced by M66 mutations that confer LEN resistance, suggesting that the acquisition of LEN resistance at this amino acid residue may be an evolutionary trap that HIV-1 struggles to escape. Our ongoing efforts aim to understand the basic mechanisms underlying HIV-1 adaptation to LEN-susceptible M66 mutants (e.g. M66L), and to characterize the adaptability of other LEN-resistant HIV-1 variants.

A NOVEL IMMATURE DELTARETROVIRAL ASSEMBLY EXHIBITS AN HTLV-1-LIKE NTD STRUCTURE, WHILE FEATURING A UNIQUE CA-CTD STABILIZATION

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The capsid CA protein of the retroviral Gag polyprotein facilitates the assembly of the viral particle and consists of the N-terminal (NTD) and C-terminal (CTD) domains. While the CTD typically stabilizes immature assembly in most retroviruses, recent findings in the deltaretrovirus Human T-cell leukemia virus type 1 (HTLV-1) reveal that this function is instead carried out by the NTD, with the CTD not being involved in interhexameric interactions. These atypical assembly features in HTLV-1 have led us to investigate additional members of the Deltaretrovirus genus to better understand the specificity of NTD-driven immature assembly.
We selected the recently identified endogenous bat Eptesicus fuscus deltaretrovirus (EfDRV) as a model for this study. Despite the absence of a functional matrix (MA) domain and any reported evidence of replication, we demonstrated EfDRV CANC's strong assembly competence in our studies.
Using an in vitro Gag-derived VLP assembly system, we determined the high-resolution structure of the immature EfDRV CA lattice using single-particle cryo-EM. Our results highlight the significance of the NTD domain for the deltaretrovirus genus, where the inter- and intra-hexameric CA-NTD interfaces between EfDRV and HTLV-1 are highly similar in their immature lattice. However, HTLV-1 and EfDRV CA-CTD domain arrangements are distinct, with EfDRV CA-CTD dimers forming strong inter and intra-hexameric interactions. In addition, at the base of the CTD region, there is a unique peptide bundle, differing from previously reported 6-helix bundle stabilization mechanisms. Hence, our data reveal a novel and unexpected mechanism of immature viral assembly for another Deltaretrovirus member.

SAME BOLTS, DIFFERENT STRUCTURE: RETROVIRUSES USE IP6 IN DIFFERENT WAYS TO BUILD DIFFERENT CAPSIDS

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Forming and stabilising a capsid core is imperative for the infectivity of all retroviruses. Retroviruses build their core from over 1,000 capsid monomers that assemble into pentameric and predominantly hexameric capsomers. For HIV1, a recent breakthrough has identified the host metabolite IP6 as an essential building block for the core. It is coordinated in the central pore of every capsomer and is sufficient to stimulate the formation of the core.
To investigate whether IP6 utilisation is conserved more broadly in retroviruses, we have taken a combined biochemical, virological, and structural approach. Our results highlight that IP6 builds and stabilises the capsid in diverse retroviral genera. Unexpectedly, however, we find that whilst the use of IP6 is conserved – the binding site is not. Some retroviruses utilise an alternative IP6 binding site located between rather than within capsomers.
Using cryoEM, we have defined the coordinating residues in capsid-like particles and confirmed by mutagenesis their involvement in IP6-driven core assembly and infectivity. Viruses vary in their sensitivity to IP6 concentration in producer- and target-cells, potentially reflecting differing requirements for assembly versus stability. Importantly, IP6 sensitivity is lost when IP6-coordinating residues are removed.
We hypothesise that retroviruses commonly exploit IP6 to assemble and stabilise their capsids. However, the site of IP6 incorporation and the nature of its interaction may determine the unique structural and functional properties of each capsid. This may underlie key biological differences, such as their ability to infect non-dividing cells and inter--species transmission.

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INTERPLAY BETWEEN DDX3 AND HIV-1 CAPSID DURING EARLY INFECTION

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HIV-1, the causative agent of AIDS, relies on a tightly regulated interplay between viral and host factors during early infection stages. Its capsid protein (CA) orchestrates critical pre-integration events, including core transport, nuclear entry, uncoating, and integration site targeting. Using CA pull-down assays combined with SWATH-MS/MS proteomics, we identified the multifunctional DEAD-box RNA helicases DDX1 and DDX3 as novel CA-interacting host factors during early HIV-1 infection in MT-4 cells. Functional validation through siRNA-mediated knockdown in Jurkat E6-1 and primary blood mononuclear cells revealed a significant decrease in HIV-1 replication, as assessed by reverse transcriptase activity. Single-round infection assays and time-of-addition studies with small-molecule DDX3 inhibitors further localized their role to early post-entry steps. Digital droplet PCR revealed impaired vDNA integration upon DDX3 inhibition, and co-immunoprecipitation confirmed a specific interaction between CA and DDX3, but not with DDX1. Importantly, molecular dynamics simulations and mutational analysis were used to map the DDX3–CA interface, revealing key amino acids in both proteins critical for the interaction. These data suggest that DDX3 facilitates early replication events preceding vDNA integration, likely by interacting with CA to promote efficient core trafficking and/or uncoating. Small-molecule inhibitors of DDX3 significantly suppressed viral replication, underscoring its potential as a therapeutic target. Our findings broaden the understanding of host factors involved in early HIV-1 replication and highlight DDX3 as a promising candidate for the development of novel antiviral strategies targeting essential host-virus interactions.

HIV CAPSID DYNAMICS AND LENACAPAVIR'S ROLE IN REFASHIONING VIRAL REPLICATION

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Understanding HIV's replication process is crucial due to its significant implications for global health. Despite over forty years of HIV research and the development of more than 30 antiretroviral drug combinations, none have succeeded in eradicating the virus. However, recent years have witnessed the introduction of a ground-breaking drug, Lenacapavir (LEN), which represents the culmination of decades of basic research on viral nuclear import and the viral capsid. Studies from our lab and others have demonstrated that the viral core can directly interact with nuclear factors such as Nup153 and CPSF6. These findings challenge the conventional belief, demonstrating that the viral capsid can enter the nucleus and persist in infected cells, paving the way for innovative therapeutic strategies such as LEN.

We recently observed that viral RNA genomes accumulate within specific nuclear niches formed through phase separation, a phenomenon induced by the nuclear entry of the viral capsid. In these HIV-induced nuclear microenvironments, called HIV-1 membraneless organelles (HIV-1-MLOs), we identified the presence of viral cores. These organelles are composed of nuclear speckle factors, CPSF6, and viral cores. Notably, HIV-1-MLOs were found to persist for extended periods *in vitro* in infected macrophages. Furthermore, we detected these structures in monocyte-derived macrophages from humanized mice following HIV infection, emphasizing their *in vivo* relevance. Whether these organelles maintain viral cores for extended periods *in vivo* within specific tissues remains an open question. Our recent data demonstrate that HIV-1-MLOs serve as hubs for nuclear reverse transcription when a reversible inhibitor of reverse transcription is removed. To further explore the role of HIV-1-MLOs, we pharmacologically disrupted these structures during viral progression using PF74, a precursor molecule of Lenacapavir. This disruption activated cGAS, a dsDNA sensor, leading to the activation of the innate immunity.

We will discuss a newly observed effect in host nuclei when a physiological dose of LEN is applied. Interestingly, our findings indicate that a physiological dose of LEN administered during viral infection does not inhibit nuclear import or the formation of HIV-1-MLOs. However, cells pre-treated with LEN exhibit full resistance to high doses of LEN *in vitro*, aligning with recent data on PrEP using LEN. Notably, when LEN is administered during viral infection, it appears to act at a step following the formation of HIV-1-MLOs. The mechanistic roles of LEN in this process will be explored, along with its impact on the viral cores that accumulate within HIV-1-MLOs. To investigate these effects, our lab employs subtomograms and high-resolution nanoscale models for cryo-electron microscopy directly within the nuclei of infected cells.

Our current research focuses on elucidating how LEN influences and remodels infected cells, with a particular emphasis on its role in activating innate immunity, the fate and composition of HIV-1-MLOs, and its impact on proviral establishment and the chromatin environment.

THE JOURNEY OF THE HIV-1 CAPSID: FROM ASSEMBLY TO NUCLEAR ENTRY

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Retroviruses such as human immunodeficiency virus type 1 (HIV-1) possess a capsid that encloses the viral RNA genome along with essential enzymes and accessory proteins. The processes of capsid assembly, maturation, and intracellular transport are critical for successful viral replication. Moreover, the capsid surface serves as a dynamic interface for interactions with host cellular components, engaging both antiviral restriction factors and viral dependency factors. As a result, the capsid has emerged a promising target for antiviral therapy, exemplified by the recent development of the long-acting drug lenacapavir. In this presentation, I will discuss the mechanisms of HIV-1 capsid assembly and maturation, as well as its interactions with key host factors, including IP6 and cyclophilin A, highlighting novel interactions that are essential for the stabilization of the HIV-1 capsid. Additionally, I will present our recent in situ structural studies on the nuclear import of HIV-1 cores and the architecture of native chromatin fibres in T cells during HIV-1 nuclear transport, utilizing correlative light and electron microscopy.

DNA CONDENSATE INDUCED BY HIV-1 NUCLEOCAPSID PROTEIN RESISTS DISASSEMBLY TO PERSIST BEYOND FUNDAMENTAL TIMESCALE OF PROTEIN-DNA INTERACTION

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Recent studies have shown that intact, mature HIV-1 capsids can enter the host nucleus and that uncoating events correlated with successful integration occur after the completion of reverse transcription of the full-length viral genome. The nucleocapsid protein (NC) binds nucleic acids with high affinity and we have shown it condenses double--stranded (ds)DNA into compact conformations compatible with the tight confinement of the capsid. What happens to these NC-dsDNA complexes after capsid rupture releases both its nucleic acid and protein contents, however, is unclear. To probe these disassembly processes, we utilize optical tweezers, confocal imaging, and atomic force microscopy to isolate single long dsDNA molecules and measure their interactions with NC. When incubated with sufficiently high NC concentrations, or at equivalent NC to dsDNA ratio, the dsDNA spontaneously forms condensed structures that sequester NC. These NC-dsDNA condensates stabilize protein binding by decreasing dissociation by orders of magnitude as compared to the binding of individual NC proteins to uncondensed dsDNA. However, other nucleic acids compete for NC binding, both limiting condensate growth and destabilizing fully formed condensates. Such inter-nucleic acid interactions occur during the viral life cycle as the fully reverse transcribed proviral dsDNA coexists with partially digested fragments of the originally packaged viral RNA dimer prior to capsid uncoating and is exposed to the host genome after capsid uncoating. Thus, we hypothesize the dynamics of the NC-dsDNA complex play an important role in the efficiency and relative timing of reverse transcription, capsid uncoating, and integration required for infectivity.

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INTERFERON-INDUCED TRANSMEMBRANE PROTEINS EXHIBIT DISTINCT HIV-1 RESTRICTION PHENOTYPES WHEN EXPRESSED IN TARGET CELLS AND INCORPORATED INTO VIRIONS

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Humans encode three IFITMs exhibiting antiviral activity: IFITM1, -2 and -3. These proteins inhibit entry of many enveloped viruses, including HIV-1. IFITMs are small type II transmembrane proteins consisting of the cytoplasmic N-terminal domain (NTD), "intramembrane" domain (IMD), conserved intracellular loop (CIL), and a transmembrane domain (TMD), followed by a short C-terminal domain (CTD). Subcellular distribution of IFITMs largely determines the spectrum of restricted viruses. We and others have shown that IFITM3 interferes with the viral fusion step, likely by rigidifying the cell membranes (a "tough membrane" model). Mutagenesis studies have mapped the functionally important conserved IFITM domains and residues. The target membrane-modifying activity of IFITM3 has been linked to the conserved amphipathic helix (residues 59-68) and the ^{91}GxxxG^{95} oligomerization motif. In addition to blocking viral entry upon expression in target cells, IFITMs incorporate into budding virions and reduce infectivity of diverse enveloped viruses (termed "negative imprinting") *via* an unknown mechanism.
To elucidate the mechanism of negative imprinting, we compared the ability of IFITM3 mutants to protect target cells from HIV-1 infection and negatively imprint progeny virions. Strikingly, IFITM3 mutants that lacked antiviral activity in target cells effectively reduced HIV-1 infectivity upon incorporation into virions. This included IFITM3 variants with deleted amphipathic helix and mutated oligomerization motif that are critical for cell protection activity. The negative imprinting activity of IFITM3 mutants was not caused by disruption of HIV-1 Env incorporation or processing. To identify the IFITM domains essential for reducing virion infectivity, we swapped domains of cat IFITM1, which lacked negative imprinting activity, with the active human IFITM1. Chimeras exhibited varied levels of negative imprinting suggestive of a role for the TMD/CTD IFITM domains. Co-incorporation of human IFITM3 and inactive dog IFITM1 that formed mixed oligomers in virions, as evidenced by co-immunoprecipitation experiments, did not alter the human IFITM3's negative imprinting activity, suggesting a dominant phenotype of the antiviral IFITM3 that is independent of homo-oligomerization. To elucidate the basis for the lack of HIV-1 restriction by the IFITM3 mutants expressed in target cells, we analyzed their subcellular distributions and found that inactive mutants appear to be mislocalized compared to wild-type IFITM3, which is the likely explanation for their lack of antiviral activity. Together, our findings reveal the utility of a tractable pseudovirus system for defining the mechanism of IFITM-mediated restriction and demonstrate a critical role of IFITM trafficking and subcellular distribution in their antiviral activity.

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CORRELATIVE FLUORESCENCE LIFETIME AND ELECTRON MICROSCOPY TO UNVEIL FUNCTIONAL STATES OF HIV-1 MATURATION

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The Human Immunodeficiency Virus-1 (HIV-1) requires a maturation process enabling entry and infection into host cells. HIV-1 maturation occurs outside the producer cell and depends on an orchestrated cleavage of Gag, a viral precursor protein, by the viral protease.
In this study, we have developed a Förster Resonance Energy Transfer (FRET)-based biosensor to detect single virus maturation. The biosensor consists of a donor Fluorescent Protein (FP) and a Fluorescence-Activating and absorption--Shifting Tag (FAST) acceptor with affinity to various fluorogenic ligands. The FRET pair is introduced between the matrix and the capsid domains of HIV-1 Gag, flanked by protease cleavage sites, showing a decrease of FRET signal upon maturation. We have validated the biosensor by using different donor FP's and FAST ligand acceptor pairs covering the visible spectrum and a large range of lifetimes, enabling multiplexing with other fluorescent markers.
Importantly, this system preserves viral infectivity allowing visualization of the complete viral replication cycle. Fluorescence lifetime measurements of viruses bearing the biosensor were further conducted at physiological and cryogenic conditions, and subsequent correlation of FLIM with cryo-electron tomography (CryoET) analyses (termed functional CLEM) confirmed the correspondence between the biosensor lifetime, virus morphology and the efficiency of viral fusion and infectivity.
Functional CLEM constitutes a powerful tool for correlative studies of structure and dynamics of HIV-1 maturation. Furthermore, this system opens the door to other biological contexts, such as the study of protein-protein interactions in cells with genetically encodable FRET-based biosensors to inform correlative studies with CryoET.

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THE MULTIPLE ROLES OF CA IN HIV-1 REPLICATION

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The CA domain of the HIV-1 Gag plays many roles in viral replication. As a domain within the HIV-1 Gag precursor, CA governs the size and morphology of immature HIV-1 particles, while as an autonomous mature virion protein, CA directs events after viral entry such as nuclear import and integration site selection. The HIV-1 CA is comparatively conserved among viral proteins and exhibits a rather extreme intolerance to mutation. Perhaps for this reason CA has recently been proven to be an effective target for host antiviral proteins, and also for drugs that may transform HIV-1 prevention. In this presentation I will review my laboratory's past and present work that focuses on HIV-1 CA, in the areas of assembly, post entry events and as a target for host proteins and therapeutic agents with antiviral activity.

HIGHLY EFFICIENT HIV REVERSE TRANSCRIPTION AND INTEGRATION IN A CELL-FREE SYSTEM

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The mature, infectious HIV-1 virion contains a conical core, which comprises an outer capsid shell of CA protein subunits that surround the viral genome and enzymes. Upon infection, the core is released into the newly infected cell where the viral RNA genome is reverse transcribed into dsDNA, transported to the nucleus, and integrated into the host chromosome. The viral capsid is also the target of the potent inhibitor, Lenacapavir.
We previously described the development of a cell-free system for HIV reverse transcription and integration (Christensen et al.: Science 370 (2020) eabc8420). We will now describe improvements in this system that have led to greatly increased efficiency of reverse transcription (~70 % late RT products/input core) and integration (~20 % integration events/late RT product), starting with fully purified viral cores. We are now applying this system to investigate the mechanistic details of the first half of the viral life cycle, from reverse transcription through integration.

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LYSYL-TRNA SYNTHETASE SUPPRESSES CGAS–STING SIGNALING TO FACILITATE HIV-1 IMMUNE EVASION

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Viral infection is capable of activating the cGAS–STING-mediated immune pathway, which senses foreign double--stranded (ds) DNA and RNA:DNA (R:D) hybrids and induces type-I interferons. While the intact HIV-1 capsid shields viral nucleic acids until nuclear import, premature cytoplasmic uncoating exposes them to immune detection. Lysyl-tRNA synthetase (LysRS), which is responsible for tRNA aminoacylation, has non-canonical functions that facilitate HIV-1 replication, including a role in packaging host $tRNA^{Lys}$ into viral particles; this tRNA serves as the primer for HIV-1 reverse transcription, a process that produces dsDNA via R:D intermediates. HIV-1 infection triggers LysRS S207 phosphorylation, promoting LysRS dissociation from the multi-aminoacyl-tRNA synthetase complex (MSC) and enabling its non-canonical functions. Prior studies showed that components of the, Msc., including LysRS, bind R:D hybrids and suppress cGAS–STING activation; the role of aminoacyl-tRNA synthetases in HIV-1 immune evasion has not been investigated. In this work, we demonstrated LysRS binding to 20-bp R:D *in vitro*. Binding depended on the oligonucleotide/oligosaccharide-binding (OB)-fold domain of LysRS but was independent of S207 phosphorylation. Knockdown of LysRS in THP-1 cells significantly increased IFN-β expression, consistent with cGAS–STING suppression. Endogenous reverse transcription assays were performed to test whether LysRS affected cGAS-mediated detection of exposed reverse transcripts *in vitro*. We found that LysRS reduced cGAS activity towards HIV-1 viral DNA in a concentration-dependent manner. These findings reveal a new role for LysRS in HIV-1 immune evasion, which may lead to new therapeutic strategies that target host factors essential for viral replication.

UNVEILING A NEW ROLE FOR NUCLEOCAPSID IN REGULATING CGAS IMMUNE SENSING OF HIV-1

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HIV1 evades innate immune sensors in most target cells. However, our knowledge of how viral factors prevent detection of HIV-1 nucleic acids remains incomplete. HIV1 nucleocapsid (NC) condenses viral RNA inside the capsid lattice and facilitates steps of reverse transcription. As specific mutations in the zinc finger motifs within NC are linked with premature reverse transcription and defective virus particles, we hypothesized that these mutations might also affect innate immune sensing. We generated HIV1 virions containing mutations in NC and confirmed that these virions packaged ~100-fold more DNA than wild-type (wt) HIV1. Strikingly, NC mutant virions activated interferon responses in myeloid cells and were severely crippled in infectivity. We found that interferon responses persisted in the presence of a reverse transcriptase inhibitor, unlike what is observed for wt HIV1. To identify which innate immune sensing pathway is activated by NC mutant viruses, we generated CRISPR knockouts of key pathway components and discovered that loss of cGAS, STING, or IRF3 eliminated interferon activation, indicating that NC mutants are sensed through the canonical cGAS-STING pathway. To determine whether NC mutations alter virus core morphology, we visualized wt and mutant virions by electron microscopy and discovered that NC mutants displayed abnormal capsid lattice structures with less internal density, suggesting that reverse transcripts rupture the capsid lattice if not condensed properly. Together, our data indicate that NC has a role in regulating innate immune sensing of HIV1 and that decondensation of reverse transcripts can be exploited to activate interferon signaling in target cells.

ADAM17 IS AN HIV-1 RESTRICTION FACTOR ANTAGONIZED BY NEF

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Nef is an HIV-1 accessory protein critical to viral pathogenesis. While its role in immune evasion is well established, the mechanism by which Nef enhances virion infectivity remains incompletely understood. A disintegrin and metalloprotease (ADAM) family of proteins regulates the shedding of surface receptors, cytokines, and growth factors. ADAM17, also known as TNF-α converting enzyme (TACE), is responsible for cleaving pro-TNF-α to release its active, pro-inflammatory form. Here, we identify ADAM17 as a host restriction factor through affinity purification–mass spectrometry. ADAM17 associates with HIV-1 Env in the endoplasmic reticulum (ER), reducing Env expression and incorporation into virions, leading to an approximately 100-fold reduction in infectivity. This restriction is independent of ADAM17's metalloprotease activity and instead depends on its prodomain, which alters Env intracellular trafficking and broadly inhibits both laboratory-adapted and circulating HIV-1 strains. Nef counteracts this restriction by decreasing cellular ADAM17 levels through $Rab11^+$ endosome – and $CD63^+$ exosome–mediated extracellular export. Our results align with earlier work showing that Nef traffics ADAM17 into extracellular vesicles but extend those findings by clarifying the molecular requirements and the functional consequence for virion infectivity. Because ADAM17 activates TNF-α during secretion into extracellular vesicles, its removal by Nef from infected cells not only lifts a potent viral restriction but may also enhance inflammatory signaling in bystander cells. Thus, Nef appears to simultaneously promote viral replication and inflammation, "killing two birds with one stone."

WHY DOES THE MATRIX PROTEIN OF MASON-PFIZER MONKEY VIRUS INTERACT WITH CALMODULIN?

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Some viruses, including retroviruses, exploit host calcium signaling, particularly involving calmodulin (CaM), to regulate key stages of their life cycle such as entry, replication, and assembly. In HIV-1, proteins like Env, Nef, and Gag interact with CaM in a calcium-dependent manner[1]. The matrix protein (MA) binds CaM, triggering conformational changes and a myristoyl switch that exposes a lipid anchor[2]. This step is critical for membrane targeting and assembly of immature particles at the plasma membrane.
In contrast, Mason-Pfizer monkey virus (M-PMV), a betaretrovirus, assembles immature capsids in the cytoplasm before transporting them to the membrane for budding. Our studies show M-PMV MA also binds CaM, and CaM interacts with Gag in host cells. This interaction appears to promote particle maturation, possibly by inducing structural changes in MA. We provide indirect evidence that CaM regulates the myristoyl switch in M-PMV, as shown by protease-mediated cleavage of MA from Gag, a step dependent on myristoyl exposure and membrane interaction, serving as an indicator of switch activation.
Given their distinct assembly pathways, CaM likely plays different regulatory roles: promoting membrane association and multimerization at the plasma membrane in HIV-1, and coordinating maturation and membrane targeting after cytoplasmic assembly in M-PMV.
To be honest, the answer to the title question is: "we do not know yet", but we can hypothesize that the interaction of CaM with MA likely acts as a dynamic regulator of structural transitions essential for efficient retroviral assembly and maturation, tailored to the unique life cycle strategies of different retroviruses.

1 Front Microbiol. 2012 Feb 17:3:55.
2 J Biol Chem. 2010 Oct 18;285: 41911–41920

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HISTONE MODIFICATIONS GUIDING PROTYPE FOAMY VIRUS INTEGRATION SITE SELECTION

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It has long been observed that retroviruses display distinct integration site selection. In the case of lenti and gamma--retroviruses, this preference is guided by their cognate cellular cofactors (LEDGF/p75 and BET proteins) which preferentially bind select histone modifications. This allows lentiviruses, including Human Immunodeficiency Virus (HIV-1), to target active transcriptional units, and gamma-retrovirus, such as Murine Leukemia Virus (MLV), to target transcriptional start sites. What remains unclear is how retroviruses, such as prototype foamy virus (PFV), interact with chromatin in the absence of a cellular cofactor. PFV exhibits a more random integration pattern compared to HIV-1 or MLV, due partially to tethering vial viral Gag. We have performed innovative, modified ChIP-Seq with recombinant PFV integration complexes (intasomes) to map the histone post-translational modifications (PTMs) interactome. This analysis identified a strong preference for PTM H3K9me3 over other PTMs and compared to HIV-1 intasomes. Using an advanced protocol for integration site mapping, Rapid Amplification of Integration Sites without Interference by Genomic DNA contamination (RAISING), we have been able to map PFV integration sites. Surprisingly, this revealed that PFV displays a strong preference for ALU repeats compared to other retroviruses. The integration of PFV in ALU elements is correlated with H3K9me3, a PTM commonly found at these elements. Our results, for the first time, reveal how PFV integrase interacts with select PTMs, allowing PFV to integrate into transcriptionally inactive intergenic regions. This could explain why PFV is non-pathogenic in humans, as well as in its natural host, with reduced risk of insertional mutagenesis.

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CIS- AND *TRANS*-ACTING FACTORS THAT MEDIATE NUCLEAR RETENTION OF INTRON-CONTAINING HIV-1 TRANSCRIPTS

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How intron containing HIV-1 transcripts are retained in the nucleus is a long-standing question. The majority of cellular pre-mRNAs are alternatively spliced, and recruitment of mRNA export factors is closely coupled with splicing. Accordingly, the first widely accepted model proposes that lack of splicing leads to a block in deposition of RNA export factors on the unspliced and partially spliced HIV-1 transcripts. However, it is noteworthy that ~5% of protein-coding genes in humans do not contain introns. Hence, splicing is not a strict pre-requisite for RNA export. The second model proposes that intron-containing HIV-1 transcripts are actively retained in the nucleus due to distinct features present within these RNAs. This model is supported by the finding that codon-optimization of unspliced and partially spliced viral mRNAs overcomes nuclear retention independent of splicing modulation. On the other hand, the minimal sequence features necessary and sufficient for nuclear retention and whether *trans*-acting host factors are involved remain unknown.
A distinguishing feature of the HIV-1 genome is its unusually biased nucleotide composition, rich in adenosines (~36%) and poor in cytosines (~18%). We found that altering the codon usage of multiple reporter genes (e.g. GFP, mCherry, firefly luciferase) to resemble HIV-1 codon usage, hence making them adenosine-rich, results in their nuclear retention and dependence of reporter gene expression on Rev/RRE. We will present ongoing work on the minimal features of these RNAs that result in nuclear retention. We will also present preliminary findings from ongoing genome-wide CRISPR and siRNA screens where we leveraged these minimal HIV-1-like reporter RNAs to identify putative host factors that mediate nuclear retention of HIV-1 unspliced and partially spliced transcripts.

LENACAPAVIR DISRUPTS HIV-1 CORE INTEGRITY WHILE STABILIZING THE CAPSID LATTICE

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**These authors contributed equally to this work.*

Lenacapavir (GS-6207; LEN) is a potent HIV-1 capsid inhibitor approved for treating multidrug-resistant infection. LEN binds to a hydrophobic pocket between neighboring capsid (CA) proteins in hexamers and stabilizes the capsid lattice, but its effect on HIV-1 capsids in vitro and in infected cells is not fully understood. To assess LEN's impact on HIV-1 capsids, we labeled the HIV-1 cores with green fluorescent protein fused to CA (GFP-CA) or a fluid-phase GFP content marker (cmGFP). HIV-1 cores labeled with GFP-CA, but not cmGFP, could be immunostained with an anti--GFP antibody and were less sensitive to the capsid-binding host restriction factor MX2, demonstrating that GFP--CA is incorporated into the capsid lattice and serves as a reporter for capsid lattice stability whereas cmGFP is an indicator of core integrity. LEN treatment of isolated HIV-1 cores resulted in a dose-dependent loss of cmGFP signal while preserving the GFP-CA signal, indicating that LEN disrupts core integrity but stabilizes the capsid lattice. In contrast, capsid inhibitor PF-3450074 (PF74) induced loss of core integrity and the capsid lattice. Electron microscopy of LEN- or PF74-treated viral cores revealed frequent breakage at the narrow end of the capsid and other morphological changes. Our results suggest that LEN treatment does not prevent nuclear envelope docking but inhibits nuclear import of cores with or without loss of core integrity. In contrast, PF74 treatment blocks nuclear import by inhibiting the nuclear envelope docking of viral cores, highlighting their different mechanisms of nuclear import inhibition.

HIV-1 UNCOATING INSIDE NUCLEAR SPECKLES STRONGLY ENHANCES INTEGRATION INTO SPECKLE-ASSOCIATED CHROMATIN DOMAINS

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HIV-1 cores enter the nucleus, localize to nuclear speckles (NSs), and uncoat near their integration site following reverse transcription. However, the spatial relationship between the uncoating site and integration site remains poorly understood. Using fluorescently labeled HIV-1 cores and NS markers, we found that loss of viral core integrity and capsid disassembly occur predominately within NSs. Treatment of infected cells with capsid inhibitors PF-3450074 (PF74) or lenacapavir (LEN) after nuclear entry disrupted capsid binding to cleavage and polyadenylation specificity factor 6 (CPSF6), leading to rapid displacement of viral cores from NSs. Both inhibitors induced loss of viral core integrity; however, PF74 caused capsid rupture primarily after cores exited NSs, whereas LEN triggered rupture before core exit. To determine location of proviral integration, we synchronized uncoating by disrupting viral cores containing small genomes with PF74 or LEN and visualized HIV-1 transcription sites. Despite differences in location of viral core rupture, proviruses resulting from PF74- or LEN-induced uncoating were located at similar distances from NSs (~1.0 µm), consistent with viral DNA release occurring outside NSs. Notably, these proviruses were positioned ~0.4 µm further from NSs than proviruses derived from full-length vectors in untreated control cells. This modest spatial shift correlated with a dramatic reduction in integration into gene-rich, transcriptionally active speckle-associated chromatin domains (SPADs), the preferred target of HIV-1 integration. Together, our findings reveal that the location of uncoating and viral DNA release critically influences HIV-1 integration site selection, and that capsid inhibitors alter integration preferences by shifting the site of uncoating.

DECIPHERING THE BIOGENESIS OF HIV-INDUCED CPSF6 PUNCTA

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Understanding HIV's replication process has crucial implications for global health. Our investigations focused on the HIV's post-nuclear entry steps, which involve the engagement of HIV elements with several nuclear factors, favouring the viral replication. Our study targeted Cleavage and Polyadenylation Specificity Factor subunit 6 (CPSF6) puncta, induced by HIV nuclear entry, to identify the crucial CPSF6 domains for the clustering. Exploiting genetic manipulation, we produced CPSF6 mutant proteins lacking different intrinsically disordered domains (IDRs) potentially responsible for HIV-induced CPSF6 puncta. Using confocal microscopy, we showed that only the depletion of CPSF6 FG motif inhibits HIV-induced CPSF6 puncta formation. This enlightens FG peptide involvement in the clustering of CPSF6 proteins which is mainly prompted by the interaction of the FG peptide with the hydrophobic capsid pocket along adjoining hexamers, while the mixed charged domain is dispensable for the clustering. Interestingly, in absence of HIV-induced CPSF6 puncta (cells KO for CPSF6 or expressing CPSF6 deleted for FG peptide), the viral release and the viral expression are reduced. Investigating the dynamics of the HIV-induced CPSF6 puncta, we were also able to elucidate the role of Nuclear Speckles (NS) factors, such as SRRM2, in CPSF6 puncta biogenesis. The results demonstrated a significant role of the IDR of SRRM2 in promoting and stabilizing the biogenesis of HIV-induced CPSF6 puncta in NS. Overall, these findings significantly contribute to our understanding of HIV replication and challenge existing paradigms providing clear evidence of the nuanced roles played by NS factors and CPSF6 domains in the viral life cycle.

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P01
HIGH-RESOLUTION STRUCTURAL INSIGHTS INTO IMMATURE MURINE LEUKEMIA VIRUS

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Murine leukemia virus (MLV) is a member of the gamma retrovirus family. In its immature form, the main structural Gag protein consists of MA, p12, CA and NC. The *in-situ* structure of the immature CA was previously resolved by subtomogram averaging to a resolution of 7 Å. We have investigated the structure and arrangement of immature MLV particles by a combination of cryo electron microscopy and tomography, subtomogram averaging and single particle analysis. Among our results, we have resolved the structure of the CA layer to a resolution of 3.6 Å, revealing a well-ordered N-terminal domain, a less ordered C-terminal domain, and a predicted 50 Å long six-helix bundle at the C-terminus. I will present our current data on the structure and arrangement of immature MLV.

P02
CALMODULIN INVOLVEMENT IN THE LATE PHASE LIFE CYCLE OF MASON-PFIZER MONKEY VIRUS

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Some viruses exploit host calcium signaling pathways to regulate key steps in their life cycle. Calmodulin (CaM), a ubiquitous calcium-sensing protein, is known to interact with several viral proteins, modulating processes such as viral replication and assembly. Although the interaction between CaM and HIV-1 matrix (MA) protein has been characterized in detail, the exact role of CaM in the replication of retroviruses remains unclear.
In this study, we investigated the interaction between the MA protein of Mason-Pfizer monkey virus (M-PMV) and CaM. Using microscale thermophoresis (MST), we demonstrated that M-PMV MA binds CaM in a calcium-dependent manner. Co-immunoprecipitation assays from HEK293T cells co-expressing the M-PMV structural polyprotein Gag and HA-tagged CaM confirmed the relevance of this interaction *in vivo*. Furthermore, we found that CaM enhances proteolytic cleavage of myristoylated M-PMV MAPP, an MA construct extended with a downstream region of the phosphoprotein that mimics the M-PMV Gag precursor. This suggests that CaM binding facilitates the myristoyl switch of M-PMV MA. This is supported by liposome binding experiments showing that CaM promotes MA association with liposomes mimicking the cytoplasmic membrane. Formation of CaM–MA complexes was further confirmed by protein crosslinking followed by mass spectrometric analysis. Together, our findings provide new insights into the interaction between CaM and M-PMV MA, highlighting a potential regulatory mechanism during the late phase of the retroviral life cycle.

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P03
IMPACT OF MYRISTOYL SWITCH-TRIGGERED MATRIX DOMAIN CLEAVAGE ON THE LATE STAGE OF MASON-PFIZER MONKEY VIRUS LIFE CYCLE

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Retroviral particles formed during the late phase of the viral life cycle must undergo maturation to become infectious. This process involves proteolytic cleavage of the Gag and Gag-Pol polyproteins by the viral protease at specific cleavage sites. In C-type retroviruses like HIV, which assemble at the plasma membrane (PM), maturation could be triggered by PM interaction and protease dimerization during particle assembly. In contrast, D-type retroviruses such as Mason-Pfizer monkey virus (M-PMV) assemble in the cytoplasm. Immature particles are transported to the PM in an unprocessed state, where budding and maturation occur. Thus, maturation must be tightly regulated, and protease dimerization alone is unlikely to trigger the maturation process.
We previously showed that *in vitro* cleavage of the myristoylated matrix (MA) domain from the downstream phosphoprotein (PP) domain of Gag by M-PMV protease is induced by MA-liposome interaction. This cleavage is facilitated by a myristoyl switch-triggered conformational change at the cleavage site.
To assess the role of this cleavage in viral maturation *in vivo*, we generated vectors encoding noninfectious particles with amino acid substitutions affecting MA myristoylation, myristoyl switch, or MA-PP cleavage. β-branched amino acids, typically used to inhibit HIV-1 protease, did not block MA cleavage in M-PMV. However, M100P, A101K and A102K substitutions effectively prevented cleavage. Mutant particles were produced in HEK293T cells, and the effects on viral maturation were evaluated.

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P04
MISMATCH RECOGNITION AND PROOFREADING DYNAMICS WITHIN THE SARS-COV-2 REPLICATION-TRANSCRIPTION COMPLEX

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The replication–transcription complex (RTC), responsible for RNA synthesis, is a prime target for antiviral drug development against SARS-CoV-2 and other coronaviruses. The RTC comprises the RNA-dependent RNA polymerase (RdRp) complex, composed of the catalytic subunit nonstructural protein (nsp) 12 and the cofactors nsp7 and nsp8, along with other accessory proteins. Crucially, it also incorporates a 3′ – 5′ exonuclease (ExoN) that performs proofreading and can remove antiviral nucleotide analogs.
Despite the importance of RdRp-ExoN regulation for designing novel antivirals, the precise molecular mechanism of mismatch recognition and removal remains unknown.
In this study, we investigated the effect of misincorporated ribonucleotides at the 3′ end within the context of the dsRNA–RdRp complex, using a combination of enzymatic and biophysical methods. This revealed that dsRNA length and mismatch identity significantly affect the dsRNA–RdRp complex stability. Subsequent catalytic activity evaluation showed that mismatches can either slow polymerase kinetics, inhibit RdRp activity, or be ignored, correlating with prior stability assessment.
We then assessed whether ExoN can cleave the RNA bound to RdRp and restore the polymerase activity. Consistent with previous reports, cleavage was significantly reduced, while the activity was restored only to a very limited extent, suggesting the involvement of additional regulatory proteins.
Finally, we explored the impact of additional RTC components, specifically the nsp13 helicase and RNA-binding protein nsp9. Notably, nsp13 further stabilized the RNA, preventing cleavage while also suppressing the RdRp activity.
Altogether, this study highlights the intricate regulatory interplay within the RTC and provides insights for future mechanistic investigations.

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P05
THE IMPORTANCE OF THE G-PATCH MOTIF OF MASON-PFIZER MONKEY VIRUS IN THE RECRUITMENT AND ACTIVITY OF CELLULAR DHX15

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The G-patch motif is a glycine-rich region composed of 45 amino acid residues that occurs exclusively in G-patch proteins of eukaryotic cells. G-patch proteins participate in fundamental cellular processes, particularly those associated with RNA metabolism. Specifically, the G-patch mediates protein-RNA or protein-protein interactions, specifies the target, or enhances the enzymatic activity. Interestingly, despite no cells different from eukaryotic cells contain G-patch proteins, betaretroviruses evolutionarily preserved this motif. Last year, we discovered a direct relationship between the G-patch localized within Gag-Pro and Gag-Pro-Pol polyproteins of the betaretrovirus Mason-Pfizer monkey virus (M-PMV) and the presence of the RNA helicase DHX15 in M-PMV virions. DHX15 was revealed to be involved in two essential processes in the M-PMV replication cycle: gRNA packaging and reverse transcription. Our study documented that DHX15 is recruited by the G-patch via a mode similar to that published for the interaction of DHX15 with the cellular G-patch protein NF-κB Repressing Factor (NKRF). The secondary structure of the M-PMV G-patch corresponds to that of the cellular G-patch and includes two structural elements: a brace helix (BH) and a brace loop (BL). To investigate the interchangeability and importance of the M-PMV G-patch motif, we substituted M-PMV BH for NKRF BH and examined its impact on the incorporation of DHX15 into the virions, M-PMV gRNA packaging and helicase activity. Surprisingly, the substitution negatively affected the recruitment of DHX15 more than we expected. Our results indicate that the betaretroviral G-patch interacts and activates DHX15 more effectively than its native cellular interaction partner.

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P06
A STOCHASTIC COUNTING METHOD FOR MEASURING THE DISTRIBUTION OF PLASMIDS BEING TRANSCRIBED IN INDIVIDUAL CELLS AFTER STANDARD TRANSFECTION

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Transfection efficiency is an important factor in retroviral research, particularly when optimizing experimental protocols to control the number of plasmids incorporated into a cell. Plasmid uptake and transcription acts as a key confounding variable influencing the relationship between pre-transfection plasmid concentration and subsequent gene expression levels. Accurately accounting for plasmid uptake is crucial for replicating experimental findings and validating methodologies across the retroviral research field. To address this, we developed a quantitative approach to estimate the average number of plasmids taken up and transcribed by cells during transient chemical co-transfection. Using flow cytometry analysis, we co-transfected cells with three HIV-1 plasmids derived from NL4.3 encoding green fluorescent protein (GFP), red fluorescent protein (mCherry), and a non-fluorescent control plasmid (Dark). By applying statistical models, including Poisson and Gaussian distributions, we fit the experimental data to estimate plasmid uptake and distribution within the cell population. Our methods were tested for reproducibility using three cell lines, HeLa (TZM-BL)*, Jurkat-Tag and HEK293 cells. This study demonstrates the importance of accounting for plasmid uptake in retroviral research and provides a robust method for quantifying and controlling this variable to improve experimental consistency and reliability across studies.

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P07
MODULATION OF HIV-1 GAG SELF-ASSEMBLY TIME COURSE BY SUBSTRATE STIFFNESS

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HIV-1 Gag self-assembly in living cells has been proposed to occur in 5 minutes in many different studies. This is a surprising long time when compare to other budding event occurring in cells, such as clathrin coated pits formation that last less then 30 s. Up to now only one paper revisited this long time experimentally and suggested that the self-assembly occurs in less than 30 s when not observed on cell membranes close/bound to a rigid substrate (1).
Self-assembly depends on the free energy needed to add each single Gag to the on-going cluster and a mechanical contribution due to membrane bending. We've previously observed in living T cells that this free energy is low ($<4k_BT$) suggesting reversibility at each step of the reaction, making the assembly long to achieve (2). In addition, self-assembly depends on the nucleation step which is directly sensitive to the diffusion of each Gag monomers. We have shown that diffusion restriction of Gag by actin meshwork strongly impact the rate of nucleation and decrease the surface density formation of self-assembly (3). Budding on a membrane facing a rigid substrate such as glass can result in additional energetic contribution (detaching a patch of membrane from the surface) or an additional diffusion restriction (due to friction), both leading to longer assembly time course and reduced density.
Here we first monitored the time-course of HIV-1 Gag on supported lipid bilayers with controlled lipid molar composition using QCM-d, confocal and STED microscopy and AFM. Then, we started to fabricate and characterize lipid bilayers on top of polyacrylamide gels with different stiffness, as monitored by force spectroscopy. First results obtained on these substrates will be presented in addition to the whole work on glass.
This work will unravel how the time course of HIV-1 Gag self-assembly can be modulated by substrate stiffness.

(1) Rapid formation of human immunodeficiency virus-like particles. Bednarska J, Pelchen-Matthews A, Novak P, Burden JJ, Summers PA, Kuimova MK, Korchev Y, Marsh M, Shevchuk A. PNAS 2020
(2) Single molecule localisation microscopy reveals how HIV-1 Gag proteins sense membrane virus assembly sites in living host CD4 T cells. **Sci Rep**. 2018 8(1):16283
(3) HIV-1 diverts cortical actin for particle assembly and release. **Nat Comms.** 2023 14(1):6945

P08
PURIFICATION OF HIV-1 CORE PARTICLES THAT MAINTAIN FULL ACTIVITY FOR ENDOGENOUS REVERSE TRANSCRIPTION AND INTEGRATION

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During the first half of the HIV-1 life cycle, the virus reverse transcribes its RNA genome and integrates the double--stranded DNA copy into a host cell chromosome. These processes are challenging to study in mechanistic detail as they occur deep inside the cell when one virus infects a single cell. To address these limitations, we previously reconstituted endogenous reverse transcription (ERT) and integration in a cell free system, starting with isolated HIV-1 virions.[1]
Here, we report that we have improved our cell free assay by isolating pure HIV-1 core particles using chromatographic techniques. These pure HIV-1 core particles are fully active for ERT and integration. We have quantitated the efficiency of ERT and integration using flow virometry to count the number of core particles in solution and droplet digital PCR (ddPCR) to measure viral RNA (vRNA) and ERT and integration products. We show that pure HIV-1 core particles contain 2 vRNA copies/core particle and we can achieve 60-100% efficiency of late ERT per core. In the presence of HeLa cell nuclear lysate, we observe an integration efficiency of up to 7% events/core, and up to 20% efficiency of integration events per late RT product. We are applying our ERT/Integration assay with purified HIV-1 cores to investigate the mechanistic details of the first half the viral life cycle from reverse transcription through integration.

1) Christensen, et al. *Science* **370**: 197 (2020)

P09
INVESTIGATING THE ROLE OF TRANSCRIPTION START SITE USAGE ON HIV-1 TRANSLATION INITIATION

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The 9.2 kb HIV-1 unspliced viral RNA serves a dual purpose; it functions as the viral genome, packaged into new viral particles as a dimer, and as an mRNA template for the synthesis of Gag and Gag-Pol. HIV-1 RNA transcription by host cell RNA polymerase II uses neighboring transcription start sites to produce unspliced RNAs containing one (1G), two, or three (3G) 5′ guanosines and a modified 5′ cap. Despite 3G being the predominant species in the cell, 1G is selectively packaged as genomic RNA (gRNA). We have previously shown that the 5′ UTR of 1G RNAs adopt distinct conformational ensembles that favor selective packaging by exposing structural elements required for efficient gRNA dimerization, Gag binding, and Gag multimerization. Mutations that eliminate structural differences between the 1G and 3G 5′ UTR abolish selective 1G packaging. Recently, we reported that while both 1G and 3G RNAs can be translated, 3G RNAs are translated more efficiently *in vitro* and in cells; translation efficiency of HIV-1 unspliced RNA is also regulated by 5′ UTR conformation. However, the underlying mechanisms that govern HIV-1 translation initiation are not well understood. Electrophoretic mobility shift assays and preliminary single-molecule FRET experiments suggest that both 1G and 3G RNAs are bound similarly by eukaryotic initiation factor 4 F, the heterotrimeric complex responsible for 5′ cap recognition and mRNA activation. Ongoing work is aimed at testing the hypothesis that distinct 5′ UTR conformational ensembles result in altered host factor interactions, ribosome scanning efficiencies, and distinct ribosome profiles.

P10
TO CLEAVE OR NOT TO CLEAVE: EFFECTS OF CLEAVAGE SITE MUTATIONS ON M-PMV REVERSE TRANSCRIPTASE ACTIVITY AND VIRAL INFECTIVITY

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Reverse transcriptase (RT) plays a central role in retroviral replication by converting viral RNA into DNA. While RTs from most betaretroviruses are monomeric, the RT of Mason-Pfizer monkey virus (M-PMV) appears to form a heterodimer, resembling the lentiviral RT of HIV-1. In M-PMV, the RT is initially translated as a part of a Gag-Pro--Pol polyprotein and during maturation it undergoes proteolytic cleavage not only at its N- and C-terminus, as well as between the polymerase and RNase H domains. This internal cleavage occurs in only one subunit, yielding a functional heterodimer. To investigate the role of precise proteolytic processing between the polymerase and RNase H domains, we introduced point mutations into the reverse transcriptase cleavage site. HEK293 cells were transfected with these mutant constructs, and viral particles were harvested. RT activity was quantified using a qPCR-based assay, protein processing was analyzed by western blotting, and virion infectivity was assessed by measuring GFP fluorescence in target cells using flow cytometry.
Although none of the mutations completely blocked the internal cleavage, two appeared to shift the cleavage site, as indicated by changes in the molecular weights of the polymerase and RNase H fragments. One mutation resulted in the complete loss of enzymatic activity, despite RT expression, while other mutations caused a partial reduction in RT activity and infectivity. Altogether these data suggest that the accuracy of the internal cleavage is critical for RT function.

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P11
INVESTIGATING THE RNA STRUCTURE OF THE HIV-1 CENTRAL POLYPURINE TRACT (CPPT) REGION: IMPLICATIONS FOR VIRAL FITNESS, ANTIVIRAL DRUG DESIGN AND GENE THERAPY VECTOR BIOLOGY

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HIV-1 is known for the high mutation rate of its RNA genome, which can lead to changes in the RNA structure. Mathematical analyses conducted by Skittrall et al. (PMID:31545786) showed the cPPT to display an evolutionarily conserved stem-loop structure together with the surrounding nucleotides. This RNA sequence is coding within HIV, hence we worked with lentiviral vectors to enable suitable mutagenesis to investigate the RNA structure's function without affecting protein function. Some vectors lack the nucleotides 5' to the cPPT, meaning that they cannot form this proposed cPPT stem-loop. Here, we compared the transduction performances of lentiviral transfer vectors with (M1) or without (parent) the cPPT stem-loop-forming nucleotides, alongside a panel of mutants that were designed to stablise/destabilise the stem-loop structure, based upon M1.
Transduction results demonstrated an overall negative effect of the introduction of the cPPT stem-loop on viral transduction, while its over-stabilisation further impaired the viral infectivity. Virion production by various mutants were similar but all were lower than the parent. In-gel SHAPE probed the RNA structure of the cPPT in parent, M1 and M5 (designed to have a stabilised stem-loop) and suggested structural flexibility in parent and M1 but the formation of a stable stem-loop in M5.

P12
CO-PACKAGING OF TBEV PROTEASE AND MINIGENOME IN FLAG-TAGGED VIRUS-LIKE PARTICLES AND EXTRACELLULAR VESICLES

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Tick-borne encephalitis virus (TBEV) is a positive-sense RNA virus belonging to the flavivirus genus: It is the causative agent of tick-borne encephalitis, which is a serious disease affecting the central nervous system.
Virus-like particles (VLPs) derived from TBEV represent a promising tool for studying viral assembly and RNA packaging due to their structural similarity to native virions and ability to encapsulate genetic material without being infectious. We engineered TBEV VLPs by expressing TBEV structural proteins: capsid (C), premembrane (prM), and envelope (E) proteins, in mammalian cells. To facilitate detection and tracking, we inserted a FLAG-tag into the C protein of these VLPs.
We generated a series of TBEV-derived minigenomes of varying lengths and sequence compositions, focusing on the presence or absence of untranslated regions (UTRs). Our results demonstrate that minigenomes containing an intact 3' UTR or 5' UTR are incorporated into VLPs more efficiently than truncated variants lacking these UTRs or those extended with both UTRs. This system enables us to study the critical role of UTRs in selective RNA packaging, providing a versatile system to dissect the contributions of specific genomic elements to RNA encapsidation.
Additionally, we conducted a detailed analysis of the packaging and intracellular co-localization of TBEV-derived minigenomes, together with the protease complex (NS2B–NS3) or TBEV structural proteins (C, prM, E) within extracellular vesicles (EVs) secreted by producer cells. The observed association of viral components with EVs suggests a potential pathway for RNA transport and intercellular communication that may contribute to viral dissemination or immune modulation.

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P13
ENGINEERED ZYMOGENS OF THE SARS-COV-2 MAIN PROTEASE FOR INHIBITOR EVALUATION

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The main protease (M^{pro}, also known as 3CLproor NSP5) of SARS-CoV-2 is essential for viral replication and a validated drug target. Several M^{pro} inhibitors are in clinical use. Paxlovid, approved by the FDA and EMA, contains nirmatrelvir, a covalent reversible inhibitor of M^{pro}, boosted by ritonavir. Simnotrelvir, also ritonavir-boosted, is marketed in China as Xiannuoxin. Ensitrelvir, a non-covalent inhibitor, is approved in Japan as Xocova.
M^{pro} is initially synthesized as part of the viral polyproteins pp1a and pp1ab. It becomes catalytically competent only after autocatalytic cleavage from the polyprotein, followed by homodimerization. The coupling of autoprocessing and dimer formation is not fully understood. Targeting the early steps of M^{pro} maturation could inform the development of innovative antiviral strategies.
To investigate this, we designed a series of artificial Mpro zymogens, including mutants impaired in autoprocessing. We used multiple approaches: bacterial expression, in vitro biochemical assays with purified zymogens, and a mammalian fluorescence-based reporter system. Our experiments revealed distinct sensitivities of *cis*- and *trans*--cleavage to inhibition, both by clinically approved drugs and novel small molecules. Surprisingly, under specific conditions, the zymogens exhibited a propensity for activation rather than inhibition.
These findings underscore the complexity of M^{pro} maturation and suggest that artificial zymogens can serve as powerful tools to probe protease activation mechanisms and evaluate inhibitors targeting pre-mature forms of the enzyme.

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P14
MANIPULATING CELLULAR INOSITOL PHOSPHATE LEVELS HAS DIFFERENT EFFECTS ON A RANGE OF RETROVIRUSES

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A wide variety of lentiviruses have been shown to use Inositol hexakisphosphate (IP6) to build and stabilise their capsids. We have been able to study this requirement through manipulation of the cellular enzymes required for inositol phosphate (IP) biosynthesis. Such manipulation not only changes the absolute levels of IP6, but also changes the landscape of inositol phosphates in the cell. By using these as tools, we are able to study the requirement, for not only IP6, but also other inositol phosphate species in a range of retroviruses not limited to lentiviruses.
We have previously shown that HIV specifically packages IP6 during assembly, in order to use it to build and stabilise the mature capsid. As such, there is a requirement for sufficient levels of IP6 during production, with levels in target cells having little impact for WT virus. Other retroviruses, in contrast, show a requirement for IPs both in target cells and producer cells. We have recently discovered that retroviruses are able to exploit the use of IP6 to build their capsids by binding at different sites in the mature capsid. This difference is reflected in the impact IP depletion has on production and infection of virions.

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P15
PHARMACOLOGIC HYPERSTABILISATION OF THE HIV-1 CAPSID LATTICE INDUCES CAPSID FAILURE

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The HIV-1 capsid has emerged as a tractable target for antiretroviral therapy. Lenacapavir is the first capsid-targeting drug approved for medical use. Here we investigate the effect of Lenacapavir on HIV capsid stability and uncoating. We employ a single particle approach that simultaneously measures capsid content release and lattice persistence. We demonstrate that Lenacapavir's potent antiviral activity is predominantly due to lethal hyperstabilisation of the capsid lattice and resultant loss of compartmentalisation, leading to a loss or reverse transcription. This study highlights that disrupting capsid metastability is a powerful strategy for the development of novel antivirals.

P16
INTERACTION OF CELLULAR HELICASE DHX15 AND THE MASON-PFIZER MONKEY VIRUS GENOME

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RNA helicase DHX15 is involved in numerous steps of the cellular metabolism. Its activation by protein cofactors containing a glycine-rich sequence known as the G-patch domain (GPD) is crucial for its proper function. GPD has been identified in Mason-Pfizer monkey virus (M-PMV), in which it positively influences reverse transcriptase activity and viral infectivity. Similarities in the sequence and secondary structure of M-PMV GPD and cellular GPD proteins known to interact with DHX15 (e.g., NF-kappa-B-repressing factor) suggest shared mode of action. This is confirmed by the finding of DHX15 in purified M-PMV particles, but only those containing intact GPD, not in those with GPD-deleted version.
Using Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation, a potential binding site of DHX15 was identified at the 3' end of M-PMV genome. This region consists of the constitutive transport element, an RNA element essential for the export of unspliced viral RNA from nucleus, and its upstream sequence. Binding of DHX15 to this region was confirmed using microscale thermophoresis. Mutations in this region led to a decrease in the amount of DHX15 in purified M-PMV virions and also to a decrease in the virion's gRNA content.
A more profound comprehension of the interaction between DHX15 and M-PMV genome could provide a novel perspective on the metabolism of viral RNA during the retroviral infection.

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P17
NUCLEAR ENTRY OF MASON-PFIZER MONKEY VIRUS CORE

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Retroviruses need to deliver their genetic material into the host cell nucleus for integration. HIV-1 (Human Immunodeficiency Virus 1) can enter the nucleus via the nuclear pore complex through active transport. Recent research suggests that the HIV-1 core remains largely intact during nuclear entry, with uncoating occurring only after reaching the nucleus. However, the nuclear entry mechanism of other retroviruses remains poorly understood. Mason-Pfizer monkey virus (MPMV), a D-type retrovirus, serves here as a model for further understanding of retroviral nuclear entry, and offers insights into retroviral biology. We prepared GFP-CA (green fluorescent protein – capsid protein) labeled MPMV virions, which enable a direct visualization of the retroviral core within the cell. To visualize MPMV cores post infection, we used HEK293 (Human Embryonic Kidney 293) cells expressing mScarlett--tagged RNA helicase DHX15 (DEAH-box helicase 15) with predominantly nuclear localization. We visualized the course of entry of GFP-labeled MPMV capsid cores into the nucleus using confocal microscopy and live imaging. We also documented the nuclear localization of MPMV cores at various times post infection. We observed colocalization of the viral cores with DHX15-rich spots, which are considered as splicing and transcription sites. The MPMV core nuclear entry was independent of reverse transcription as shown by the use of reverse transcription MPMV mutant. In conclusion, MPMV can enter the nucleus as an intact core. Once inside, it associates with transcriptionally active nuclear domains.

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P18
A CORRELATIVE LIGHT ELECTRON MICROSCOPY APPROACH TO STUDY HIV CAPSID UNCOATING *IN VITRO*

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Fusion of the viral and cellular membranes releases the HIV-1 conical capsid (CA) core into the target cell. Capsid disassembly, or "uncoating", is a prerequisite to successful genomic integration of the viral DNA into host chromatin. However, the molecular mechanisms of uncoating, and the accompanying morphological changes in the CA core, remain unknown. Using time-lapse fluorescence microscopy and cryo-electron tomography (cryo-ET), we are developing a correlative light electron microscopy (CLEM) workflow to study HIV-1 core uncoating *in vitro*. Fluorescently tagged HIV-1 virions are adhered to grids, and saponin is added to strip the viral membrane and initiate the capsid uncoating process. Treatment of permeabilized cores with small molecules, such as IP_6, Lenacapavir, or PF74 allowed us to study their respective effects on capsid morphology, by cryo-ET. Fluorescence images that were collected before vitrification was used to enable identification of HIV-1 cores by CLEM under the TEM, for subsequent cryo-ET tilt series data collection, 3D reconstructions and structure determination. on uncoating particles that are identified via fluorescence imaging and subsequently perform 3D reconstructions from the tilt series data. Our preliminary results indicate distinct core morphologies between untreated virions and those treated with small molecule inhibitors, such as IP6, including inhibitors PF74 and LEN. We will present the results demonstrating the utility of our CLEM workflow that allows us to correlate virions and small-molecule treated cores from fluorescence microscopy to cryo-ET and image reconstruction. These *in vitro* assays will facilitate differentiating intact from partially assembled CA cores and elucidate structural changes during the uncoating process. An understanding of core morphologies will in turn enable identifying distinct types of core assemblies *in situ*.

P19
MULTIMERIZATION INDUCES THE ORDERING OF THE INNER LEAFLET IN GAG ASSEMBLY IN THE PLASMA MEMBRANE

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HIV-1 lipid envelope has been reported to be enriched in host sphingomyelin and cholesterol. Viral Gag protein plays a central role in virus assembly and budding. Our previous study showed that Gag brings sphingomyelin-rich and cholesterol-rich domains in close proximity in a multimerization- and curvature-dependent manner. However, the molecular mechanism by which Gag in the inner leaflet selectively enriches the outer leaflet lipid domains is still unknown. To better understand this mechanism, it is essential to know the physical properties of the inner leaflet membrane in the Gag assembly. Here, we used Gag/Gag-Halo expression, a recently developed environment--sensitive dye, NR-Halo, and ratiometric and FLIM imaging techniques to measure the local membrane order of the inner leaflet in the assembly. Our results suggest that the membrane order in wild-type Gag assembly was higher than in multimerization-deficient mutant assembly. In addition, mutants defective in membrane curvature formation also increase the membrane order to a similar extent to wild-type Gag. This study suggests that Gag multimerization, rather than membrane curvature, increases the membrane order at the assembly.

P20
PREDICTED INTERACTIONS OF HIV-1 GAG AND IBAR IRSP53

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Although it has been shown that IBAR (Inverse BAR-domain) protein IRSP53 plays a role in budding the immature HIV-1 virion, the structural details and mechanisms are still unclear (Inamdar et al.: Elife. 2021;10. Epub 20210611.). IRSP53 has been shown to co-locate with the growing Gag lattice and it is thought that it helps to bend the membrane and thus create the local curvature necessary for budding. We evaluated the hypothesis that there is a direct interaction between the Gag lattice and IBAR IRSP53. This mechanism should be unique to IRSP53 as another IBAR, IRTKS, was shown to be a negative control experimentally.
We used molecular dynamics simulations to examine if there is a direct protein-protein interface between IRSP53 and HIV-1 Gag. We find that there are protein-protein interactions between the SH3 domain of IRSP53 and both the CA and MA domains of HIV-1 Gag. The SH3 domain fits nicely between the MA and CA domains of HIV-1 Gag when the Gag is multimerized and constrains the conformation of the MA and CA domains when Gag is not multimerized. These interactions involve many residues present in the SH3 domain of IRSP53, but not IRTKS in accordance with earlier experiments.

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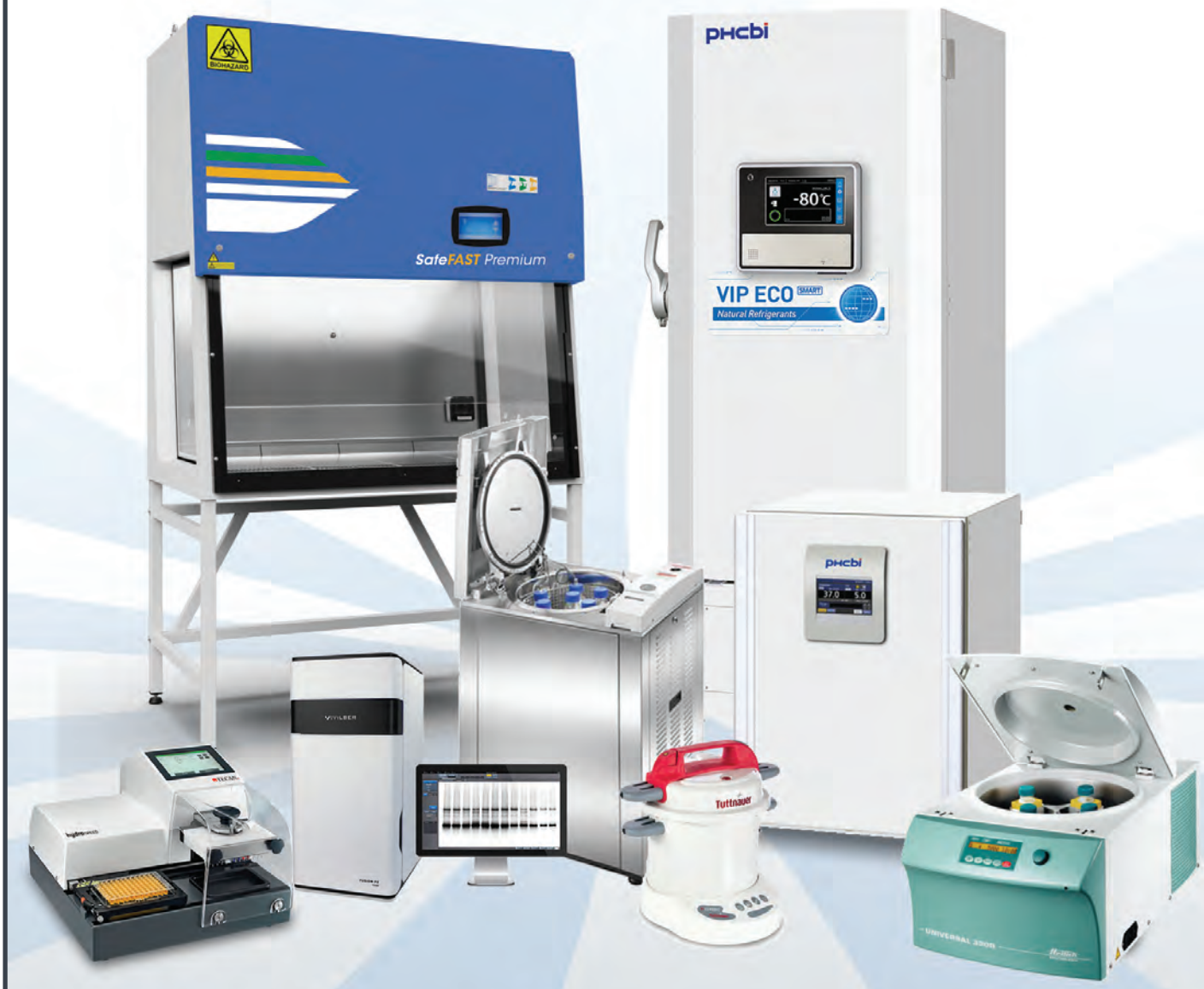

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